ADVANCES IN VIRUS RESEARCH, VOL 69

THE INNATE ANTIVIRAL RESPONSE: NEW INSIGHTS INTO A CONTINUING STORY

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I. Introduction

The pathological manifestation of viral infection is the result of complex interactions between the direct cytopathic effect of viral infection and the local and systemic immune responses to infection. Antiviral cytokines (interferons or IFNs) and chemokines activated as an early response to infection play an important role both in the outcome of the viral infection and in its virulence. Interferons can inhibit viral replication directly by inducing an antiviral state in the host cells. Indirect inhibition occurs due to increasing the expression of major histocompatibility complex (MHC) antigens, initiating inflammatory responses and stimulating the development of cytotoxic T cells as well as the differentiation of B cells into antibody-producing plasma cells. Interferons also play a crucial role in the activation of macrophages and natural killer (NK) cells. Two families of the transcriptional

0065-3527/07 $35.00
DOI: 10.1016/S0065-3527(06)69001-5

factors play a major role in the transcriptional activation of these genes: the well-characterized family of NF-κB factors and the more recently described family of interferon regulatory factors (IRFs). The IRFs play a critical role in the induction of Type I IFN and chemokine genes, as well as genes mediating antiviral, antibacterial, and inflammatory responses (Barnes *et al.*, 2002a; Kunzi and Pitha, 2003; Taniguchi *et al.*, 2001).

The ability of the host to detect invasion by a pathogenic intruder and to activate defense mechanisms to eliminate an infection is essential for survival. The innate immune response has developed as a rapid and regulated defense mechanism in which the recognition of an invading pathogenic organism can occur on binding to a specific cytoplasmic receptor or to a Toll-like receptor (TLR). These TLRs recognize the conserved patterns of proteins, lipoproteins, double-stranded (ds) RNA, or unmethylated CpG DNA that are pathogen-associated molecular patterns (PAMPs) (Akira *et al.*, 2001). These interactions induce multiple signaling pathways leading to the activation of transcription factors which control the expression of a diverse set of genes coordinating the immune responses. Although cascades of multiple kinases usually mediate activation of the transcription factors, their functional diversity is modulated by interaction with other transcription factors and cofactors. Indeed, these regulatory networks are critical components of the host defense against invading pathogens, including viruses.

A new dimension of complexity is added to the virus-mediated immune response by the finding that certain viruses have evolved mechanisms allowing them to overcome some of the components of the host-induced antiviral response. This can be through the production of proteins which mimic cytokines or their receptors. Other viruses have developed defense mechanisms that enable them to interfere with the transcriptional activation of interferon genes or with their mechanisms of action. Thus, many of the viral genes that are not required directly for viral replication may be essential for pathogenicity of the virus *in vivo* (Garcia-Sastre and Biron, 2006).

II. Interferons

Type I IFNs are the earliest defense mounted by the host during viral infection, with activity against many viruses, and thus play a critical role in the innate antiviral response (Biron, 2001; Samuel, 2001; Sen, 2001). A deficiency in the IFN system can lead to fulminant

viral disease (Levin and Hahn, 1985). Type I IFN can both directly inhibit viral replication as well as activate immune cells; therefore this family of cytokines has been extensively studied in the context of host immune antiviral defense (Sen, 2001). Human Type I IFN genes lack introns and map to the short arm of human chromosome 9. The Type I IFN family consists of more than 20 IFN-*A* genes and pseudogenes, 5 IFN-*W* genes, but only 1 IFN-*B* and IFN-*G* gene (Table I) (Diaz, 1995). All these proteins induce signaling pathway through a common receptor complex (IFNAR1 and IFNAR2). IFN-*A* genes show about 96% homology and, although they all have antiviral functions, some functional specificity between the individual IFN-*A* proteins is starting to emerge (Hilkens *et al.*, 2003). The single IFN-*G* gene (Type II IFN) contains three introns and maps to the long arm of human chromosome 12 and signals through a distinct receptor complex IFNGR (Kalvakolanu, 2003). In addition, a new group of IL-10-related antiviral proteins was described and designated IFN-λ and consisting of λ1–3 (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003). Genes encoding these proteins are localized on human chromosome 19. The IFN-λ proteins bind to a receptor complex composed of IL-10R2 and IFN-λR1 and signal through JAK–STAT pathway (Kotenko *et al.*, 2003). In addition, in mouse another antiviral protein, limitin, was identified (Hardy *et al.*, 2004). Whereas IFN-α is a family of nonglycosylated, monomeric proteins, IFN-β, IFN-ω, and IFN-γ are *N*-glycosylated (Allen and Fantes, 1980). Functionally, IFN-β is a dimer, whereas IFN-γ is a tetramer. Synthesis of IFN-α takes place mainly in cells of lymphoid origin, such as B cells and dendritic cells (DCs), and the expression profile of the individual IFN-α subtypes is both virus and cells specific. IFN-β is induced as an immediate early gene in infected fibroblasts, epithelial cells, and macrophages. Interestingly, IFN-β seems to play an essential role in the expression of IFN-*A* genes, since in nonlymphoid cells the synthesis of IFN-α is induced by a feedback mechanism mediated by IFN-β (Marie *et al.*, 1998) and the IFN-*B* knockout mice are not able to express IFN-α genes (Deonarain *et al.*, 2000). IFN-ω is expressed in leukocytes and trophoblasts, and IFN-κ in keratinocytes and DCs (LaFleur *et al.*, 2001; Nardelli *et al.*, 2002). Production of IFN-γ is a specialized function of T lymphocytes, although NK cells and macrophages can produce IFN-γ as well (Samuel, 2001; Sen, 2001; Weissmann and Weber, 1986). Since Type I IFN plays a major role in the antiviral immune response, this chapter will concentrate on IFN-α/β.

On binding to their respective receptors, IFN-α/β exert their multiple effects through receptor-mediated signaling pathways, resulting in the induction of IFN-stimulated genes (ISGs) (Darnell *et al.*, 1994).

TABLE I

HUMAN INTERFERONS

Name	Genes	Proteins	Producer cells	Effect
IFN-α	>20	Nonglycosylated	Lymphoid cells	Antiviral state
	No introns	166aa	Macrophages	ISG induction
	Chromosome 9	Monomer		MHC I induction
IFN-β	1	*N*-Glycosylated	Fibroblasts	Antiviral state
	No introns	166aa	Epithelial cells	ISG induction
	Chromosome 9	Dimer	Macrophages	MHC I induction
IFN-ω	5	*N*-Glycosylated	Leucocytes	Presumably similar to IFN-α
	No introns	172aa	Trophoblasts	
	Chromosome 9	–		
IFN-γ	1	*N*-Glycosylated	T cells	2′,5′-OAS induction
	3 introns	146aa	Macrophages	IL-1 enhancement
	Chromosome 12	Tetramer	NK cells	MHC I induction
				MHC II induction
IFN-λ	3	Possible *N*-glycosylation of IFNl1	Ubiquitous—including mDC	Antiviral state
	5 exons	215–225aa		MHC I induction
	Chromosome 19			
IFN-κ	1	207aa	DC	Induces cytokines from monocytes and DC
	Chromosome 9		Keratinocytes	Antiviral state
			Monocytes	Induces ISG

The signaling pathway involves the activation of two JAK kinases (JAK1 and Tyk2) that are associated with IFN receptors and the consequent tyrosine phosphorylation of preexisting signal transducer and activator of transcription (STAT). On phosphorylation, STAT1 and STAT2 assemble together with interferon regulatory factor 9 (IRF-9) into a multimeric complex (ISGF3) that is transported to the nucleus, where it interacts with interferon-responsive elements (ISRE) present in the 5′ flanking region of ISG (Levy, 1995). While ISGF3 seems to be the main transcription factor regulating transcription of ISG, Type I IFN also stimulates formation of STAT1 homodimers that bind to a slightly different DNA domain, the IFN-γ-activated site (GAS), present in the promoters of ISG that can be induced both by Type I IFN and IFN-γ. In addition, it was shown that during *Lymphocytic choriomeningitis virus* (LCMV) infection, Type I IFN signaling depends only on STAT2 not STAT1 (Ousman *et al.*, 2005), and that the STAT2–IRF-9 heterodimer is also an activator of transcription (Kraus *et al.*, 2003). The signaling by Type I IFN is not limited to the JAK–STAT pathway as this receptor can also activate both the MAPK and PI3K pathways (Platanias, 2005). How much these two pathways contribute to the antiviral response *in vivo* is not clear. Although all IFN-αs and IFN-β use the same receptor the binding affinity of IFN-β and IFN-α to the two components of the IFNAR may be distinct (Platanias *et al.*, 1996). Interestingly, although IFN-λ signals through a different receptor to IFN-α/β, they also signal through the JAK–STAT pathway to the ISRE domain (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003). IFN-α/β activate a large profile of ISG, some of which encode proteins with diverse function including antiviral properties, proapoptotic functions and modulators of ubiquitination pathways (de Veer *et al.*, 2001). Expression of ISG has been demonstrated to be distinct in different cell types (Hilkens *et al.*, 2003).

Type I IFNs are not only essential for antiviral defense but they also exert a number of immunoregulatory effects. They modulate the expression of MHC antigens, and it is via this mechanism that IFN-α/β increase susceptibility of *Vaccinia virus* (VV) or LCMV-infected fibroblasts to lysis by cytotoxic T lymphocytes (CTLs) (Bukowski and Welsh, 1985). IFN-α/β can also downregulate expression of IL-12 in human DC and monocytes (Karp *et al.*, 2000), stimulate expression of IFN-γ in response to influenza virus infection (Sareneva *et al.*, 1998), and induce expression of IL-15 (Durbin *et al.*, 2000). This immunostimulatory effect of Type I IFN enhances activation of NK cells (Biron *et al.*, 1999) and memory $CD8^+$ T cells during the early steps of infection (Zhang *et al.*, 1998). Human Type I IFN promotes differentiation of DC (Santini *et al.*, 2000),

cytotoxicity of NK cells, upregulation of IFN-γ expression, and stimulation of the differentiation of B cells in both DC-dependent and -independent manner (Biron, 2001). Recent data demonstrate that Type I IFN can directly stimulate the B-cell response during the early stages of influenza virus infection (Coro *et al.*, 2006) and protect $CD8^+$ T cells from antigen-induced cell death (Marrack *et al.*, 1999). IFN-α was also shown to induce differentiation of human monocytes-derived DC (mDC) that were able to induce Th1 polarization both *in vitro* and *in vivo* (Santini *et al.*, 2000).

A. Antiviral Effect of IFN: Induction of ISG

Type I IFN inhibits the replication of a large number of both RNA and DNA viruses *in vitro*. The use of genetically modified mice deficient for the Type I IFNAR or components of the IFN-signaling pathway, such as STAT1, clearly establishes the importance of Type I IFN in the resistance to viral infection *in vivo*. Both IFN-*A*/*B* or STAT1 knockout mice are highly susceptible to viral infection and unable to establish an antiviral state (Angel *et al.*, 1999; Hwang *et al.*, 1995; Meraz *et al.*, 1996; Muller *et al.*, 1994). Initial experiments in cell culture suggested that IFNs inhibit viral replication at the level of viral protein synthesis (Biron, 2001; Samuel, 2001; Sen, 2001). However, it was shown later that IFN-mediated inhibition of the replication of paramyxoviruses and rhabdoviruses occurs at the level of primary transcription (Staeheli *et al.*, 1993), and the inhibition of retroviruses and lentiviruses is posttranslational, at the level of virus assembly and release (Okumura *et al.*, 2006; Pitha, 1994; Pitha *et al.*, 1979).

Several of the ISG encode proteins that have direct antiviral activities (Samuel, 2001). The best characterized of these are 2′,5′-oligoadenylate synthetase (2′,5′-OAS), RNA-dependent protein kinase (PKR), Mx-GTPase, and the RNA-specific adenosine deaminase (ADAR). The 2′,5′-OAS pathway leads to RNA degradation and consists of three enzymes; 2′,5′-OAS, on activation by dsRNA, polymerizes ATP into pppA(2′p5′A)*n*, (2′,5′A oligoadenylates) (Kerr and Brown, 1978). 2′,5′A oligoadenylates in turn activate a cellular endonuclease, 2′,5′-OAS-dependent RNase L, which is present in cells in an inactive form (Silverman, 1985). When activated by 2′,5′-OAS, it is converted from the inactive monomeric form to an active homodimer (Dong and Silverman, 1995). Homodimeric RNase L then degrades both cellular and viral RNAs at UU or AU nucleotides. 2′,5′-phosphodiesterase catalyses the degradation of 2′,5′-OAS (Lengyel, 1982). Expression of 2′,5′-OAS in cells leads to the establishment of an antiviral state which

results in the selective inhibition of the replication of picornaviruses such as *Mengovirus* and *Encephalomyocarditis virus* (EMCV); no inhibition of *Vesicular stomatitis virus* (VSV) or *Herpes simplex virus 1* (HSV-1) has been observed (Chebath *et al.*, 1987). Homozygous RNase L knockout mice succumb more rapidly in response to EMCV infection than wild-type mice (Zhou *et al.*, 1999). Another interferon-induced antiviral gene is PKR, which is activated by autophosphorylation mediated by dsRNA (He, 2006). Activated PKR catalyses phosphorylation of several substrates including the α subunit of the initiation factor eIF-2 (eIF-2α) (Samuel, 1979), which has been implicated in the inhibition of viral protein synthesis (Pathak *et al.*, 1988). Phosphorylation of the transcription factor inhibitor IκB stimulates NF-κB activation (Kumar *et al.*, 1994), and the HIV-1 encoded Tat transactivator was shown to interact with PKR (McMillan *et al.*, 1995). As described later, the crucial role of PKR in the antiviral effect of IFN is indicated by the observation that a number of viruses have developed mechanisms to negate PKR function. PKR-deficient mice exhibit an increased susceptibility to VSV infection (Stojdl *et al.*, 2000), whereas the antiviral response to influenza virus (FluV) and VV was not impaired, again demonstrating some viral specificity among the ISG (Abraham *et al.*, 1999).

The Mx proteins are GTPases which are induced by IFN-α/β but not by IFN-γ. There are two Mx genes in both the human and mouse genome encoding MxA and MxB or Mx1 and Mx2, respectively. Their antiviral activity is through GTPase activity. Overexpression of Mx confers a high degree of antiviral activity and resistance to infection by orthomyxoviruses (influenza A and C, *Thogoto virus*), Bunyavirus (*Hantanavirus*), Paramyxovirus (measles), Rhabdovirus (VSV), and Togavirus (*Semliki Forest virus*) but not picornaviruses (Haller *et al.*, 1998; Pavlovic *et al.*, 1995). The inhibition of influenza virus replication by Mx proteins is due to the inhibition of primary transcription mediated by a virion-associated polymerase, while *Thogoto virus* inhibition is due to an impairment of viral nucleocapsid transport to the nucleus and consequent inhibition of primary transcripton (Pavlovic *et al.*, 1992). However, Mx proteins were also shown to suppress replication of influenza virus via the stimulation of a proapoptotic pathway (Mibayashi *et al.*, 2002).

Two other IFN-stimulated proteins also have a direct antiviral activity. ISG20 is nuclease specific for single-stranded RNA (ssRNA) and when overexpressed, inhibits replication of VSV, influenza virus, EMCV, and HIV-1 (Espert *et al.*, 2003). Another ISG that can downmodulate infectivity of RNA viruses is A to I editing adenosine deaminase acting

on RNA (ADAR). Transition of nucleotides from A to I disrupts base pairing, with the AU base pair becoming the less stable IU pair, destabilizing the dsRNA (Bass and Weintraub, 1988). This A–I editing has been found in multiple viral RNA sites of negative strand RNA viruses, and it has been associated with persistent infection (Murphy *et al.*, 1991). This hypermutation has been observed in the matrix protein of *Measles virus* (Billeter *et al.*, 1994), parainfluenza virus (Murphy *et al.*, 1991), and *Bornavirus* (Formella *et al.*, 2000).

Two, possibly three ISG, were identified as being able to inhibit HIV-1 infection *in vitro*. IFN-α/β inhibit HIV-1 in at least two steps in its life cycle. In *de novo* infection in T cells, the inhibition is predominantly at the early steps of HIV-1 replication, possibly at the level of reverse transcription (Shirazi and Pitha, 1993). Acute SIV infection in the brain has been shown to induce IFN-β synthesis which suppresses SIV replication and induces latency (Barber *et al.*, 2006). It was shown that this inhibition occurs at the transcriptional level by interferon-induced expression of a dominant negative form of the C/EBR-β transcription factor, which was also shown to inhibit HIV-1 replication in macrophages (Honda *et al.*, 1998). The second level of IFN-mediated inhibition of HIV-1 occurs after infection is established and, in chronically infected cells, at the level of virus assembly (Pitha, 1994). This step in HIV-1 replication is mediated by the interferon-induced, ubiquitin-like protein ISG15, which is conjugated to cellular proteins. ISG15 mimics the IFN effect and inhibits release of HIV-1 virions without affecting the synthesis of viral proteins. Elimination of ISG15 expression by specific siRNA reverses the IFN inhibition and allows release of virions. Addressing the molecular mechanism of this inhibition, it was shown that ISG15 expression interferes with the ubiquitination steps that are critical for assembly and release of HIV-1 virions. The HIV-1 Gag polyprotein interacts through p6 PTAP motif with Tsg101, which functions in sorting of the vacuolar proteins, and this interaction is dependent on p6 ubiquitination. The effect of ISG15 was at least partially related to the inhibition of Gag and Tsg101 ubiquitination and the disruption of the interaction of the Gag domain with Tsg101. This association is prevented in cells expressing ISG15, resulting in inhibition of virion release (Okumura *et al.*, 2006). The precise mechanism by which ISG15 interferes with the HIV-1-Vps pathway is unclear, but it was shown that ISGylation of one of the E2 enzymes of the ubiquitin pathway suppresses conjugation of ubiquitin (Takeuchi and Yokosawa, 2005). Inhibition of virus assembly and maturation is also the major mechanism by which IFN is able to block the replication of murine retroviruses (Pitha, 1980). A number of retroviruses and negative strand RNA viruses

contain the L domain which has a role in the endosomal trafficking pathway (Morita and Sundquist, 2004); thus ISG15 may affect replication of a broad group of viruses.

Expression of two cellular proteins APOBEC3G and TRIM5α restricts HIV-1 replication. APOBEC3G contains a cytosine deaminase domain and converts cytidine to uridine in the single-stranded proviral DNA, which results in hypermutation of the HIV-1 genome (Yu *et al.*, 2004). The expression of APOBEC3G was shown to be upregulated by IFN-α in both hepatocytes and HIV-1-infected macrophages (Tanaka *et al.*, 2006). The elimination of APOBEC3G by specific siRNA was shown to inhibit the IFN-mediated inhibition of HIV-1 replication. HIV-1-encoded Vif mediates degradation of APOBEC3G through a ubiquitin-mediated proteasome-dependent pathway and prevents its incorporation into virions (Yu *et al.*, 2003). This Vif-mediated degradation is inhibited by IFN-α; however, whether this IFN effect is mediated by ISG15 is unknown. These results suggest that APOBEC3G, like ADAR, is an IFN-induced antiviral protein that can induce hypermutation of the viral genome and decreases viral fitness. TRIM5α is another protein that has been shown to block the early steps of HIV-1 replication, possibly by the ubiquitination of HIV-1 capsid protein (Wu *et al.*, 2006). While its status as an ISG is unclear, Trim5α belongs to a family of proteins with a tripartite motif, expression of which is stimulated by IFN-α/β (Asaoka *et al.*, 2005). Thus, the IFN-α/β-mediated targeting of the ubiquitination pathway may have a major impact on the attenuation of HIV-1 replication. IFN induction may, however, also contribute to the immunopathogenicity of HIV-1 infection. The detection of high levels of IFN-α in serum of AIDS patients is a predictor of rapid disease progression, and it has been suggested that IFN contributes to $CD4^+$ T cell depletion by stimulation of the TRAIL-mediated apoptotic pathway (Herbeuval *et al.*, 2006).

Analysis of the transcription signature of IFN-induced genes shows that IFN treatment results in a major upregulation of cellular gene expression (Der *et al.*, 1998). Although the antiviral function of the majority of ISG has not been yet determined, it has been shown in cell culture that Type I IFN inhibition is at the level of synthesis of viral proteins, as shown for VV, reovirus, VSV, influenza virus, and *Mengovirus* (Sen, 2001). However, IFN has also been shown to block other steps of the viral life cycle. IFN-α inhibits Cytomegalovirus (CMV) replication at the level of transcription of viral immediate-early (IE) genes, in particular the major genes *IE1*, *IE2*, and *IE3* and, consequently, steady state levels of late transcripts are reduced, while early gene expression is only partially affected (Martinotti *et al.*, 1992).

During the early days of IFN research, it was assumed that the antiviral effect is mediated by a common mechanism that is able to inhibit a large number of viruses. Instead, it has become clear that the antiviral effect is due to the combinatory effects of many proteins and that any given antiviral protein may show specificity for a distinct group of viruses.

III. Induction of Type I IFN Genes: Transcription Factors of the IRF Family

The molecular mechanism of the virus-mediated induction of Type I IFN genes has been under intensive investigation for the last 30 years. The identification of IRF-3 and IRF-7 and their role in the transcriptional activation of IFN genes had a major impact on the field (Au *et al.*, 2001; Juang *et al.*, 1998; Lin *et al.*, 2000; Sato *et al.*, 2000; Schafer *et al.*, 1998). The importance of IRF-3 and IRF-7 in the induction of IFN genes is underlined by the observation that many viruses prevent the IFN-induced antiviral response by targeting the function of IRFs (Katze *et al.*, 2002; Lubyova and Pitha, 2000; Ronco *et al.*, 1998; Talon *et al.*, 2000).

Induction of Type I IFN in infected cells is primarily due to transcriptional activation. The sequence domain in the 5′ region of the gene, termed the virus responsive elements (VREs), contains multiple GAAANN repeats that are highly conserved in both IFN-*A* and IFN-*B* gene promoters (Au *et al.*, 1995). The stimulation of IFN-*B* gene transcription by viral infection or dsRNA is mediated by complex enhanceosome consisting of NF-κB, IRF-3 and IRF-7, and activated protein 1 (AP-1) which is recruited to the VRE of the IFN-*B* promoter (Kim and Maniatis, 1997; Merika *et al.*, 1998; Wathelet *et al.*, 1998). The expression of the individual IFN-*A* gene subtypes is also regulated at the transcriptional level (Bisat *et al.*, 1988). The VRE of IFN-*A* promoters do not contain an NF-κB site but show multiple repeats of AANNGAAA sequences that can bind members of IRF family (Au *et al.*, 1995; Lin *et al.*, 2000; Yeow *et al.*, 2000). IRF-1, IRF-3, and IRF-7 together with histone transacetylases are part of the transcriptionally active IFN-*A* enhanceosome (Au and Pitha, 2001). The differential expression of individual IFN-α subtypes was shown to be due to distinct nucleotide substitutions in these domains (Au and Pitha, 2001; Au *et al.*, 1993; Lopez *et al.*, 1997) as well as the presence of negative regulatory sequences (DNRE) located in the upstream promoter regions of some IFN-*A* subtypes (Lopez *et al.*, 2000). Thus, while the

activation of IFN-*B* gene transcription is regulated by both NF-κB and IRF-3, activation of the IFN-*A* genes seems to depend mostly on IRFs.

To date, nine human cellular IRFs (IRF-1, IRF-2, IRF-3, IRF-4/Pip/ICSAT, IRF-5, IRF-6, IRF-7, ICSBP/IRF-8, and ISGF3γ/p48/IRF-9) as well as virus-encoded analogues of cellular IRFs have been identified (Barnes *et al.*, 2002a). These factors can function as transcriptional activators, repressors or both. All IRFs share significant homology in the N-terminal 115 amino acids, which comprises the DNA-binding domain, characterized by five tryptophan repeats. Three of these repeats contact DNA with specific recognition of the GAAA and AANNN-GAA sequences (Escalante *et al.*, 1998). However, the unique function of a particular IRF is accounted for not only by the DNA-binding specificity but also by cell type-specific expression, its intrinsic transactivation potential, and an ability to interact with other members of the IRF family or other transcription factors and cofactors.

IRF-3 and IRF-7 function as direct transducers of virus-mediated signaling and play a crucial role in the expression of Type I IFN genes and some ISG (Au *et al.*, 2001; Barnes *et al.*, 2002b; Marie *et al.*, 1998). While IRF-3 is constitutively expressed in most cell types (Au *et al.*, 1995), IRF-7 is constitutively expressed only in some lymphoid cells and plasmacytoid DC (pDC) with IFN-inducible expression in many cell types (Au *et al.*, 1998; Marie *et al.*, 1998). The transcription of IRF-7 is stimulated by the IFN-α/β-induced transcription complex ISGF3, viral infection, and liposaccharide (LPS) (Au *et al.*, 1998; Lu *et al.*, 2000). It was also shown that *Epstein-Barr virus* (EBV)-encoded latent membrane protein 1 (LMP-1) can induce IRF-7 and thus stimulate IFN synthesis, attenuate viral infection, and promote EBV latency (Xu *et al.*, 2006). In uninfected cells, IRF-3 and IRF-7 reside predominantly in the cytoplasm, but on virus infection they are phosphorylated on specific serines in the C-terminal regulatory domain. Phosphorylation of IRF-3 on Ser 386 is required for its transcriptional activation and dimerization (Mori *et al.*, 2004). Activated IRF-3 interacts with the transcription coactivator p300/CBP, translocates to the nucleus, and binds to the VRE domain of the IFN-*B* promoter (Lin *et al.*, 1998; Schafer *et al.*, 1998; Weaver *et al.*, 1998; Yoneyama *et al.*, 1998). Replacement of both serines and threonines in the IRF-3(5D) construct between aa395 and aa407, and replacement of Ser 477 and 479 of IRF-7 (2D) by phosphomimetic Asp resulted in constitutively active forms of these two proteins. While the constitutively active IRF-3 (IRF-3–5D) accumulates in the nucleus and induces expression of endogenous IFN genes in uninfected cells, its function is not completely identical to virus-activated IRF-3. Overexpression of IRF-3–5D led to the

expression of endogenous IFN-*A* genes in the absence of IRF-7, while wild-type IRF-3 alone was not sufficient to activate IFN-*A* genes in infected cells. Also the profile of IFN-*A* subtypes induced by constitutively active mutants was distinct from that activated by viral infection via IRF-7 (Au *et al.*, 2001). These data revealed that the transcriptional specificity of constitutively active IRF-3(5D) and IRF-7(2D) were not identical to the virus-mediated activation. Since the constitutively active mutant of IRF-3 is often used in replacement of viral infection, this difference should be noted.

Phosphorylation and activation of IRF-3 and IRF-7 in infected cells is mediated by two noncanonic IκB kinases, IKK and TBK1 (Fitzgerald *et al.*, 2003a; Sharma *et al.*, 2003). There is a high degree of homology between the two kinases, and studies with specific siRNA indicate that these kinases are involved in polyI:C and *Sendai virus* (SeV)-mediated activation of IRF-3 in cultured cells. Phosphorylated IRF translocate to the nucleus, where they bind to the transcriptional coactivator p300/CBP and IRF-E elements in the VRE region of the IFN-*A* and IFN-*B* promoters. TBK1, which is constitutively expressed, can phosphorylate IRF-3 *in vitro* not only on Ser 386 but also on Ser 396, 398, 402, and 405 (McWhirter *et al.*, 2004). IKK expression is limited to hematopoietic cells, and it is inducible. Both of these kinases play a critical role in the activation of IRF-3 in response to viral and bacterial infection (Fitzgerald *et al.*, 2003a; Hemmi *et al.*, 2004; Sato *et al.*, 2003), suggesting that the innate response to viral and bacterial infection proceeds through a common mechanism—activation of the IRF-3- and IRF-7-mediated transcription (Pitha, 2004).

In infected cells, ubiquitously expressed IRF-3 mediates induction of IFN-β and some ISG, including IRF-7, the expression of which is critical for the induction of the IFN-*A* genes. Reconstitution of IRF-7 expression in infected human fibroblasts that can express only IFN-β confers expression of a distinct subtype of IFN-*A* genes (Yeow *et al.*, 2000). In contrast, mouse fibroblasts expressing only low levels of IRF-7 were able to induce the IFN-*A4* gene but not the other murine IFN-*A* genes (Marie *et al.*, 1998). Mice deficient for IRF-3 show an impairment in *Newcastle disease virus* (NDV)-mediated induction of IFN-α/β and an increased susceptibility to EMCV infection, while the antiviral response to VSV and HSV-1 was unchanged (Sato *et al.*, 2000). In contrast, in IRF-7-deficient mice, viral infection was unable to stimulate expression of Type I IFN genes. Altogether, these data, together with *in vitro* experiments (Yeow *et al.*, 2000) suggest that in both human and murine cells, IRF-7 is a vital transcription factor, directly involved with the regulation of IFN-*A* gene expression; indeed it has been

suggested that IRF-7 is the master regulator of Type I IFN (Honda *et al.*, 2005b). While the critical role of IRF-7 in the induction of Type I IFN genes cannot be disputed, the role of IRF-3 in the antiviral response should not be underestimated. *In vitro* experiments clearly show that expression of IRF-3 alone results in the induction of IFN-*B* gene *in vitro* (Juang *et al.*, 1999; Yeow *et al.*, 2001). A number of viruses target the activation and function of IRF-3 as a part of viral mimicry to overcome the cellular antiviral response (as will be discussed later), and thus it is unlikely that IRF-3 is only a bystander in the antiviral response.

The dependence of IFN-α induction on synthesis of IFN-β and expression of IRF-7 in nonlymphoid cells suggested that Type I IFN induction proceeds through two steps, involving an autocrine feedback loop (Marie *et al.*, 1998). First, virus activates constitutively expressed IRF-3 leading to synthesis of low levels of IFN-β, and subsequently this IFN-β induces the transcription of IRF-7 which, after virus-mediated activation, stimulates transcription of IFN-*A* genes, synthesis of IFN-α, and further enhancement of IFN-β synthesis (Sato *et al.*, 2000). However, cells which produce high levels of IFN, such as pDC2, express high levels of IRF-7 constitutively (Fitzgerald-Bocarsly *et al.*, 2000; Gibson *et al.*, 2002; Siegal *et al.*, 1999). IFN-α synthesis in these cells is not dependent on IFN-β (Izaguirre *et al.*, 2003; Prakash *et al.*, 2005). A defect in IFN synthesis was observed in mouse embryonic fibroblasts (MEFs) from IFNAR1-deficient mice, while the IFN production was not decreased in splenocytes, which would include pDC (Erlandsson *et al.*, 1998). Also of relevance is the observation that IFN-*B* knockout mice are not able to express IFN-*A* genes (Deonarain *et al.*, 2000). These data further indicate the importance of IFN-mediated induction of IRF-7 and amplification of IFN synthesis in cells unable to express IRF-7 without stimulation, as compared to cells such as splenic pDC which constitutively express IRF-7.

Human IRF-5 has also been implicated in the innate antiviral response. *In vitro* IRF-5, like IRF-7, rescued IFN-α induction in fibroblast cells, which expressed only IRF-3 and IFN-β. Although IRF-5 was activated by viral infection and transported to the nucleus, it showed some properties that are distinct from IRF-3 and IRF-7. The IRF-5 polypeptide contains two nuclear localization signals whereas IRF-3 or IRF-7 have only one, and nuclear IRF-5 could also be detected in uninfected cells (Barnes *et al.*, 2001, 2002a,b). The activation and phosphorylation of IRF-5 by viral infection could be detected in cells infected with NDV or VSV but not in cells infected with SeV or treated with polyI:C, suggesting that the activation of IRF-5 is virus-specific. Transcription of IRF-5 is induced not only by IFN-α/β and viral infection

(Mancl *et al.*, 2005) but also by the tumor suppressor p53 (Mori *et al.*, 2002), and overexpression of IRF-5 induces $p21^{WAF1/CIP1}$ and arrests cells in the G2/M phase of the cell cycle (Barnes *et al.*, 2003). Like IRF-3, IRF-5 induces expression of several proapoptotic genes and also induces apoptosis. Interestingly, a high percentage of primary lymphocytic malignancies show a lack of IRF-5 expression, suggesting that expression of the *IRF-5* gene may be silenced in these cells (Barnes *et al.*, 2003). *In vitro* experiments suggest that both IRF-5 and IRF-7 bind to the VRE of IFN-*A* genes and activate expression of IFN-α. However, the subtypes of IFN-α induced by IRF-5 and IRF-7 were distinct. While IFN-α1 was the major subtype induced by NDV in IRF-7-expressing cells, IRF-5-expressing cells expressed IFN-α8 as the major subtype, further confirming that not all induction of IFN-*A* genes is mediated by IRF-7. The transcriptional signatures of IRF-5 and IRF-7 in NDV-infected B cells are therefore both overlapping and distinct (Barnes *et al.*, 2002a, 2004). Gene array analysis revealed a significant increase in the transcription of a number of ISG in NDV-infected B cells overexpressing IRF-5 which were not expressed in IRF-7-expressing cells. Interestingly, IRF-5 overexpression specifically upregulated a number of early inflammatory proteins including RANTES, MIP-1β, I-309, MCP-1, and IL-8, suggesting an important role for IRF-5 in the regulation of the expression of these early inflammatory cytokines and chemokines (Barnes *et al.*, 2003, 2004). These results indicate that IRF-5 has a nonredundant function in infected cells.

While the critical role of IRF-7 in induction of Type I IFN genes was confirmed *in vivo* (Honda *et al.*, 2005b), IRF-5-deficient mice did not show any defect in CpG or polyI:C-mediated induction of Type I IFN, although the response to viral infection was not analyzed in these mice (Takaoka *et al.*, 2005). In correlation with our *in vitro* results, the expression of inflammatory cytokines, TNFα, IL-6, and IL-12 was significantly downregulated in IRF-5 knockout mice (Barnes *et al.*, 2003; Takaoka *et al.*, 2005). The IRF-5 null mice we have generated show a decrease not only in these inflammatory cytokines but also a decrease in the relative levels of IFN-α/β in the serum and spleen following NDV infection as compared to wild-type mice (unpublished results). We have also observed that unlike the human IRF-5, which is expressed in multiple spliced variants (Mancl *et al.*, 2005), in C57BL/6J mice we can detect only one IRF-5 splice variant expressed at very low levels in bone marrow, but not in spleen or mouse cell lines (unpublished results). The discordant effect of IRF-5 on the activation of IFN-*A* genes *in vitro* and in TLR-stimulated induction *in vivo* is unexpected. Activation of IRF-5 by TLR7 and TLR9 MyD88-dependent

pathways has been clearly demonstrated (Schoenemeyer *et al.*, 2005; Takaoka *et al.*, 2005). It was also shown that activation of IRF-5 by TLR7 involves the formation of a tertiary complex consisting of MyD88, TRAF6, and IRF-5 (Takaoka *et al.*, 2005). Thus it seems that in the presence of high levels of TLR-activated IRF-7, the contribution of IRF-5 to the induction of IFN genes is negligible, and its role is limited to the induction of inflammatory chemokines and cytokines. Since the expression of IRF-5-induced inflammatory cytokines is NF-κB dependent, it is possible that IRF-5 activation involves cooperation with NF-κB, similar to that observed for IFN-β activation by IRF-3 and NF-κB. On the other hand, the role of IRF-5 in the stimulation of Type I IFN genes may be limited to cells which do not express or activate IRF-7 and may depend on the distinct, concentration-dependent activity of IRF-5 and IRF-7. Further analyses of the functional interactions of IRF-5 with NF-κB are clearly warranted.

IRF-1 and IRF-2 were originally identified through their ability to bind to the PRDI domain in the VRE of the IFN-*B* gene and proposed to function as an activator and repressor of IFN-*B* gene, respectively. However, homozygous deletion of IRF-1 in mice did not impair activation of IFN-*A* or IFN-*B* genes in infected MEFs, while dsRNA-mediated induction of Type I IFN was diminished (Matsuyama *et al.*, 1993; Reis *et al.*, 1994). Subsequent studies have revealed that IRF-1 is involved in the broad spectrum of an antiviral defense mediated by IFN-γ, and the induction of NOS synthetase, guanylate-binding protein, and 2′,5′-OAS was impaired in IFN-γ-treated IRF-1-deficient MEFs (Kamijo *et al.*, 1994; Kimura *et al.*, 1994). IRF-1 is effectively induced by IFN-γ and IFN-γ-stimulated expression of NO synthetase genes is mediated by IRF-1 (Coccia *et al.*, 2000; Saura *et al.*, 1999). While IRF-1 does not have a critical role in the virus or dsRNA-mediated transcription of Type I IFN genes, the presence of IRF-1 was detected in the IFN-*B* enhanceosome binding to the IFN-*B* promoter region (Thanos and Maniatis, 1995) as well as in the IFN-*A* enhanceosome (Au and Pitha, 2001). Furthermore, analysis of the repertoire of lymphoid cells from IRF-1-deficient mice has shown defects in the maturation of $CD8^+$ T cells as well as a defective Th1 response, impaired production of IL-12 in macrophages, and defective NK cell development (Duncan *et al.*, 1996). These data indicate that IRF-1 has essential functions in the development and activity of various immune cells. In addition, IRF-1 also plays a critical role in the inducible expression of MHC class I gene expression and apoptosis, with cells from IRF-1-deficient mice being resistant to UV- and drug-induced apoptosis (Reis *et al.*, 1994). IRF-2 was identified as a factor

binding to the same sequence as IRF-1 but suppressing its transcriptional activation. Overexpression of IRF-2 in NIH/3T3 cells resulted in the oncogenic transformation of these cells; however, the role of IRF-2 in the antiviral defense is not clearly established. IRF-2 was also found to have a role in the development of myeloid DC (Honda *et al.*, 2004).

IRF-4 and IRF-8 are expressed primarily in lymphocytes, macrophages, B cells, and DCs. These two proteins have a high degree of homology but demonstrate only a weak DNA-binding affinity; their binding to DNA is increased by complexing with other transcription factors (Marecki *et al.*, 1999; Tailor *et al.*, 2006). IRF-4 binding is stabilized on heterodimerization with the transcription factor PU.1, and this heterodimer activates expression of the immunoglobulin (Ig) light-chain in B cells. IRF-4 has a critical role in the maturation of B and T cells (Lu *et al.*, 2003). IRF-4-deficient mice do not form germinal centers in B-cell follicles of the spleen and lymph nodes and have defects in myeloid DC. While the major role of IRF-4 is in B-cell differentiation, it was also found to stimulate expression of the myeloid-specific *gp91phox* gene that affects the development of myeloid cells. IRF-8 shares a number of similar properties to IRF-4; it binds DNA after interaction with transcription factors of IRF family, including IRF-1 and IRF-2 as well as PU.1 and E47. While the IRF-8/IRF-1 complex generally functions as a suppressor of transcription, the IRF-8/IRF-4 heterodimer activates transcription of ISG15 (Meraro *et al.*, 2002). The IRF-8/IRF-1 complex also induces numerous genes that are important for macrophage differentiation and macrophage-induced inflammation (Dror *et al.*, 2007; Liu *et al.*, 2004). IRF-8-deficient mice show major defects in $CD8^+$ DC and pDC (Tamura and Ozato, 2002; Tamura *et al.*, 2005). These mice also displayed increased susceptibility to infection with VV and *Leishmania major*. The impaired resistance to infection was shown to be due to a defect in Th1 immune response and inability to express IL-12. Consequently, IRF-8 was shown to stimulate the transcription of IL-12p40 (Meraro *et al.*, 1999). The fact that IRF-8 affects differentiation of the high IFN-producing pDC indicates its importance in the innate antiviral response as well as in TLR-mediated signaling.

IRF-9 plays a major role in the antiviral effect of Type I IFN. It is a component of the tertiary complex, ISGF3, formed in IFN-treated cells and binding to the ISRE elements of ISG and stimulating their transcription. In this complex, which also contains STAT1 and STAT2, IRF-9 is the major DNA-binding component. IRF-9 can also form a DNA-binding complex with the STAT1 homodimer and with STAT2,

and these complexes bind to DNA with the same specificity as ISGF3 (Kraus *et al.*, 2003). MEFs from IRF-9 knockout mice are deficient in both Type I and II IFN responses (Harada *et al.*, 1996; Kimura *et al.*, 1996). Furthermore, since the MEFs from these mice show an impaired induction of IRF-7 stimulation, transcription of IFN-*A* genes was also inhibited. These data indicate that IRF-9 also plays an essential role in the innate antiviral response.

IV. Pathogen Recognition Systems

A. Toll-Like Receptors

Recognition of invading pathogens is mediated by a relatively small number of cellular receptors that recognize viral nucleic acids or membrane glycoproteins associated in molecular patterns (Medzhitov, 2001). The TLRs are evolutionarily conserved, and in human and mouse they are constitutively expressed on immune cells, including antigen presenting cells such as DC and macrophages, but also on B cells and epithelial cells. The level of TLR expression can be modulated by stress and cytokines. The binding of a specific viral ligand to a TLR results in the activation of signaling pathways, which leads to the activation of an antiviral and inflammatory response. TLR are membrane glycoproteins containing leucine-rich repeats, an extracellular domain, and a signaling cytoplasmic domain (TIR) that is homologous to the interleukin 1 receptor (IL-1R). Of the 12 TLR identified in mammals, TLR3, TLR7/8, and TLR9 recognize viral nucleic acids and are generally expressed in endosomes, while the other TLR, which recognize lipids, lipopolysaccharides, and viral glycoproteins, are expressed on the cellular surface.

The signaling pathway mediated by the recognition of a specific ligand binding to a given TLR is generally dependent on the adaptor protein MyD88, which is recruited to the TIR domain of the TLR (Akira *et al.*, 2001). Recruitment of MyD88 to the TLR occurs after ligand-mediated TLR dimerization. Three additional adaptors associated with the TLR-mediated pathways have also been identified: the MyD88-associated adaptor (MAL/TIRAP), TIR domain-containing adaptor (TRIF/TICAM), and TRIF-related adaptor (TRAM) (Fitzgerald *et al.*, 2003b; Yamamoto *et al.*, 2004). Some of these adaptors are TLR specific, for example, association of MyD88 with MAL is necessary for the recruitment of MyD88 to the TIR domain of TLR2 and TLR4. However, binding of LPS to TLR4 activates signaling not only through the MyD88 adaptor but also by an MyD88-independent pathway that

utilizes two adaptors, TRIF and TRAM, to activate both the IRF and NF-κB pathways (Fitzgerald *et al.*, 2003b). TRAM, which is myristoylated, recruits MyD88 to the plasma membrane and TLR4 (Rowe *et al.*, 2006). TRIF also plays a critical role in TLR3 signaling. TLR3 is the only TLR that does not associates with MyD88; the binding of a ligand to TLR3 instead activates the IRF and NF-κB pathway through the TRIF adaptor. In contrast, TLR7/8- and TLR9-mediated signaling is dependent only on MyD88, and no other adaptors associated with these TLRs have been yet identified. Thus, the TLR family shows distinct degrees of signaling complexity, and it is not without interest that the TLRs recognizing the viral and bacterial nucleic acids are the simple ones, using either TRIF or MyD88 and not the other known adaptors (Miggin and O'Neill, 2006).

Activation of TLR3, TLR7/8, and TLR9 receptors by nucleic acids, as well as activation of TLR4 by LPS results in the induction of the inflammatory response via the NF-κB-signaling pathway and the antiviral response by the IRF pathway. The inflammatory response, which is predominantly generated by the NF-κB pathway, is initiated by the recruitment of two kinases, IRAK4 and IRAK 1, to MyD88 death domain and subsequent association of the phosphorylated IRAK1 with TRAF6. TRAF6, which functions as a ubiquitin ligase E3, forms a tertiary complex with TAK1 and the TAK-binding proteins (TAB1, TAB2, and TAB3), catalyzing the ubiquitination of NEMO (IKKγ). Ubiquitinated NEMO associates with IKKα and IKKβ to form the IKK complex. Phosphorylated IKKβ undergoes ubiquitination followed by proteasome-mediated degradation. Interestingly, while NEMO ubiquitination is K63 linked, IKKβ degradation is K48 linked. TAK1 also activates JNK and p38 which, together with NF-κB, activate expression of inflammatory genes.

The Type I IFN-mediated antiviral response can be induced through both MyD88-dependent and -independent pathways. Binding of the ssRNA or dsDNA to TLR7 and TL9, respectively, stimulates formation of a TLR-associated tertiary complex containing MyD88, IRAK1 and IRAK4, TRAF6, and IRF-7. TRAF6 mediates ubiquitination of IRF-7 (Kawai *et al.*, 2004), and IKKα was shown to be required for IFN induction (Chuang and Ulevitch, 2000; Du *et al.*, 2000). While unmethylated CpG DNA (Hemmi *et al.*, 2000), HSV-1 and HSV-2 genomic DNA (Krug *et al.*, 2004) specifically stimulate TLR9, methylated RNA is not recognized by this receptor (Ishii and Akira, 2005). Viral ssRNA and synthetic siRNA are recognized by TLR7 and TLR8. In mice, TLR7 is activated by infection with ssRNA viruses, including influenza virus and VSV (Diebold *et al.*, 2004). In addition to ssRNA, the synthetic imidazoquinoline, imiquimod, and its derivative resiquimod (R848)

activate TLR7/8 in both humans and mice. Both imiquimod and R848 elicit robust antiviral and antitumor immune responses *in vivo*, which correlate with a strong induction of Type I IFN (Megyeri *et al.*, 1995; Stanley, 2002; Testerman *et al.*, 1995). As a consequence of this activity, imiquimod is used successfully for the treatment of external genital warts caused by *Human papillomavirus* (von Krogh, 2001). Binding of the respective ligands to TLR7 or TLR9 activates IRF-7, the master regulator of Type I IFN gene expression (Honda *et al.*, 2005b); however, neither TLR7 or TLR9 activates IRF-3 (Kato *et al.*, 2004; Schoenemeyer *et al.*, 2005).

Our data show that signaling through TLR7/8 activates not only IRF-7 but also IRF-5 and induces its nuclear translocation. Binding of R848 to TLR7 stimulated IFN-α expression as well as some inflammatory genes in PBMC and monocytes (Megyeri *et al.*, 1995; Schoenemeyer *et al.*, 2005). Stimulation of TLR3 by polyI:C did not result in the activation of IRF-5, while IRF-7 was activated both by TLR3 and by TLR7/8. In human monocytes, TLR7 signaling activated Type I IFN genes and induced synthesis of biologically active IFN only in the presence of IRF-5. The activation of IRF-5 required MyD88, IRAK1, and TRAF6 (Schoenemeyer *et al.*, 2005). TLR9 stimulation also induced nuclear translocation of IRF-5 and its association with MyD88 and TRAF6. Overexpression of IRF-5, MyD88, and TRAF6 activated transcriptional activity of a promoter containing multiple ISRE repeats. However, while IRF-5 knockout mice have shown impairment in the TLR9-mediated induction of inflammatory genes such as TNFα, IL-6, and IL-12, the induction of Type I IFN was not affected (Takaoka *et al.*, 2005).

The MyD88-independent induction of Type I IFN mediated by TLR3 and TLR4 induced by binding of dsRNA or LPS, respectively, is stimulated by TRIF (Yamamoto *et al.*, 2003). TRIF alone can stimulate TLR3-induced signaling pathway, while in the TLR4-signaling pathway, TRIF associates with MAL (Fitzgerald *et al.*, 2003b). Mice that are TRIF-deficient (Yamamoto *et al.*, 2003) or have a mutation in the TRIF gene (Hoebe *et al.*, 2003) have a profound defect in IRF-3 activation and fail to produce IFN-β. Activation of TLR3 or TLR4 induces association of TRIF with TRAF3, and this interaction is critical for recruitment of TBK1 and phosphorylation and activation of IRF-3 and IRF-7. However, stimulation of TLR3 does not activate IRF-5 (Schoenemeyer *et al.*, 2005).

Two noncanonical IκB kinases, IKK and TBK1, which have been implicated in the phosphorylation and activation of IRF-3 and IRF-7 in infected cultured human cell lines, are also involved in TLR3- and

TLR4-mediated activation of IRF-3 and IRF-7. The activation of IRF-3 and stimulation of transcription of IFN-β and RANTES in infected, dsRNA- or LPS-treated cells does not occur in TBK1-deficient cells (McWhirter *et al.*, 2004). Rather unexpectedly, the TLR9-stimulated induction of IFN-α also requires IKKα function. However, while induction of IFN-α was impaired in IKKα-deficient pDC, induction of inflammatory cytokines was not affected (Hoshino *et al.*, 2006). Whether IKKα rather than TBK1 phosphorylates and activates IRF-7 in response to TLR9 activation in pDC or whether IKKα is required for the activation of MyD88 or TRAF6 is yet to be determined. It should be noted that TRIF can also activate the NF-κB pathway via its interaction with receptor-interacting protein-1 (RIP-1) (Meylan *et al.*, 2004). Thus, there are distinct differences between the MyD88-dependent and -independent TLR-signaling pathways, leading to the antiviral response and expression of IFN (Fig. 1). The TRIF-mediated, MyD88-independent pathway relies on TRAF3 and TBK1 for the activation of IRF-3 and IRF-7, which results in expression of both IFN-α and IFN-β. In contrast, the MyD88-dependent activation of the antiviral response employs TRAF6 for the activation of IRF-5 and IRF-7 and results predominantly in the induction of IFN-α. TLR7/8 induction of IFN-α seems to depend on both the activity of TBK1 and IKKα, but whether IKKα directly phosphorylates and activates IRF-7 and IRF-5 or another component of the signaling pathway remains to be determined (Hoshino *et al.*, 2006; Schoenemeyer *et al.*, 2005).

1. Negative Regulators of TLR

The inflammatory immune response generated by the activation of TLR-mediated stimulation of antiviral gene transcription, while critical for the host response against pathogens, may result in pathogenic manifestations when unregulated, resulting in fatal sepsis/septic shock and autoimmune disease (Cook *et al.*, 2004; Uematsu and Akira, 2006). It is therefore important to control the magnitude of the TLR response, and several distinct mechanisms have been employed to help regulate the duration of TLR-mediated response. Soluble analogues of TLR2 and TLR4 were identified that work as antagonists of the TLR (sTLR2) or block its interaction with MyD88 (sTLR4) (Hyakushima *et al.*, 2004; LeBouder *et al.*, 2003). Ubiquitin-mediated proteolytic degradation of TLR4 and TLR9 is facilitated by the ubiquitously expressed ubiquitin E3 ligase, TRIAD3A, which specifically targets these two TLRs but has no effect on TLR2 (Chuang and Ulevitch, 2004). Deubiqitination of TRAF6 by LPS-induced A20 is involved in the

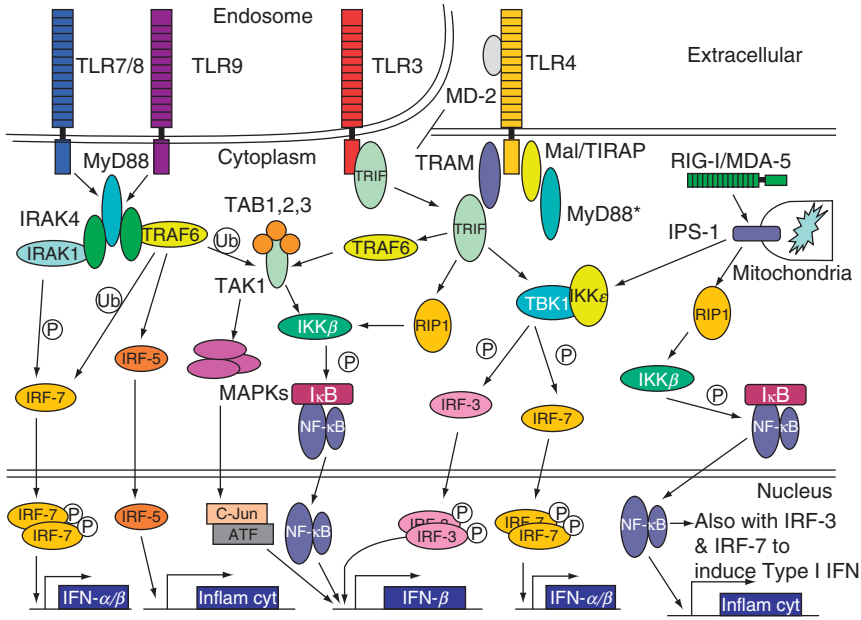

FIG 1. Mechanisms of viral detection by cellular sensors. Recognition of viral nucleic acids by TLR3, TLR7/8, and TLR9 occurs in the endosome; viral glycoproteins are recognized by TLR4 and TLR2 on the cell surface, and the cytosolic sensors RIG-I and MDA-5 recognize dsRNA replication intermediates. TLR7/8 and TLR9 activation recruits the adaptor protein MyD88 to the TIR domain. MyD88 in turn interacts with IRAK4 and TRAF6. TRAF6 activates TAK1 (via ubiquitination), and TAK1 together with TAB1, TAB2, and TAB3 can activate MAP kinases leading to the activation of ATF2/c-Jun. TAK1/TAB can also activate IKKα, IKKβ, and IKKγ/NEMO, leading to IκB phosphorylation and degradation and translocation of NF-κB to the nucleus. An additional inflammatory cytokine pathway has recently been proposed via MyD88- and TRAF6-mediated activation of IRF-5. These pathways control the expression of inflammatory cytokines. IRAK4 can also recruit IRAK1 and TRAF6 which results in activation of IRF-7, leading to Type I IFN expression. In the MyD88-independent pathway TLR3 activation recruits the adaptor protein TRIF to the TIR. TRIF can activate IRF-3 via TBK1/IKKϵ and, together with NF-κB activated via the RIP-1 pathway or via TRAF6, can mediate expression of IFN-β. TRIF and TBK1/IKKϵ can also mediate Type I IFN expression via IRF-7 activation and inflammatory cytokines through TRAF6, TAK1, and MAP kinases. TLR4 is able to signal through the same pathways as TLR3 through recruitment of TRIF by TRAM, or via MyD88-dependent pathways by recruitment of the adaptor protein TIRAP/MAL (denoted by *). The dsRNA sensors RIG-I/MDA-5 interact with IPS-1 via their CARD domains, and this interaction leads to the activation of IRF-3 and IRF-7 via TBK1/IKKϵ phosphorylation. NF-κB can be activated through the FADD and RIP-1 pathway.

negative regulation of TLR2, TLR3, and TLR4 signaling (Boone *et al.*, 2004). Constitutively expressed tumor necrosis factor-related apoptosis inducing ligand (TRAIL) inhibits TLR2-, TLR3-, and TLR4-mediated signaling by stabilization of NF-κB inhibitor IκBα (Diehl *et al.*, 2004). Another constitutively expressed protein TOLLIP inhibits TLR2 and TLR4 signaling by autophoshorylation of IRAK1 (Burns *et al.*, 2000). Phosphorylation of IRAK1 is also inhibited by the LPS-induced analogue of IRAK1, IRAKM (Kobayashi *et al.*, 2002). Some inhibitors are induced following TLR activation; LPS- and cytokine-induced SOCS1, which inhibits cytokine signaling mediated by JAK–STAT signaling cascade (Alexander and Hilton, 2004; Naka *et al.*, 1997), inhibits TLR4 signaling by inducing degradation of phosphorylation-activated MAL (Mansell *et al.*, 2006). Therefore, SOCS1 plays an important role not only in cytokine signaling but also in TLR2- and TLR4-mediated signaling. A splice variant of MyD88 (MyD88s), which lacks the death domain, inhibits TLR signaling by binding of the death domain of IRAK (Janssens *et al.*, 2003), while two TIR-containing proteins, ST2 and SIGGIR, sequester MyD88 by TIR–TIR interaction (Li and Qin, 2005). A new cellular factor, peptidylpropyl isomerase (Pin1) was shown to attenuate the transcriptional activity of activated IRF-3. The interaction of Pin1 with IRF-3 facilitated ubiquitination and proteasome-mediated degradation of IRF-3 (Saitoh *et al.*, 2006). These negative regulators block the continuous stimulation of TLR and contribute to the induction of the refractory state that protects the host from an excessive inflammation.

B. Cytoplasmic CARD Receptors

dsRNA, a replication intermediate, is a by-product of many RNA viruses, and it has long been implicated as the target of cellular sensors of viral infection and an inducer of the antiviral response (Vilcek and Kohase, 1977). After the recognition of dsRNA by TLR3 was firmly established, it also became clear that Type I IFN can be induced in cells which are not expressing TLR3 and that the first response to viral infection is not mediated by IRF-3 (Lopez *et al.*, 2004). The cytosolic receptors, RNA helicase retinoic inducible gene-I (RIG-I) and melanoma differentiation-associated antigen-5 (MDA-5/Helicard), were found to participate in the recognition of the intracellular dsRNA and serve as positive regulators of IFN gene expression (Andrejeva *et al.*, 2004; Yoneyama *et al.*, 2005). Both proteins belong to the DExD/H box helicase family of proteins and contain two caspase recruitment domains in the N-terminal region and a helicase domain in the C-terminal region. Binding of dsRNA to the helicase domain

requires ATPase activity and induces a conformational change that allows association of CARD domain with cellular adaptors. RIG-I-mediated signaling results in the activation of TBK1 and IKKϵ kinases with consequent phosphorylation and activation of IRF-3 and the IκB kinase complex formation. Another helicase, LGP2, which shows identity to the helicase domains of RIG-I and MDA-5 but lacks the CARD domain, functions as a dominant negative mutant of RIG-I and MDA-5 (Rothenfusser *et al.*, 2005; Yoneyama *et al.*, 2005). Activation by CARD domain involves its association with another CARD containing adaptor designated IPS-1 (IFN-*B* promoter stimulator) (Kawai *et al.*, 2005), also known as MAVS (mitochondrial antiviral signaling) (Seth *et al.*, 2005), VISA (virus-induced signaling adaptor) (Xu *et al.*, 2005), and Cardif (CARD adaptor inducing IFN-*B*) (Meylan *et al.*, 2005) was independently identified by four laboratories. IPS-1 (MAVS/VISA/Cardif) shows several interesting properties. This protein contains an N-terminal CARD domain and C-terminal transmembrane domain which localizes this protein to the mitochondrial membrane. It interacts with Fas-associated death domain adaptor FADD and the kinase RIP-1. Both FADD and RIP-1 were shown to play a role in virus-mediated induction of IFN-*B* (Balachandran *et al.*, 2004; Meylan *et al.*, 2004). Thus, the RIG-I/MDA-5-signaling pathway employs FADD, RIP-1, TBK1, and IPS-1 to activate IRF-3 and induce IFN-β. However, a few points require further clarification; while there seems to be some dispute as to whether IPS-1 activation of IRF-3 requires TBK1 (Kawai *et al.*, 2005; Xu *et al.*, 2005), the role of IPS-1 in the TLR3/TRIF pathway also needs further clarification (Meylan *et al.*, 2005; Xu *et al.*, 2005), leaving the mechanism by which IPS-1 activates IRF-3 unexplained.

Since dsRNA is recognized by TLR3, RIG-I, and MDA-5, the question arises whether there is specificity in the RNA recognition or whether the functions of TLR3 and cytoplasmic CARD helicases are redundant. Few differences in the signaling pathways have been recognized. The extracellular dsRNA seems to be preferentially recognized by TLR3, while the dsRNA intermediate formed during viral replication is recognized by RIG-I or MDA-5. The generation of RIG-I and MDA-5 null mice has shown further distinctions. While the majority of RIG-I-deficient mice were embryonic lethal, the MDA-5-deficient mice were healthy. RIG-I was shown to play a critical role in the virus-mediated antiviral response in majority of the cells including fibroblast, epithelial cells, and cDC, but the recognition of virus in pDC is mediated by TLR7 and TLR9 (Kato *et al.*, 2005). Furthermore, an observation from the Akira laboratory has shown that the recognition of viral infection by RIG-I and MDA-5 are distinct (Kato *et al.*, 2005).

The induction of Type I IFN was significantly impaired in RIG-I-deficient MEFs infected with the majority of RNA viruses examined, indicating that RIG-I is critical for the antiviral response induced by NDV, VSV, and SeV. In contrast, IFN induction in MDA-5-deficient MEFs was impaired only in EMCV-infected cells, indicating that MDA-5 recognition was essential in the induction of the antiviral response by a picornavirus. What determines the distinct recognition of picornavirus and paramyxovirus RNA by RIG-I and MDA-5, and whether these two helicases induce a different profile of IFN-α subtypes remains to be determined. Like the TLR pathways, RIG-I signaling has to be tightly regulated, with RIG-I-mediated activation of IRF-3 being limited by A20 and Pin1 (Boone *et al.*, 2004; Saitoh *et al.*, 2006) and by the RNA helicase LGP2 (Rothenfusser *et al.*, 2005).

C. TLR9 Independent Recognition of dsDNA

While both TLR receptors and cytoplasmic RNA helicases can recognize ssRNA and dsRNA, the recognition of foreign dsDNA has been associated with recognition by TLR9, which is expressed primarily by pDC. However, several observations suggest that dsDNA can be recognized independently of TLR9, suggesting that a new pathway, possibly analogous to the RIG-I/MDA-5 pathway, may also exist for DNA. It has been demonstrated that HSV can activate the antiviral response by both TLR9-dependent and -independent pathways in a cell type specific manner (Hochrein *et al.*, 2004). There are few differences between recognition of dsDNA by TLR9-dependent and -independent mechanisms; TLR9 recognition depends on the presence of unmethylated CpG sequences, while the cytoplasmic recognition of B-form DNA is sequence independent, but the presence of the sugar phosphate backbone is critical for recognition (Stetson and Medzhitov, 2006). While TLR9 is expressed in endosomes and activation by CpG DNA occurs in the endosomal compartment, the cytoplasmic recognition occurs in the cytosol. Cytoplasmic DNA does not activate NF-κB and MAP kinases, which are effectively induced by TLR9 (Stetson and Medzhitov, 2006). B-DNA activates IRF-3, while TLR9 activates IRF-7 and IRF-5, and both TBK1 and IKK are required for the activation of the innate antiviral response. The transcription signature induced by B-DNA shows a strong profile of interferon-induced genes. Whether this TLR9-independent, B-DNA-stimulated antiviral and inflammatory response contributes to autoimmune disorders still needs to be evaluated.

D. Virus Recognition by Cellular Sensors

Viral infection is recognized by cellular sensors and activates signaling pathways leading to the activation of IRF-3, IRF-5, and IRF-7 and an induction of the inflammatory and antiviral response. A body of experimental evidence indicates that both viral nucleic acids and glycoproteins can be recognized by the cellular receptors (Table II). However, it is not clear what determines the specificity of the recognition of viral nucleic acid or glycoprotein by a distinct TLR or CARD receptor and whether the receptors recognize both the viral genome and the replication intermediates. The herpesviruses represent a good example of the complexity of viral recognition. The DNA genomes of herpesviruses are unmethylated and contain CpG-rich motifs. HSV-1 was shown to be a very effective inducer of IFN-α in human pDC, and HSV-1 and HSV-2 induction of IFN-α was shown to be dependent on TLR9 and yet did not require HSV-1 replication (Krug *et al.*, 2004; Lund *et al.*, 2003). However, IFN-α synthesis could also be induced in human mononuclear cells by HSV-1 glycoprotein D alone (Ankel *et al.*, 1998). Furthermore, two different receptors may recognize HSV-1 in macrophages, since the induction of inflammatory cytokines is TLR9-dependent, but the induction of Type I IFN is TLR9-independent (Hochrein *et al.*, 2004). Another virus of the herpes group, Murine cytomegalovirus (MCMV) induces Type I IFN and inflammatory cytokines through recognition of TLR3 and TLR9 (Krug *et al.*, 2004), while the recognition of Human cytomegalovirus (HCMV) was through TLR2 (Compton *et al.*, 2003).

There are several cellular receptors which recognize RNA viruses. The observation that TLR3 can be activated by dsRNA and the assumption that viral dsRNA, either genomic or replication intermediate, is critical for the activation of the antiviral response lead to expectation that TLR3 would have a critical role in the recognition of RNA viruses. Therefore, the observation that VSV, MCMV, or LCMV replicated at similar levels in the TLR3-deficient and wild-type mice was unexpected (Edelmann *et al.*, 2004). There are, however, several indications that the inflammatory response induced by several viruses in CNS is mediated by TLR3. The inflammatory response induced by the Theiler's murine encephalomyelitis virus (TMEV) infection in astrocytes was dependent primarily on TLR3 and not on TLR7, yet TLR3 did not effect viral replication in these cells (So *et al.*, 2006). The infection of human neurons with *Rabies virus* leads to the expression of IFN-β and several chemokines and, since neurons expressed high levels of TLR3, the authors concluded that the induction was mediated by TLR3 (Prehaud *et al.*, 2005). The inflammatory response induced by infection

TABLE II
RECOGNITION OF VIRUSES BY CELLULAR SENSORS

Sensor	PAMP	Virus	References
TLR2	Viral glycoproteins	Measles	Bieback *et al.*, 2002
		HCMV	Compton *et al.*, 2003
		HSV-1	Kurt-Jones *et al.*, 2004
		VZV	Wang *et al.*, 2005
TLR3	dsRNA	MCMV	Tabeta *et al.*, 2004
		TMEV	So *et al.*, 2006
		Rabies	Prehaud *et al.*, 2005
		West Nile virus	Wang *et al.*, 2004
		RSV	Rudd *et al.*, 2005
		FluV	Guillot *et al.*, 2005
TLR4	Viral glycoproteins	RSV	Kurt-Jones *et al.*, 2000
		MMTV	Burzyn *et al.*, 2004
TLR7	ssRNA	VSV	Lund *et al.*, 2004
		FluV	Lund *et al.*, 2004
		Sendai virus	Melchjorsen *et al.*, 2005
		Coxsackie B virus	Triantafilou *et al.*, 2005
TLR9	DNA	HSV-1 and HSV-2	Hochrein *et al.*, 2004; Krug *et al.*, 2004
		MCMV	Krug *et al.*, 2004; Tabeta *et al.*, 2004
RIG-I	dsRNA	HCV	Sumpter *et al.*, 2005
		Sendai virus	Melchjorsen *et al.*, 2005; Yoneyama *et al.*, 2004
		VSV	Kato *et al.*, 2006
		Ebola	Cardenas *et al.*, 2006
MDA-5	dsRNA	ECMV	Kato *et al.*, 2006

VZV, Varicella-zoster virus.

with *West Nile virus* (an ssRNA virus) is initiated by TLR3. The inflammation leads to the disruption of the blood–brain barrier and the entry of the virus into the brain (Wang *et al.*, 2004). Thus in CNS, where it is expressed at high levels, TLR3 may participate in the recognition of infection by RNA viruses. Infection of lung epithelial cells with Respiratory syncytial virus (RSV) or influenza virus induced a profound inflammatory response that appeared to be TLR3-dependent (Guillot *et al.*, 2005; Rudd *et al.*, 2005). In these cells, RSV induced synthesis of IL-8 and RANTES, but only the synthesis of RANTES was mediated by TLR3; in contrast, IL-8 synthesis was found to be MyD88-dependent. However, TLR3-mediated signaling did not effect RSV replication, suggesting the absence of Type I IFN induction. On the basis of the data from TLR3 knockout mice, the role of TLR3 in the recognition of viral infection and induction of the innate antiviral response has been not generally accepted (Schroder and Bowie, 2005). However, one indicator which supports the role of the TLR3 in the antiviral response is the observation that several viruses, such as VV and HCV, target TRIF to block the antiviral response.

ssRNA that is enriched either in uridine or in guanosine residues is recognized by TLR7 or TLR8 (Heil *et al.*, 2004). However, the first indication that the ssRNA is natural ligand during the viral infection was presented by Lund *et al.* (2004) who demonstrated that the recognition of the ssRNA of VSV and influenza virus by pDC is mediated by TLR7 and results in pDC activation and induction of cytokines. Interestingly, the recognition and induction of cytokine production by SeV (a paramyxovirus) indicates cell type specificity. While in non-immune cells recognition of the SeV dsRNA is by the CARD receptor RIG-I, in myeloid cells viral ssRNA is recognized by TLR7 and TLR8 (Melchjorsen *et al.*, 2005). The cardiac inflammatory response induced by coxsackie B virus infection, which is associated with myocardial damage, is also mediated by TLR8 and TLR7 (Triantafilou *et al.*, 2005). Viral recognition is not limited to TLR, and many RNA viruses that actively replicate in the cytoplasm are recognized by the RIG-I in almost all cell types except pDC. RIG-I has a critical role in the recognition of SeV infection and activation of IRF-3 (Yoneyama *et al.*, 2004). The MEFs and mDC derived from RIG-I-deficient mice were unable to induce IFN-β, ISG, or IL-6 in response to VSV or SeV infection, yet induction of the antiviral response was unchanged in pDC (Kato *et al.*, 2005). Interestingly while the RIG-I plays a major role in the recognition of paramyxoviruses, the picornavirus EMCV was recognized by MDA-5 but not by RIG-I (Kato *et al.*, 2006). RIG-I was also shown to play a major role in induction of the antiviral response via

recognition of HCV RNA replicative intermediates in cultured hepatocytes (Sumpter *et al.*, 2005). While HCV RNA triggered IRF-3 phosphorylation and induction of IFN-β during the early stages of infection, this response was absent in cells that are permissive for HCV replication (Foy *et al.*, 2003).

Finally, the enveloped viruses can also be recognized by TLR2 or TLR4, which are the primary receptors for LPS. TLR4 was activated by the fusion protein (F) of RSV (Kurt-Jones *et al.*, 2000) and by the envelope protein of *Mouse mammary tumor virus* (MMTV) (Burzyn *et al.*, 2004), while *Measles virus* hemagglutinin protein and glycoprotein of CMV activated TLR2 (Bieback *et al.*, 2002; Compton *et al.*, 2003). Altogether these results indicate that both viral nucleic acids and glycoproteins are capable of triggering the antiviral response and that the abundance in pattern recognition may be needed to boost the antiviral response and its duration.

V. Antigen Presenting Cells: Dendritic Cells

Although Type I IFN can be induced in most infected cell types in human peripheral blood cells only two populations of cells produce Type I IFN. These are monocytes and a rare subset of human peripheral blood cells, designated natural IFN-α-producing cells, which are able to stimulate profoundly higher levels of Type I interferon in response to HSV-1 than other blood cells (Fitzgerald-Bocarsly, 1993; Fitzgerald-Bocarsly *et al.*, 1988). Further characterization of these cells revealed that these cells were $CD4^+$ $CD11c^+$ Lin^- subset of DC referred to as pDC (Cella *et al.*, 1999; Siegal *et al.*, 1999). These cells lack myeloid markers, but express high levels of IL-3 receptor α chain (CD123) and pDC specific markers such as a transmembrane protein BDCA-2 and neuropilin-1/BDCA-4 (Colonna *et al.*, 2004; Dzionek *et al.*, 2001). The same subset of pDC was also identified in mouse; however, the mouse pDC do not express CD123 but can be recognized as $CD11b^-$ $CD11c^{low}$ $B220^+$ cells that also express Ly6C. In addition, a number of novel antibodies have been generated which specifically recognize mouse pDC (Asselin-Paturel *et al.*, 2005; Blasius *et al.*, 2004). The Flt-3 ligand (Flt-3L) stimulates development of pDC *in vitro* from bone marrow precursors as well as *in vivo*, while GMCSF (for mouse) or GMCSF and IL-4 (for human) stimulate development of mDC (Brawand *et al.*, 2002; Gilliet *et al.*, 2002). The pDC differ from the mDC by their migration pattern (Kamath *et al.*, 2002; Penna *et al.*, 2002), with immature mDC being present in the peripheral tissue

and after maturation migrating to T cell zones of lymphatic tissue. In contrast, pDC are recruited to the sites of inflammation, where they become activated (Jahnsen *et al.*, 2002).

The mDC and pDC express distinct TLR repertoires and therefore recognize different sets of pathogens. While the relative levels of TLR1, TLR2, and TLR3 in mDC are high, expression levels of TLR5, TLR6, TLR8, and TLR10 are much lower. In contrast pDC express relatively high levels of TLR7, TLR8, and TLR9, but the levels of TLR1 and TLR6 are much lower (Kadowaki *et al.*, 2001). Murine pDC do not seem to express TLR8. The TLRs expressed on pDC are only those associated with the recognition of viral or bacterial DNA and viral RNA, and do not respond to bacterial components such as LPS, peptidoglycan, and extracellular dsRNA (Bauer *et al.*, 2001; Boonstra *et al.*, 2003; Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001).

The relative levels of IFN-α and the subtypes of IFN-α expressed in pDC appear to be virus-specific. While HSV-1 induced much (10–100 fold) higher levels in pDC than in mDC, the difference between the IFN levels induced by SeV in pDC and mDC was much smaller (5–10 fold) (unpublished results). Furthermore, the subtypes of IFN-*A* genes induced in SeV and HSV-1-infected pDC were distinct. In HSV-1-infected pDC, IFN-α1 was the major subtype expressed, whereas IFN-α5 was the major subtype induced by SeV. In addition, both viruses induced low levels of about five other IFN-α subtypes, some of which were distinct (Izaguirre *et al.*, 2003). Since HSV-2 and SeV are recognized by TLR9 in pDC (Lund *et al.*, 2003) or RIG-I in other cells, respectively (Melchjorsen *et al.*, 2005), these data confirm that in pDC, TLR9 recognition predominates (Kato *et al.*, 2005). High IFN-α levels can also be induced in pDC by infection with ssRNA viruses, such as VSV and influenza, being recognized by TLR7 (Diebold *et al.*, 2004; Lund *et al.*, 2004).

The reasons why the pDC are much more efficient producers of IFN-α than mDC or other cells has not been yet answered unanimously, but several factors may contribute to the high IFN synthesis in these cells (Fitzgerald-Bocarsly, 1993; Izaguirre *et al.*, 2003; Siegal *et al.*, 1999). The majority of cells that can synthesize IFN constitutively express only IRF-3; an autocrine feedback loop is required for the IRF-7-mediated transcription of IFN-α and enhancement of synthesis of IFN-β (Marie *et al.*, 1998). In contrast, pDC constitutively express high levels of IRF-7 mRNA and protein and thus are able to respond rapidly to viral infection by synthesis of IFN-α, without the requirement for the autocrine feedback loop (Coccia *et al.*, 2004; Dai *et al.*, 2004; Izaguirre *et al.*, 2003). In addition, pDC produce a broader variety of IFN-α subtypes (Izaguirre *et al.*, 2003). The degradation of

IRF-7 in pDC also seems to be attenuated (Prakash *et al.*, 2005) and while the localization of the CpG ligand in the endosomal compartment in pDC triggers IFN-α induction, in mDC this ligand is rapidly transported to lysozomes and degraded (Honda *et al.*, 2005a).

Two other IRF, IRF-4 and IRF-8, were also found to be expressed in human pDC and not in monocytes or mDC (Izaguirre *et al.*, 2003), with mouse pDC only expressing high levels of IRF-8 but not IRF-4 (Tamura *et al.*, 2003). IRF-8 has been shown to play a critical role in the development of pDC (Tamura *et al.*, 2005); however, it is not clear whether it has any role in the direct transcriptional activation of IFN genes (Tailor *et al.*, 2006). Although there is no evidence that IRF-4 directly participates in the innate antiviral response and induction of Type I IFN genes, it was identified as a negative regulator of TLR-mediated signaling (Negishi *et al.*, 2005). IRF-4 knockout mice, on TLR9 activation, produced higher levels of inflammatory cytokines than wild-type mice. In addition IRF-4 was shown to compete with IRF-5, but not with IRF-7, for the binding to MyD88 in the TLR-activated pathway. These results indicate that the activity of IRF-5 in pDC may be downregulated both by IRF-7 and by IRF-4, although at different levels. While the activated IRF-7 may compete with IRF-5 for the binding to VRE elements in the promoters of IFN-*A* genes (Barnes *et al.*, 2001), IRF-4 competes IRF-5 binding to MyD88, thus blocking its activation and transcriptional activity (Negishi *et al.*, 2005). These data indicate that the transcriptional activity of IRF-5 is under strong negative control in macrophages and DC cells.

Viral infection of pDC induces not only Type I IFN but also secretion of IL-12 and IL-6, which cooperate with Type I IFN and induces maturation of DC, enhancing their ability to present antigens and enabling pDC to serve as a link between innate and adaptive immunity (Kadowaki *et al.*, 2000; Le Bon and Tough, 2002). Maturation of DC results in the production of proinflammatory cytokines such as IL-12 and upregulation of MHC class II and costimulatory molecules (CD40, CD80, and CD86). Type I IFN and IL-12 stimulate IFN-γ production in NK cells, $CD8^+$ T cells, and $CD4^+$ T cells which results in the subsequent activation of cellular immunity. Virus-activated pDC elicit potent Th1 polarization (Cella *et al.*, 2000). Production of Type I IFN and IL-6 stimulates the differentiation of memory B cells into antibody-producing plasma cells (Jego *et al.*, 2003).

The impact of pDC on innate and acquired immunity *in vivo* is variable for different viruses. During MCMV infection, pDC are major producers of Type I IFN and IL-12 (Dalod *et al.*, 2002). Infection with MCMV induces pDC maturation and stimulates pDC activation of NK cells.

In contrast, mDC did not produce Type I IFN or activate NK cells and produced only low levels of other cytokines (Dalod *et al.*, 2003). This DC interaction with NK cells is not limited to pDCs and is vital for the establishment of an efficient early response to viral infection (Andoniou *et al.*, 2005; Andrews *et al.*, 2005). MCMV has been shown to infect DC and that this leads to functional impairment of these cells (Andrews *et al.*, 2001). The types of DC which MCMV preferentially infects *in vivo* can impact the host response to infection (Dalod *et al.*, 2003). Thus, in response to MCMV infection, pDC activated the innate immune system during the early stages of viral infection. The production of IFN-α/β was required for the maturation of $CD8^+$ DC which present MCMV antigens and stimulate $CD8^+$ T cells. During MCMV infection, pDC therefore not only stimulate innate antiviral responses but also promote the adaptive immune response. In contrast, pDC did not contribute to the IFN-α/β synthesis in mice infected with LCMV suggesting that different populations of cells stimulate the antiviral response to this virus (Dalod *et al.*, 2002).

The number of DC also seems to be modulated during some viral infections. Decreased numbers of pDC were detected during HIV-1 infection and were found to increase again during the antiviral therapy (Finke *et al.*, 2004). Hepatitis B- and C-infected individuals also show decreased numbers of pDC (Duan *et al.*, 2004; Siegal and Spear, 2001). *Measles virus* has been shown to inhibit DC development through the induction of a novel STAT2-dependent, STAT1-independent pathway (Hahm *et al.*, 2005). Interestingly, it has been shown that RSV and measles infections of human pDC cells are able to switch on both TLR-independent and -dependent (specifically TLR7 and TLR9) interferon production (Schlender *et al.*, 2005). However, RSV infection also induced IFN-mediated suppression of $CD4^+$ T cells (Chi *et al.*, 2006). Thus pDC, through the production of Type I IFN and inflammatory cytokines, direct various arms of the T cell-mediated response. Further understanding the role of pDC in the cross talk between the innate and adaptive immunity could have major implications for their clinical regulation.

A. IFN-Mediated Cross Talk Between Innate and Acquired Immune Response

The use of the mice that have defects in IFN signaling, such as IFNAR or STAT1 knockout mice have demonstrated that the major role of IFN-α/β in the antiviral defense is to modulate the immune response to viral infection (Biron, 2001). Viral infection of pDC induces not only Type I IFN but also secretion of IL-12 and IL-6, which cooperate with

Type I IFN to stimulate maturation of DC. Maturation of DC results in the induction of more proinflammatory cytokines, including IL-12, and upregulation of both MHC I and II and costimulatory molecules (CD40, CD80, and CD86). Virus-activated pDC were found to elicit potent Th1 polarization (Cella *et al.*, 2000).

The most important cellular compartment involved in the restriction of viral infection are cytotoxic $CD8^+$ T lymphocytes. Viral infection activates both MHC I -restricted $CD8^+$ T cells and MHC II-restricted $CD4^+$ T cells. Both Type I and II IFN stimulate the expression of MHC I, but only IFN-γ stimulates MHC II, and thereby increases the efficiency of the cellular immune response. MHC-restricted killing of infected cells by activated T cells is critical for recovery from viral infection (York and Rock, 1996). Both IFNAR and STAT1 knockout mice show a strong defect in basal and inducible expression of MHC class I (Lee *et al.*, 1999). On the part of the virus, mutations in the CTL epitopes of viral proteins known as antigenic drift (e.g., NP protein of *Influenza A virus*) also inhibits MHC I presentation and allows escape from recognition by CTL (Voeten *et al.*, 2000). Antigenic drift that allows escape from CTL surveillance is characteristic of viruses that cause chronic infections such as HCV and HIV-1 (McMichael and Phillips, 1997; Weiner *et al.*, 1995). In mice, IFN-α/β control T cell-mediated viral clearance in acute cutaneous and ocular infection with HSV-1 and in poxvirus infection. In macrophages, IFN-γ inhibits HSV-1 and VV replication by inducing the production of NO in infected cells (Karupiah *et al.*, 1993). The growth of mouse hepatitis virus is restricted in mice by IFN-γ-activated macrophages (Vassao and Pereira, 1994). Intraperitoneal infection with MCMV or HSV-1 results in inflammatory infiltrates, consisting mainly of macrophages with increased expression of IFN-α/β and TNFα. *In vitro* studies also suggest that in addition to Type I IFN, IL-15 and NK cells play an important role in the antiviral response to HSV-1 infection (Mossman and Ashkar, 2005). CMV titers are increased in IFN-α-depleted mice and in mice with severe combined immunodeficiency (SCID) (Heise and Virgin, 1995). The antiviral effect of IFN-α has also been demonstrated by the observation that the replication of recombinant VV expressing IFN-α is markedly inhibited compared to that of wild-type virus (Ramshaw *et al.*, 1992).

In addition to the antiviral effect against a large number of viruses, human Type I IFN promotes the differentiation of DC (Santini *et al.*, 2000), promotes NK cell cytotoxicity, upregulates IFN-γ expression, and stimulates DC-dependent and -independent differentiation of B cells (Biron, 2001). IFN-α was also shown to induce differentiation of human monocytes to DC, which were able to induce Th1 polarization both *in vitro* and *in vivo* (Santini *et al.*, 2000). Furthermore, Type I IFN were

found to protect $CD8^+$ T cells from antigen-induced cell death (Marrack *et al.*, 1999) and to stimulate clonal expansion of antigen-specific $CD8^+$ T cells in response to viral infection (Kolumam *et al.*, 2005). Thus while in the past, the IFN system has only been considered as a part of host innate immunity, data indicate that activation of Type I IFN represents a defense mechanism that bridges innate and T cell-mediated immunity (Biron, 2001).

VI. Viral Evasion of the Interferon Response

In order to replicate and establish infection, viruses have developed different strategies for evading the host innate immune response (summarized in Tables III and IV). The major replicative intermediate for many viruses, dsRNA, is recognized by a number of cellular sensors resulting in activation of signaling pathways and production of IFN-β, as well as many other ISGs, which then coordinate the antiviral response. Many viruses encode proteins which specifically inhibit the interferon pathways (Fig. 2). The majority of these inhibitors are encoded by viral nonstructural genes. Many of these genes are vital for viral pathogenesis. Infection by mutant viruses lacking these genes is often attenuated when faced with a functional interferon response in the host. The ever-growing list of virus-encoded interferon inhibitory proteins has been found to interfere at nearly every level of the interferon response. Many of these proteins are multifunctional and serve as interferon inhibitors at multiple levels. Indeed, major pathogens for humans including *Hepatitis C virus* (HCV), herpesviruses, and paramyxoviruses tend to encode multiple antagonists of the IFN pathway to increase their chances of successfully establishing infection. Here, we will discuss how viruses inhibit the innate immune response generated against them in order to establish infection and permit viral replication.

A. Inhibition of Type I IFN Induction

Viral recognition begins at the level of TLRs and CARD helicases recognizing viral DNA or RNA. dsRNA is a replication by-product of many viruses that can be detected by the host TLR or cytoplasmic sensors, leading to the induction of the IFN-α/β. To combat this recognition, both the NS1 protein of influenza virus (Li *et al.*, 2004) and E3L protein of VV (Davies *et al.*, 1993) are able to bind and sequester dsRNA to prevent recognition of dsRNA by the cytosolic sensors RIG-I or MDA-5 and to

TABLE III
INHIBITORY FACTORS ENCODED BY DNA VIRUSES

Virus	Viral protein	Target	References
AdV	VA	Inhibition of PKR	Kitajewski *et al.*, 1986
	E1A	Inhibition of STAT1	Look *et al.*, 1998
		Inhibition of IRF-3 via binding of p300/CBP	Juang *et al.*, 1998
CMV	IE1	Blocks ISGF3 association with chromatin	Paulus *et al.*, 2006
	?	Downregulates induction of IRF-1 and IRF-4	Browne *et al.*, 2001
EBV	BZLF-1	Inhibition of IRF-7	Hahn *et al.*, 2005
	EBER	Binding of PKR	Elia *et al.*, 1996
	LMP-1	Regulation of STAT1 phosphorylation via NF-κB	Najjar *et al.*, 2005
HPV16	E6	Inhibition of IRF-3	Ronco *et al.*, 1998
	E7	Inhibition of IRF-1 through histone deacetylase	Park *et al.*, 2000
HPV18	E6	Inhibition of Tyk2, STAT1, and STAT2 phosphorylation	Li *et al.*, 1999
	E7	Inhibition of IRF-1	Um *et al.*, 2002
HSV-1	ICP27	Inhibition of IRF-3 and NF-κB	Melchjorsen *et al.*, 2006
	ICP0	Blocks IRF-3- and IRF-7-mediated activation of ISGs	Lin *et al.*, 2004
	UL13 and UL41	Induction of SOCS3	Yokota *et al.*, 2004, 2005
HSV-2	vhs	Regulation of IFN-α/β in a non-PKR-dependent pathway	Murphy *et al.*, 2003

KSHV	vIRF-1	Binds cellular IRFs	Burysek *et al.*, 1999
		Blocks IRF-3 recruitment of p300/CBP	Lin *et al.*, 2001
	vIRF-2	Inhibits PKR activation	Burysek and Pitha, 2001
		Inhibits IRF-1 and IRF-3 activation of IFN-β	Fuld *et al.*, 2006
	vIRF-3	Inhibits NF-κB nuclear translocation	Seo *et al.*, 2004
		Blocks IRF-3 and IRF-7 activation	Lubyova and Pitha, 2000
	vIRF-4	Blocks IRF-3 and IRF-7 activation	Fuld *et al.*, 2006
	RTA	Targets IRF-7 for degradation	Yu *et al.*, 2005
VV	E3L	dsRNA sequestration	Davies *et al.*, 1993
		Block of IRF-3 and IRF-7 phosphorylation	Smith *et al.*, 2001
		Inhibits RNase L 2′,5′-OAS system	Xiang *et al.*, 2002
	K3L	Inhibition of PKR	Carroll *et al.*, 1993
	B18R	Soluble and cell surface receptor for Type I IFN	Alcami *et al.*, 2000
	A46R	TIR homologue interfering with MyD88, TRIF, and TRAM	Stack *et al.*, 2005
	A52R	TIR homologue blocking NF-κB via IRAK2 and TRAF6	Harte *et al.*, 2003

TABLE IV
INHIBITORY FACTORS ENCODED BY RNA VIRUSES

Virus	Viral protein	Target	References
Ebola	VP35	Inhibits activation of IRF-3	Basler *et al.*, 2003
FluV	NS1	Inhibits IRF-3 activation	Talon et al, 2000
		Inhibits NF-κB activation	Wang *et al.*, 2000
		dsRNA sequestration	Li *et al.*, 2004
HCV	NS5a	Inhibition of IRF-1 and PKR	Pflugheber *et al.*, 2002
	E1	Inhibition of NF-κB nuclear translocation	Lasarte *et al.*, 2003
	NS3/4a	Inhibition of IRF-3	Foy *et al.*, 2003
		Binding of TBK1	Otsuka *et al.*, 2005
		Cleavage of TRIF	Li *et al.*, 2005a
		Degradation of STAT1	Lin *et al.*, 2005
		Cleavage of IPS-1	Meylan *et al.*, 2005
	Core	Degradation of STAT1	Lin *et al.*, 2005
		Induction of SOCS3	Bode *et al.*, 2003
Hendra virus	V	Prevents STAT1 and STAT2 nuclear translocation	Rodriguez *et al.*, 2003
HPiV2	V	STAT2 degradation	Parisien *et al.*, 2001

Measles	V	Interferes with STAT1–STAT3 phosphorylation	Palosaari *et al.*, 2003
Mumps	V	Inhibition and degradation of STAT1	Kubota *et al.*, 2005
		Degradation of STAT3	Ulane *et al.*, 2003
Nipah virus	V	Prevents STAT1 and STAT2 nuclear translocation	Rodriguez *et al.*, 2002
	V, W, P	Binding of STAT1 in nucleus and cytoplasm	Shaw *et al.*, 2004
Paramyxovirus	V	Inhibition of MDA-5	Andrejeva *et al.*, 2004
Polio	3A	Inhibition of IFN-β secretion	Dodd *et al.*, 2001
	3C	Cleavage of NF-κB p65-RelA subunit	Neznanov *et al.*, 2005
Rabies	P	Inhibition of TBK1 phosphorylation of IRF-3	Brzozka *et al.*, 2005
		Retention of phosphorylated STAT1/2 in cytoplasm	Brzozka *et al.*, 2006
Rhinovirus 14	?	Retardation of IRF-3 dimerization	Peng *et al.*, 2006
RSV	NS1/NS2	Inhibition of IFN induction	Spann *et al.*, 2004
	NS2	Degradation of STAT2	Ramaswamy *et al.*, 2006
SARS	?	Inhibition of IRF-3 activation	Speigel *et al.*, 2004
VSV	M	TFIID inhibition leading to suppression of IFN-β transcription	Yuan *et al.*, 1998

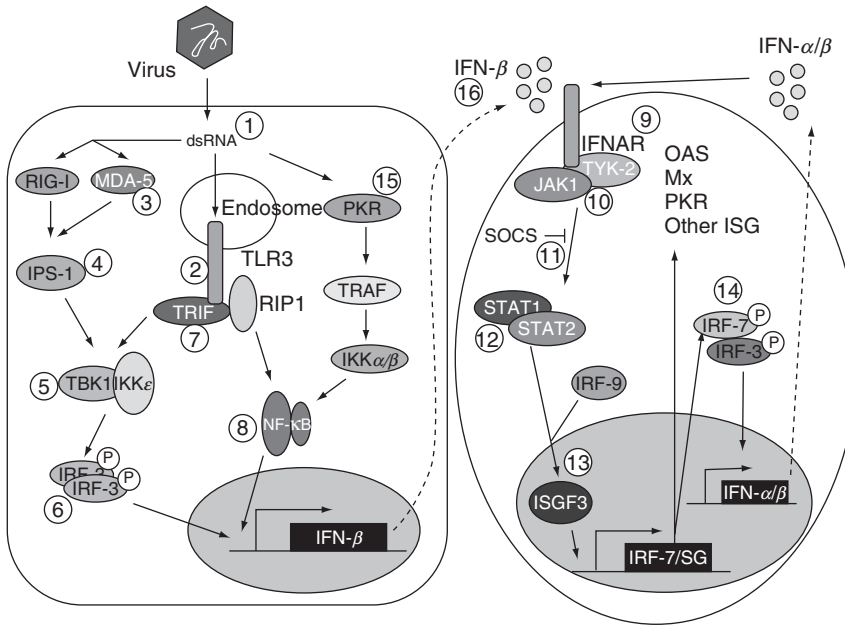

FIG 2. Viral inhibition of the innate interferon response. Viral inhibition of the antiviral response occurs at several levels both during the induction of IFN-β and the subsequent induction of ISG. The site of action of viral inhibitors from a number of viruses is illustrated on the virus-induced IFN-α/β response loop. 1: dsRNA: FluV, VV, SeV. 2: TIR mimicking: VV. 3: MDA-5 inhibition: paramyxoviruses. 4: IPS-1 inhibition: HCV. 5: TBK1/IKKϵ inhibition: SeV, Ebola virus, rotavirus, FluV, HCV. 6: IRF-3 inhibition: *Rhinovirus*, HPV 16, KSHV, AdV. 7: TRIF degradation: HCV. 8: NF-κB inhibition: FluV, KSHV, *Poliovirus*. 9: IFNAR: VV. 10: JAK1/Tyk2 inhibition: HPV18, *Measles virus*. 11: SOCS3 induction: HCV, HSV-1. 12: STAT inhibition: HCV, *Mumps virus*, *Nipah virus*, RSV, parainfluenza virus 2, *Hendra virus*, LCMV. 13: Interference with ISGF3: CMV. 14: IRF-7 inhibition: KSHV, EBV, VV. 15: PKR inhibition: HCV, EBV, KSHV, VV, reovirus, AdeV, FluV, HIV-1. 16: IFN-β secretion: *Poliovirus*.

block the antiviral response (Talon *et al.*, 2000). Rather than sequestering dsRNA, the C and V proteins of SeV inhibit the production of viral dsRNA (Komatsu *et al.*, 2004; Strahle *et al.*, 2006). As an alternative strategy to removal of the pathogen-associated ligands of these receptors, some viruses directly interfere with the function of the receptors. The VV proteins A46R and A52R both have homology to the TIR domain of TLRs and can compete for the binding of downstream adaptor molecules, including IRAK2, TRAF6, MyD88, TRIF, and TRAM, to the intracellular domain of multiple TLRs (Harte *et al.*, 2003; Stack *et al.*, 2005). This results in interruption of TLR signaling and attenuation of the IFN

response. The V protein of several paramyxoviruses including *Human parainfluenza virus 2*, SeV, *Hendra virus*, and *Mumps virus* were shown to bind MDA-5, resulting in inhibition of IFN-β induction by IRF-3 in response to dsRNA (Andrejeva *et al.*, 2004). Virally encoded inhibitors have also been shown to interfere signaling through interaction with IPS-1 (MAVS/Cardif/VISA), which is the downstream adaptor molecule of both RIG-I and MDA-5 (Kawai *et al.*, 2005). The HCV serine protease NS3/4A is able to cleave IPS-1 in a site-specific manner, removing its association with the mitochondrial membrane (Li *et al.*, 2005b; Meylan *et al.*, 2005), which interrupts the RIG-I-signaling pathway (Sumpter *et al.*, 2005). The NS3/4a protease is also able to cleave the TLR3 and TLR4 adaptor TRIF, allowing the virus to inhibit the TLR response before the activation of IRF-3 and NF-κB (Li *et al.*, 2005a). Thus, the disarmament of the antiviral signal induced by RIG-I or TLR during the initial stages of HCV infection enables the virus to replicate and is vital for viral immune evasion.

IRF-3 is an integral part of the early innate response. Signal transduction pathways from both TLRs and CARD helicases activate IRF-3 and NF-κB in order to induce IFN-β transcription and begin the IFN cascade to combat infection. Therefore, the activation of IRF-3 is crucial, and a number of viruses encode proteins which either inhibit IRF-3 activation or interfere with the transcriptional activity of IRF-3. The NS3 protein of HCV binds directly to TBK1 and inhibits it from binding to and subsequently activating IRF-3 (Otsuka *et al.*, 2005). Phosphorylation of IRF-3 by TBK1 is also inhibited by the P proteins of *Rabies virus* and *Borna disease virus* (Brzozka *et al.*, 2005; Unterstab *et al.*, 2005).

A number of other viruses also encode inhibitors of IRF-3 activation. The VP35 protein of Ebola virus has dsRNA-binding activity which appears to contribute to the antagonist function of VP35 and to impair signaling downstream of TBK1 and IKK (Basler *et al.*, 2003; Cardenas *et al.*, 2006; Hartman *et al.*, 2004). The Kaposi's sarcoma-associated herpesvirus (KSHV) contains a cluster of four genes that encode proteins with homology to cellular IRFs (vIRF) (Russo *et al.*, 1996), some of which function as dominant negative mutants of cellular IRFs and inhibit the antiviral response. Both vIRF-1 and vIRF-3 are able to inhibit IRF-3 activation (Burysek *et al.*, 1999; Fuld *et al.*, 2006; Lubyova and Pitha, 2000). In addition, vIRF-1 also inhibits the recruitment of p300/CBP by IRF-3 required for the IRF-3-mediated transcriptional activation (Burysek *et al.*, 1999; Lin *et al.*, 2001). *Rhinovirus* is able to severely retard IRF-3 dimerization in order to prevent its transcriptional activity (Peng *et al.*, 2006). The direct binding of the E6 protein

of HPV16 to IRF-3 also inhibits IRF-3 activity (Ronco *et al.*, 1998). Furthermore, it was shown that the E1A protein of adenovirus (AdV) can prevent recruitment of p300/CBP by IRF-3 and thus inhibits its transcriptional activity (Juang *et al.*, 1998).

Other cellular IRFs have also been defined as targets of viral inhibitory factors. IRF-1 is targeted by vIRF-1 (Fuld *et al.*, 2006), the E7 protein of the pathogenic human papillomaviruses 16 and 18 (Park *et al.*, 2000; Um *et al.*, 2002), NS5a of HCV (Pflugheber *et al.*, 2002), and HCMV (Browne *et al.*, 2001), resulting in a decrease in IFN-β synthesis in infected cells. IRF-7, which has been defined as the master regulator for controlling IFN-α synthesis, is also the target of a number of viral inhibitors; vIRF-1 of KSHV (Burysek *et al.*, 1999) and BZLF-1 of EBV which prevent IRF-7 activation (Hahn *et al.*, 2005) and inhibit its transcriptional activity, while RTA of KSHV, which functions as a ubiquitin E3 ligase, targets IRF-7 for proteasome-mediated degradation (Yu *et al.*, 2005). Both VV E3L (Smith *et al.*, 2001; Xiang *et al.*, 2002) and KSHV vIRF-4 (Fuld *et al.*, 2006) inhibit the phosphorylation of both IRF-3 and IRF-7, allowing the virus to inhibit the initial IFN-β response and the subsequent secondary IFN-α response.

The other transcription factor required for the induction of IFN-β as well as any inflammatory response is NF-κB, and viruses have developed strategies to inhibit the activation and action of this transactivator. Influenza NS1 inhibits the activation of NF-κB (Donelan *et al.*, 2004; Wang *et al.*, 2000), while KSHV vIRF-3 (Seo *et al.*, 2004) and E1 of HCV (Lasarte *et al.*, 2003) prevent NF-κB nuclear translocation. The *Poliovirus* 3C viral protease cleaves the p65-RelA subunit of NF-κB (Neznanov *et al.*, 2005).

B. Inhibition of Type I IFN Signaling

The viral proteins we have described so far inhibit the induction of the IFN-induced antiviral response generated during the early stages of viral infection. However, viral inhibitors can also inhibit the IFN-signaling pathway and induction of ISGs. Interestingly, some viruses target both the initial IFN-β induction as well as the subsequent IFN signaling and amplification feedback loop in order to attenuate the antiviral response.

The large DNA viruses encode proteins that can compete for the binding of IFN and other cytokines to cellular receptor and thus prevent the cytokine-mediated signaling. VV encodes the B18R protein which shares homology with the IFNAR. This protein inhibits binding

of IFN-α/β to its receptors both in infected and uninfected cells, thus preventing the autocrine amplification of IFN and blocking induction of the antiviral state in both infected and uninfected cells (Alcami *et al.*, 2000; Colamonici *et al.*, 1995; Symons *et al.*, 1995). As a consequence, the spread of viral infection is greatly facilitated.

Binding of Type I IFN activates two JAK kinases associated with IFNAR. One of the kinases associated with the IFNAR is Tyk2, and the E6 protein of HPV18 binds to the same site of the IFNAR as Tyk2 and consequently prevents phosphorylation of STAT1 (Li *et al.*, 1999). JAK1 is another IFNAR-associated kinase, the autophosphorylation of which is inhibited by the V protein of *Measles virus* leading to a reduced response to IFN-α signaling (Yokota *et al.*, 2003). An alternative approach to the impairment of IFN-α signaling is through the induction of the suppressor of cytokine-signaling protein, SOCS3, which is induced by core of HCV (Bode *et al.*, 2003). Both the UL13 and UL41 proteins of HSV-1 also function to downregulate STAT phosphorylation through induction of SOCS3 (Yokota *et al.*, 2004, 2005).

Activation of STATs by phosphorylation is essential for their homo- and heterodimerization. Phosphorylated STAT1 and STAT2 complex with IRF-9 to form ISGF3 which activates transcription of ISG containing an ISRE in their promoters. Many viruses target STATs as a way of inhibiting the interferon response (Horvath, 2004). STAT1 is degraded by a proteasome-dependent pathway by a number of viral proteins. These include two Hepatitis C-encoded proteins, core and NS3/4a (Lin *et al.*, 2005), and the V protein of *Mumps virus* (Lin *et al.*, 2005), whereas the V and W proteins of *Nipah virus* bind STAT1 in the nucleus and cytoplasm and prevent its binding with STAT2 and IRF-9 (Shaw *et al.*, 2004). STAT2 is targeted for degradation by the NS2 protein of RSV (Lo *et al.*, 2005; Ramaswamy *et al.*, 2004, 2006) and by the V protein of *Human parainfluenza virus 2* (Parisien *et al.*, 2001; Ulane *et al.*, 2005). Indeed, some viruses are able to target both STAT1 and STAT2 simultaneously. The V proteins of *Nipah virus* (Rodriguez and Horvath, 2004; Rodriguez *et al.*, 2002) and *Hendra virus* (Rodriguez *et al.*, 2003) and the P protein of *Rabies virus* (Brzozka *et al.*, 2006) are all able to prevent the nuclear translocation of both STAT1 and STAT2. *Mumps virus* is able to target STAT3 for degradation via the V protein (Ulane *et al.*, 2003, 2005) and *Measles virus* V protein is also able to interfere with the phosphorylation of STAT1, STAT2, and STAT3 and formation of the ISGF3 complex (Palosaari *et al.*, 2003; Takeuchi *et al.*, 2003; Yokota *et al.*, 2003). In contrast, binding of the IE1 protein of CMV to both STAT1 and STAT2 allows the ISGF3 complex to be assembled but interferes with the association between ISGF3 and chromatin, leading to partial inhibition

of the interferon response (Paulus *et al.*, 2006). STAT1 modulation by viral inhibitors has also been shown to affect the $CD8^+$ T cell response to LCMV infection (Gil *et al.*, 2006).

A third intracellular protein activated by dsRNA is protein kinase R (PKR), and the functions of this protein are targeted by many viral inhibitors. The NS5a protein of HCV (Pflugheber *et al.*, 2002) and EBER protein of EBV (Elia *et al.*, 1996) both inhibit PKR activation. KSHV-encoded vIRF-2 also interacts with PKR, preventing both autophosphorylation, which activates PKR, as well as phosphorylation of its downstream targets such as eIF-2α (Burysek and Pitha, 2001). Both the K3L protein of VV (Carroll *et al.*, 1993; Davies *et al.*, 1993) and VA of AdV (Kitajewski *et al.*, 1986) also inhibit PKR phosphorylation of eIF-2α. Reovirus-encoded protein sigma3 binds dsRNA and inhibits the activation of PKR by competing for dsRNA (Imani and Jacobs, 1988). PKR is also inactivated in cells infected with influenza virus by a cellular inhibitor of PKR, p58(IPK), which is induced by cellular stress or influenza virus infection (Gale *et al.*, 2002; Lee *et al.*, 1990). Inhibition of PKR activity by HIV-1 has also been suggested (Roy *et al.*, 1990).

The end effect of the functions of all of these viral inhibitors is a downregulation or even a complete suppression of the innate interferon response in virus-infected cells. This provides an environment that is permissive to virus replication and allows infection, whether acute, chronic, or latent, to be established.

Viruses have also developed other methods for manipulating the innate immune response to provide an optimal environment for their replication. Both IFN-α/β and STAT1 have been shown to be required for the induction of NK cell cytotoxicity (Nguyen *et al.*, 2002a; Roy *et al.*, 1990). Indeed, Type I IFN has been shown to be vital in activating STAT4 to induce IFN-γ in LCMV infection (Nguyen *et al.*, 2002b). Chemokines induced by IFN such as MIP-1α and MCP-1 are vital for the recruitment of immune cells to the site of infection. A lack of infiltrating lymphocytes at the site of infection can result in an increase in viral replication and pathogenicity as demonstrated by infection of MCP-1-deficient mice with MCMV (Hokeness *et al.*, 2005).

Many herpesviruses are able to establish latent infection and to achieve this must manipulate their host environment. The expression of latent membrane protein of EBV (LMP-1), a viral oncogene, is controlled by the viral protein EBNA-2. It has been shown that transcription of LMP-1 can be induced by IRF-7 binding to the ISRE in the LMP-1 promoter. Subsequently LMP-1 expression stimulates induction of IRF-7, thus generating a regulatory circuit (Ning *et al.*, 2003). It has also

been shown that IRF-5 can break this circuit, which leads to a reduction in LMP-1 expression (Ning *et al.*, 2005). Interestingly, LMP-1 has also been implicated in the maintenance of EBV latency through priming of latent cells for Type I IFN production in an NF-κB-, and possibly IRF-7-dependent manner (Xu *et al.*, 2006). Thus, IFN protects cells against an exogenous infection and may contribute to the latency of EBV in these cells.

HCV is also able to manipulate cells in an IFN-dependent manner. HCV-encoded NS5b delays cell cycle progression through the induction of Type I IFN, even in the absence of replicating viral genomes (Naka *et al.*, 2006). The NS3/4a serine protease of HCV protects infected hepatocytes from TNFα-mediated liver disease in a MAPK kinase-dependent manner (Frelin *et al.*, 2006). Both mechanisms allow for immune evasion, giving the virus additional time to establish and maintain infection. *Poliovirus*-encoded 3A inhibits protein secretion through the inhibition of ER–Golgi traffic. It not only reduces Type I IFN synthesis but also synthesis of IL-6, IL-8, and the levels of TNF receptor on the cell surface (Choe *et al.*, 2005; Dodd *et al.*, 2001; Neznanov *et al.*, 2001). Perhaps most important, for the virus to establish infection *in vivo* is the ability of the 3A protein to reduce MHC I expression, resulting in a reduction in the level of viral antigen presented to $CD8^+$ T cells and reduced CTL activity against infected cells (Choe *et al.*, 2005; Deitz *et al.*, 2000).

VII. Conclusions and Perspectives

Since the discovery of interferon and its antiviral effects in 1957 by Isaacs and Lindenmann, it has been only more recently that the importance of the innate antiviral response in the modulation of viral infection and pathogenicity has been fully recognized. The identification of TLR receptors and the CARD helicases as the cellular sensors of viral infection have enabled researchers to begin unraveling virus recognition and the signaling pathway leading to the induction of the antiviral and inflammatory responses in molecular terms. The availability of different strains of genetically modified mice and the ongoing study of both the myeloid cell compartment and DC further our understanding of how the immune system recognizes and coordinates a response against an infecting pathogen. Conversely, the study of virally encoded factors that have evolved to combat the innate immune response has revealed a multitude of proteins that are able to inhibit the interferon response at virtually every level. The finding that overcoming the innate immune response can have

dramatic effects on the subsequent adaptive immune responses was very revealing. Thus the ability to inhibit the interferon response has a large impact on the ability of a virus to establish and maintain infection, whether it be acute, chronic, or latent. Improved knowledge of both the innate immune response and the viral inhibitors which target it, will lead to new possibilities for development of vaccines and drugs not only for the elimination of viral infections but potentially also for controlling autoimmune and inflammatory diseases.

Acknowledgments

We thank K. Fitzgerald and our colleagues in the interferon field for helpful discussions on the subjects covered. This article is focused largely on the molecular mechanisms of the antiviral response. This is a rapidly expanding area, and we apologize to authors whose work we were unable to reference due to space limitations. Our own unpublished data that have been discussed were supported by National Institutes of Health grants to P.M.P.

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ADVANCES IN VIRUS RESEARCH, VOL 69

APOPTOSIS DURING HERPES SIMPLEX VIRUS INFECTION

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ABSTRACT

Herpes simplex virus (HSV) infection triggers apoptosis in infected cells. However, proteins synthesized later in infected cells prevent apoptotic cell death from ensuing. *In vivo* data showing that apoptosis accompanies herpes stromal keratitis and encephalitis suggest that apoptotic modulation plays a role in the development of herpetic disease. Tremendous progress has been made toward identifying the viral factors that are responsible for inducing and inhibiting apoptosis during infection. However, the mechanisms whereby they act are still largely unknown. Recent studies have illustrated a wide diversity in the cellular response to HSV-triggered apoptosis, emphasizing the importance of host factors in this process. Together, these findings indicate that apoptosis during HSV infection represents an important virus–host interaction process, which likely influences viral pathogenesis.

0065-3527/07 $35.00
DOI: 10.1016/S0065-3527(06)69002-7

I. Background

A. Apoptosis

Apoptosis is an energy-dependent, organized form of cell self-destruction. It was initially distinguished from other forms of cell death due to the unique morphological features displayed by the dying cells. These features include chromatin condensation, membrane blebbing, and maintenance of organelle integrity (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Certain biochemical features are now known to be involved in apoptosis. These include changes in the nucleus, cytoplasm, and membranes of apoptotic cells (Fig. 1). In the nucleus, apoptotic cells cut their DNA into subgenomic fragments (Wyllie *et al.*, 1980). This DNA fragmentation can be detected using electrophoresis or the terminal deoxynucleotidyl transferase-mediated incorporation of labeled nucleotides at DNA breakage sites (TUNEL assay) (Gavrieli *et al.*, 1992). Key mediators of apoptosis include a specific group of proteases, called caspases (Alnemri *et al.*, 1996). The caspases are synthesized as inactive precursor molecules called procaspases. When the N-terminal prodomain is cleaved from the precursor molecule, the active caspases are ready to cleave their substrates. Common methods of measuring caspase activation include immunoblot detection of the procaspase and active caspase forms and cleavage of endogenous or fluorescently labeled synthetic substrates. During apoptosis, the plasma membrane changes to allow exposure of phosphatidylserine (PS) molecules to the exterior of the cell (Fadok *et al.*, 1998). Apoptotic cells containing this surface-exposed PS can then be detected by staining with Annexin V (Koopman *et al.*, 1994; Vanags *et al.*, 1996). Exposed PS triggers nearby phagocytes or other surrounding cells to engulf and degrade the apoptotic corpse. This prevents the release of cellular contents into the extracellular space, which would, otherwise, elicit an inflammatory response.

Apoptosis is vital to homeostasis in multicellular organisms. Therefore, it is not surprising that it is controlled by multiple molecular networks. Depending on the nature of the death signal, cells activate apoptotic events through two major pathways (Green, 1998; Nagata, 1997; Sun *et al.*, 1999). The extrinsic apoptotic pathway is initiated by ligand binding to a death receptor, such as TNFα (Ashkenazi and Dixit, 1998). The active ligand–receptor complex recruits adaptor molecules that enables the binding, autocleavage, and activation of procaspase 8. This, in turn, leads to the cascade of events culminating in the activation of the effector caspases, which cleave cellular proteins

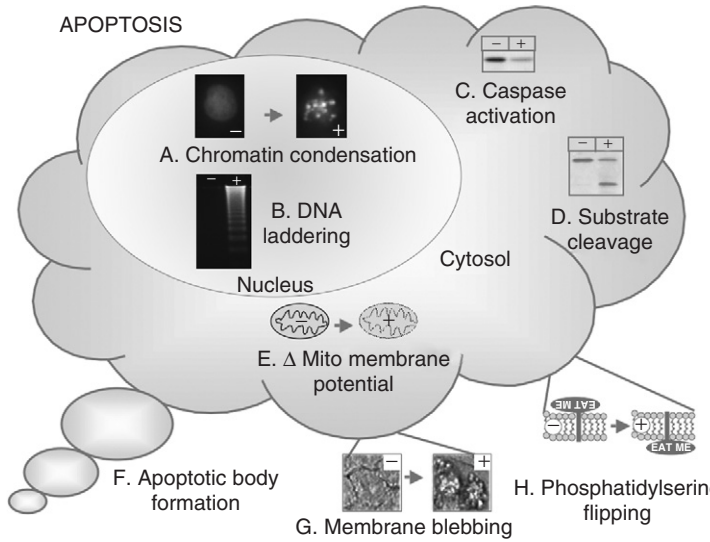

FIG 1. Apoptotic cells exhibit distinctive features in the nucleus, cytoplasm, mitochondria, and plasma membrane. (A) Use of a fluorescent DNA dye allows visualization of cellular chromatin changes accompanying apoptosis. In a nonapoptotic cell (−), chromatin is stained homogeneously throughout the nucleus. However, nuclei of apoptotic cells (+) appear smaller and brighter (reflecting condensed chromatin) and have a punctate staining pattern. (B) During apoptosis, DNA is cleaved between nucleosomes leading to an ~200 base pair ladder-like pattern when it is extracted from the cells and separated by gel electrophoresis. (C) When caspases are activated, a prodomain is cleaved from the procaspase. Using an antibody specific for the procaspase form, activation can be seen as a loss of the precursor form. (D) Caspase substrate cleavage may be assessed by monitoring the disappearance of the full-length substrate and the appearance of the cleaved product via immunoblot. (E) Mitochondrial membrane permeability is increased during apoptosis. This allows for ion exchange and causes the potential between the inner and outer mitochondrial membrane to be disrupted. (F and G) The plasma membrane of apoptotic cells exhibits protrusions, commonly referred to as blebs. (F) Blebs eventually pinch off from the cell producing membrane-bound apoptotic bodies. (H) Flipping of the orientation of PS molecules on the plasma membrane serves as a signal (EAT ME) for nearby cells to phagocytose the apoptotic corpse.

important for cell maintenance. Known substrates of effector caspases include the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP) (Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994), structural elements, lamin B and actin (Lazebnik *et al.*, 1995; Mashima *et al.*, 1995), and the DNase inhibitor DNA fragmentation factor 45, DFF-45 (Liu *et al.*, 1997). Cleavage of these targets results in the morphological and biochemical changes distinctive of apoptotic cell death.

The intrinsic apoptotic pathway is triggered by signals initiating within the cell (Green and Evan, 2002). Examples of intrinsic apoptotic inducers include DNA damage, oxidative stress, and viral infection. The apoptotic signal is transmitted to the mitochondria resulting in changes of the relative abundance of Bcl-2 family member proteins, which causes disruption of the mitochondrial membrane potential and release of cytochrome *c* into the cytoplasm (Green, 1998; Petit *et al.*, 1996). Cytochrome *c* forms a complex with Apaf-1 that recruits and activates caspase 9 (Green, 1998; Sun *et al.*, 1999). Like caspase 8, caspase 9 induces a signaling cascade, which causes the processing of downstream effector caspases. Thus, the intrinsic and extrinsic apoptotic pathways converge at this point.

B. Herpesviruses and Apoptosis

A common cellular defense mechanism against viral invasion is the elimination of infected cells through apoptotic cell death (reviewed in Koyama *et al.*, 2000). If the infected cell undergoes apoptosis before the virus produces progeny virions, virus replication will be limited. Therefore, many viruses, including herpesviruses, have evolved apoptosis evasion strategies. The *Herpesviridae* family is divided into three subfamilies (α, β, and γ) based on biological properties such as host range and length of reproductive cycle. Members of each herpesvirus subfamily are known to encode antiapoptotic genes. One γ herpesvirus, Kaposi's sarcoma-associated virus, produces a viral homologue of FLICE-inhibitory proteins, vFLIP. vFLIP blocks protease activities of caspases 3, 8, and 9 (Belanger *et al.*, 2001; Djerbi *et al.*, 1999; Thome *et al.*, 1997). The *Epstein-Barr virus* encodes two genes, which possess antiapoptotic activities, EBERs and EBNA1 (Kennedy *et al.*, 2003; Nanbo *et al.*, 2002). One β herpesvirus, human cytomegalovirus, produces vICA and vMIA that inhibit caspase 8 and the proapoptotic Bcl-2 family member, Bax, respectively (Arnoult *et al.*, 2004; Poncet *et al.*, 2004; Skaletskaya *et al.*, 2001). Among the α herpesviruses, apoptosis modulation is most well defined for Herpes simplex virus 1 (HSV-1). This chapter will summarize key studies which have contributed to our current knowledge of apoptosis during HSV-1 infection.

C. Herpes Simplex Virus Life Cycle

HSV-1 is well known as the causative agent of cold sores and other orofacial lesions. Under certain circumstances, HSV-1 infection can also cause ocular, brain, and disseminated viral disease (reviewed in

Whitley, 2001). Initial infections generally occur in toddlers and young children and over two-thirds of the US population are infected by adulthood (Schillinger *et al.*, 2004). Primary infections may be asymptomatic or lead to the formation of characteristic blistering lesions (e.g., on or around the oral mucosa). The host immune system is usually able to clear the virus from the infected epithelial tissues. However, HSV-1 establishes a latent infection in neuronal ganglia servicing the site of initial infection and persists there for the lifetime of the infected individual. HSV-1 may then reactivate from its latent state, which leads to recurrent viral replication in the epithelium and the formation of new lesions.

The surface of an HSV-1 virion is embedded with various viral glycoproteins. The virus uses a subset of these [glycoproteins B and C (gB and gC)] to attach to the heparin sulfate moieties on the surface of host cells (Shieh *et al.*, 1992; WuDunn and Spear, 1989). Subsequently, glycoprotein D (gD) binds to receptor molecules, including HVEM and nectins (Geraghty *et al.*, 1998; Montgomery *et al.*, 1996; Warner *et al.*, 1998; Whitbeck *et al.*, 1999). Next, the virion envelope fuses with the cell membrane releasing the nucleocapsid and tegument proteins into the cytoplasm (Morgan *et al.*, 1968). The nucleocapsid then travels to the host cell's nucleus where the DNA genome is threaded through a nuclear pore (Batterson *et al.*, 1983; Sodeik *et al.*, 1997). Once viral DNA is inside the nucleus, viral gene transcription commences.

HSV-1 gene expression occurs in a highly regulated cascade. Viral genes are grouped into three sets (immediate early, early, and late) based on their temporal expression pattern. In the first stage of viral gene expression, a multiprotein complex containing the viral tegument protein, VP16, and cellular proteins, such as Oct-1, HCF-1, and RNA polymerase II transcribe the immediate early genes (Campbell *et al.*, 1984; Gerster and Roeder, 1988; Kristie and Sharp, 1993). The immediate early genes, especially ICP27 and ICP4, are necessary for efficient transcription of the early genes (DeLuca and Schaffer, 1985; DeLuca *et al.*, 1985; Dixon and Schaffer, 1980; McCarthy *et al.*, 1989; Preston, 1979; Sacks *et al.*, 1985; Watson and Clements, 1980), which act mainly in viral genome replication (Boehmer and Lehman, 1997). Genome replication allows for the expression of the late genes, which predominantly encode structural proteins required for progeny virion production (Enquist *et al.*, 1998; Honess and Roizman, 1974; Mettenleiter, 2002). Therefore, disruption of either of the immediate early genes, ICP4 or ICP27, produces viruses which are attenuated for early viral expression, do not produce late gene products, and are replication defective (DeLuca and Schaffer, 1985; DeLuca *et al.*, 1985;

Dixon and Schaffer, 1980; McCarthy *et al.*, 1989; Sacks *et al.*, 1985; Watson and Clements, 1980).

HSV-1-infected cells undergo major structural and biochemical alterations termed cytopathic effect (CPE) (Roizman, 1974). CPE includes nucleolar changes, chromatin margination, plasma and organelle membrane modifications, cell fusion (polykaryocytosis), decreased cellular macromolecular synthesis, and a "rounded up" appearance due to cytoskelatal and matrix changes (Avitabile *et al.*, 1995; Hampar and Elison, 1961; Heeg *et al.*, 1986; Roizman, 1962; Roizman and Roanne, 1964; Schwartz and Roizman, 1969; Shipkey *et al.*, 1967). Such alterations ultimately lead to the destruction of cells in culture, classically described as being a necrotic cell death mechanism involving cell lysis. However, it is now clear that HSV-1 infection also triggers the apoptotic cell death pathway. If left unchecked this viral-induced apoptosis kills the host cells.

II. HSV-1's Modulation of Apoptosis

Apoptosis is first triggered and later blocked in cells infected with HSV-1. The first report of this phenomena was in 1997, when Koyama and Adachi showed that infecting the HEp-2 strain of HeLa cervical adenocarcinoma cells with HSV-1 in the presence of the protein synthesis inhibitor, cycloheximide (CHX), caused membrane blebbing, chromatin condensation, and DNA fragmentation (Koyama and Adachi, 1997). Later studies have determined that other key features of apoptosis including caspase activation, cleavage of caspase substrates, mitochondrial membrane potential change, and PS flipping are present during HSV-1-dependent apoptosis (Aubert and Blaho, 1999; Gautier *et al.*, 2003; Jerome *et al.*, 2001a; Kraft *et al.*, 2006; Nguyen *et al.*, 2005). The finding that CHX treatment reveals apoptosis in infected cells suggested a biphasic modulation of apoptotic during HSV-1 infection in which *de novo* protein synthesis is required for the prevention, but not the induction of apoptosis by the virus.

A. Prevention of Apoptosis by HSV-1

1. Wild-Type Virus

Wild-type HSV-1 infection has been shown to confer resistance to apoptosis induced by both the extrinsic and intrinsic signaling pathways (Aubert *et al.*, 1999; Galvan and Roizman, 1998; Koyama

and Miwa, 1997). This blocking ability has also been shown to be true in clinical HSV-1 isolates (Jerome *et al.*, 1999, 2001b). There is also some data to suggest that the extent of HSV-1's ability to block apoptosis may be cell-type dependent (Galvan and Roizman, 1998). However, the molecular basis for this differential response is unknown.

2. The Prevention Window

Because the lytic HSV-1 replication cycle occurs in a defined temporal order, it was possible to gain insight into the viral events involved in apoptosis modulation by examining the timing in which apoptosis induction and prevention occur. In a study performed in our laboratory, CHX was used to block protein synthesis in the HEp-2 strain of HeLa cells at various times post HSV-1 infection (Aubert *et al.*, 1999). When CHX was added between 0 and 3 hours postinfection (hpi), the HEp-2 cells underwent apoptotic cell death. This result indicated that the triggering of apoptosis by HSV-1 occurs between 0 and 3 hpi. Because the only gene products expressed at these early times are the immediate early genes, this result was consistent with triggering occurring prior to early and late viral gene expression. In contrast, CHX addition at 6 hpi or later failed to cause apoptosis in the HSV-1-infected HEp-2 cells. The major conclusion from this study was that infected cell antiapoptotic proteins are synthesized between 3 and 6 hpi. This critical time period in which proteins that block apoptosis must be produced to prevent apoptotic death during infection was termed the "prevention window." It is during the HSV-1 prevention window that factors are produced which block environmental extrinsic (Goodkin *et al.*, 2003) and intrinsic (Aubert *et al.*, 1999) stimuli. It should be noted that the kinetics of the HSV-1 prevention window are identical to that of HSV-2 (Yedowitz and Blaho, 2005).

3. Viral Antiapoptotic Factors

Determining what viral components confer antiapoptotic activity to HSV-1 has been an area of intense research in recent years. So far, seven viral genes have been shown to possess antiapoptotic activities. Evidence for these activities comes from studies of the viral factors' capacity to block apoptosis induced by both the virus itself and other environmental stimuli.

a. ICP4 In 1996, Leopardi and Roizman reported that the HSV-1 ICP4-deletion mutant, HSV-1(KOS*d*120) (referred to as *d*120), which is deleted for both copies of the gene encoding the ICP4 protein, was unable to block HSV-1-induced apoptosis in cultured Vero monkey

kidney cells (Leopardi and Roizman, 1996). As expected, infection with this virus led to the accumulation of viral immediate early proteins without synthesis of later viral proteins (DeLuca and Schaffer, 1985; DeLuca *et al.*, 1984, 1985; Knipe and Smith, 1986). Cells infected with the *d*120 virus, but not wild-type HSV-1 showed the characteristic signs of apoptosis, represented as TUNEL-positive cells and DNA laddering (Leopardi and Roizman, 1996). *d*120-induced DNA laddering was suppressed by exogenous expression of ICP4. These data led to the conclusion that the ICP4 mutant virus was unable to block apoptosis like the wild-type virus and, thus, ICP4 is essential for inhibiting virally induced apoptosis. Studies from our laboratory have confirmed the role of ICP4 in apoptosis prevention during HSV using another ICP4-null deletion virus, CgalΔ3 (Aubert and Blaho, 2003; Nguyen *et al.*, 2005; Sanfilippo and Blaho, 2006).

b. ICP27 In an attempt to study the production of immediate early proteins during HSV-1 infection, Martine Aubert in our laboratory infected HEp-2 cells with an ICP27-deletion virus, HSV-1(vBSΔ27) (Soliman *et al.*, 1997). Surprisingly, it turned out that this was not a viable system for the study of immediate early proteins because the infected cells died by apoptosis. Although HEp-2 cells infected with the wild-type virus showed the typical CPEs of HSV-1 infection, those infected with vBSΔ27 appeared shrunken, irregularly shaped, and had lost their adherence to the substratum (Aubert and Blaho, 1999). Furthermore, they displayed DNA laddering and PARP cleavage, indicating they were dying as a result of apoptosis. These findings showed that although both wild-type and ICP27 mutant viruses induce apoptosis in infected human cells, only the wild type is capable of blocking it (Aubert and Blaho, 1999). From these results, it was concluded that ICP27 is essential for the prevention of infection-induced apoptosis in HEp-2 cells.

To determine which functions of ICP27 were necessary to block infection-induced apoptosis, an extensive study was conducted using 15 recombinant ICP27 mutant viruses (Aubert *et al.*, 2001). The N-terminal region of ICP27 contains sites for the protein's RNA binding and nuclear/nucleolar localization. While these sites were necessary for efficient replication in Vero cells, they were not essential for prevention of apoptosis in HEp-2 cells. The C-terminus of ICP27 contains regions necessary for enhancing the expression of later viral gene products (Hardwicke *et al.*, 1989). All C-terminal mutations affected the virus' ability to inhibit apoptosis. It follows then, that ICP27's antiapoptotic role in the virus life cycle is likely indirect,

through the upregulation of early and late antiapoptotic viral gene products. It should therefore be expected that ICP4's role in blocking apoptosis is also indirect.

c. U_S3 In an effort to rescue the *d*120 virus, an additional mutation was found outside the gene encoding ICP4 (Leopardi *et al.*, 1997). That mutation mapped to the U_S3 gene. U_S3 is a nonessential late gene, which encodes a serine/threonine kinase (Roizman and Knipe, 2001). Vero cells infected with recombinant viruses lacking only U_S3 displayed more DNA laddering than those infected with wild-type viruses. This result lead to the proposal that the U_S3 protein is involved in the blocking apoptosis induced by the virus (Leopardi *et al.*, 1997). In other studies, it was found U_S3 expressed from a baculovirus vector blocked *d*120-induced cytochrome *c* release and procaspase 3 cleavage. This result suggested that U_S3 was blocking apoptosis upstream of mitochondrial signaling.

Using the baculovirus expression system, U_S3 has been shown to be capable of blocking caspase 3 activation stimulated by the overexpression of viral proteins (Hagglund *et al.*, 2002) and Bcl-2 family members (Benetti *et al.*, 2003; Munger and Roizman, 2001; Ogg *et al.*, 2004). Studies such as these, which express U_S3 in the absence of a viral infection, have also been used to investigate the molecular mechanism of U_S3's antiapoptotic activity. Caspase-dependent cleavage of BAD was shown to accompany apoptotic cell death in BAD-overexpressing cells. However, neither BAD cleavage nor apoptosis occurred in cells that coexpressed U_S3 and BAD (Benetti *et al.*, 2003; Munger and Roizman, 2001). This led the authors to conclude that the abrogation of BAD cleavage was responsible for U_S3's antiapoptotic function. It is not clear at this time if this is directly related to apoptosis during viral infection as BAD cleavage has not been studied in HSV-1-infected cells. For a more detailed discussion of U_S3's antiapoptotic activities, readers are referred to a review focused on this topic (Nishiyama and Murata, 2002).

d. ICP22 ICP22 is a nonessential immediate early protein. The first evidence that ICP22 might play a role in apoptosis prevention was derived from studies showing that it becomes degraded during virus-induced apoptosis (Aubert and Blaho, 1999). However, the significance of this observation was unclear, since it seemed to occur only at very late times when the cells were already dead (Munger *et al.*, 2003). A later study found that HSV-1 recombinants containing deletions in the gene encoding ICP22 exhibited an enhanced level of apoptosis compared to the parental wild-type virus. This result supports a

role for ICP22 in apoptosis prevention during infection (Aubert *et al.*, 1999).

The $U_S1.5$ protein is expressed from an alternative initiation codon in the ICP22 open reading frame (Carter and Roizman, 1996). This protein is identical to ICP22 except that it lacks the N-terminal 147 amino acids. Remarkably, in contrast to ICP22's antiapoptotic role during infection, overexpression of $U_S1.5$ using a baculovirus vector has been shown to trigger caspase 3 activation in rabbit skin cells (Carter and Roizman, 1996; Hagglund *et al.*, 2002). This result suggests that $U_S1.5$ may have proapoptotic activities.

e. gD The glycoprotein D (gD) is found in abundance on the surface of mature HSV-1 virions (Handler *et al.*, 1996) and is required for virus entry (reviewed in Roizman and Knipe, 2001). Studies have shown that a virus lacking the gD protein had a decreased ability to block apoptosis induced by infection (Zhou and Roizman, 2001; Zhou *et al.*, 2000). One potential caveat to these studies is that since gD is required for viral entry, a high multiplicity of infection is required to conduct these studies and the virus actually enters cells by endocytosis (Zhou *et al.*, 2000).

Ectopic gD expression using a baculovirus vector complements the apoptotic phenotype of the gD-null virus (Zhou *et al.*, 2000). Zhou *et al.* used this system to precisely map the antiapoptotic functional domains of gD (Zhou and Roizman, 2001, 2002a,b; Zhou *et al.*, 2000, 2003). The domains of gD needed for apoptosis prevention (Zhou *et al.*, 2003) differed from those previously shown to be required for receptor binding and membrane fusion (Connolly *et al.*, 2002; Whitbeck *et al.*, 1999).

f. gJ The glycoprotein J (gJ), also referred to as the U_S5 gene product, is a minor viral glycoprotein expressed with late kinetics (Jerome *et al.*, 1999, 2001a; Zhou *et al.*, 2000). A vector encoding the gJ protein was able to block apoptosis induced by the gD-null virus (Zhou *et al.*, 2000). Jurkat T cells, infected with a gJ-deletion virus underwent apoptosis (Jerome *et al.*, 2001a). gJ was able to partially protect T cells from Fas and granzyme B-mediated apoptosis when supplied in *trans* (Jerome *et al.*, 2001a). In another study, deletion of gJ from the viral genome led to reduction in the virus' ability to inhibit apoptosis (Jerome *et al.*, 1999). These results suggest that gJ is another HSV-1 protein that acts in apoptosis prevention.

g. LAT The latency-associated transcripts (LAT) are the only HSV-1 gene products abundantly expressed during the latent phase of HSV-1 infection (Spivack and Fraser, 1988; Stevens *et al.*, 1987). Although the

transcripts do contain potential open reading frames, whether a LAT protein is produced in latently infected cells is controversial (Doerig *et al.*, 1991; Lock *et al.*, 2001). LAT-null viruses have been reported to contain defects in the establishment, maintenance, and reactivation from latency (reviewed in Jones, 2003). The first evidence that LAT could act as an antiapoptotic agent was reported in 2000 by Perng *et al.* (2000). They found that the trigeminal ganglia of rabbits infected with a LAT-null recombinant virus possessed more apoptotic cells (as measured by TUNEL and PARP cleavage) than those infected with the wild-type parent virus. This has been confirmed in mice, as LAT-null infections of mice led to increased apoptosis in the trigeminal ganglia compared to wild-type virus (Branco and Fraser, 2005). In this study, apoptosis prevention by LAT seemed to be neural specific as the apoptosis of $CD8^+$ T cells was not affected by the LAT defect. Additionally, *in vitro* experiments support the antiapoptotic function of LAT. Caspase 9 cleavage is evident at 48 hpi when Neuro-2A cells are infected with a LAT-null virus, but not a wild-type virus (Henderson *et al.*, 2002), which suggests that LAT may block apoptosis upstream of these initiator caspases. Expression of LAT from a plasmid was also demonstrated to inhibit ceramide- and fumonisin-induced cell death in mouse and human cell lines (Henderson *et al.*, 2002). LAT expression was also shown to inhibit apoptosis induced by Bax, caspase 8, or caspase 9 overexpression (Ahmed *et al.*, 2002; Inman *et al.*, 2001; Jin *et al.*, 2003; Peng *et al.*, 2004).

The proposal that LAT possesses antiapoptotic function has not been without controversy (Thompson and Sawtell, 2000). In one paper, even though the authors found more cell death in mice infected with the wild-type virus compared to LAT-null infected, they did not observe apoptosis as measured by TUNEL staining (Thompson and Sawtell, 2001). From this result, the authors concluded that LAT's prosurvival function during infection was not due to apoptosis prevention. However, several groups have reported that the region of LAT necessary for its antiapoptotic activity are the same as those required for its reactivation function (Ahmed *et al.*, 2002; Inman *et al.*, 2001; Jin *et al.*, 2003; Peng *et al.*, 2004). Moreover, replacement of the LAT region with the antiapoptotic *Bovine herpesvirus* (BoHV) LR produced a virus capable of wild-type reactivation (Perng *et al.*, 2002). One caveat to this study was that since the BoHV LR comes from a homologous virus, it was possible that it may share functions with LAT unrelated to its antiapoptotic activity. This limitation was overcome by replacing LAT with an unrelated baculovirus antiapoptotic gene (Jin *et al.*, 2005). This replacement restored the reactivation defect of LAT-null HSV-1. Finally, micro

RNAs present in LAT (Cui *et al.*, 2006; Pfeffer *et al.*, 2005) may determine its antiapoptotic activity (Gupta *et al.*, 2006). Together, these data provide evidence that LAT possesses an antiapoptotic function that may be needed for appropriate latency regulation.

4. Cellular Genes

a. Bcl-2 Bcl-2 is an antiapoptotic mitochondrial protein that has been shown to block the intrinsic apoptotic pathway at the point of release of cytochrome *c* (Adams and Cory, 1998). In an initial study, overexpression of Bcl-2 was shown to block the cytochrome *c* release observed in *d*120 infected HEp-2 cells (Galvan *et al.*, 2000). Later, work by Connors' group showed similar results with Bcl-2 overexpression and indicated that cells infected with ICP4 and ICP27 deleted viruses possessed less Bcl-2 mRNA and protein than those infected with wild-type HSV-1 (Zachos *et al.*, 2001). The time in which this decrease in Bcl-2 levels was observed corresponded with the prevention window (Aubert *et al.*, 1999). A p38MAPK inhibitor led to increased Bcl-2 levels and reduced apoptosis in ICP27-null-infected cells, suggesting that p38MAPK activity may mediate the Bcl-2 destabilization. These results suggest that the abundance of Bcl-2 may be important for apoptosis prevention during HSV-1 infection.

b. NF-κB NF-κB is a cellular transcription factor important for the expression of genes involved in the innate immune response such as the interferons (IFNs) and both the antiapoptotic Bcl-2 and IAP family members. Inactive NF-κB is found complexed with IκB, which sequesters NF-κB in the cytoplasm until it is needed for transcription. On appropriate cell signals, IκB is targeted for degradation, which allows NF-κB to translocate to the nucleus and bind κB sites of target genes, facilitating their transcription (Gerster and Roeder, 1988; Ghosh and Baltimore, 1990). In 1998, Patel *et al.* found that during HSV-1 infection, NF-κB translocated to the nucleus. However, it did not appear to stimulate transcription of κB promoted reporter genes in their system. In 2001, Amici *et al.* reported a study suggesting that the activation of NF-κB during infection proceeded via IKK activation, or the "classical" pathway to NF-κB activation. At that time, it was not clear what the significance of NF-κB activation was during HSV-1 infection of human cells.

While deciphering the mechanism of HSV-1's apoptosis inhibition, research in our laboratory demonstrated that NF-κB translocation from the cytoplasm into the nuclei of infected cells initiated at 3 hpi, which correlated with the time in which apoptosis prevention by HSV-1 was first observed (Goodkin *et al.*, 2003). The complete inhibition of

protein synthesis at 3 hpi by the addition of CHX precluded NF-κB translocation, while CHX addition at 6 hpi or later did not elicit this effect (Goodkin *et al.*, 2003), showing that infected cell protein synthesis is required for the nuclear import of NF-κB. In cells infected with the apoptotic ICP27-null virus, NF-κB remained cytoplasmic and did not bind its DNA recognition site. Prestimulation of NF-κB by the addition of a phorbol ester prevented the ICP27-null virus-induced apoptosis (Goodkin *et al.*, 2003). Furthermore, an HEp-2 cell line expressing (IκBαDN) a dominant negative form of IκBα underwent detectable apoptosis when infected with wild-type HSV-1. Together, these results provided evidence for NF-κB's involvement in apoptosis prevention during HSV-1 infection. While other studies have supported this role for NF-κB in apoptosis prevention (Gregory *et al.*, 2004; Medici *et al.*, 2003), alternative roles for NF-κB during HSV-1 infection have also been proposed (Taddeo *et al.*, 2002, 2004).

B. Apoptosis Induction

1. ICP0 Gene Expression Is a Viral Apoptotic Trigger

Initial studies into apoptosis during HSV infection had shown that apoptosis was triggered in the absence of protein synthesis (Koyama and Adachi, 1997) prior to 3 hpi (Aubert *et al.*, 1999). Therefore, the triggering event must occur sometime between virus attachment and the translation of immediate early viral genes. Evidence that binding and entry of HSV-1 were not sufficient for apoptosis induction came from studies using HSV-1 that had been exposed to ultraviolet (UV) irradiation. Since UV damages virion DNA, viral transcription is prevented and no IE gene products are synthesized. Infection with UV irradiated virus failed to cause apoptosis (Aubert *et al.*, 1999; Sanfilippo *et al.*, 2004). Importantly, this was true even using a dose of irradiation that did not affect virion binding and entry (as measured by VP16's ability to act as a transcriptional activator) (Sanfilippo *et al.*, 2004). On the basis of these findings, it was concluded that the processes of binding and entry of HSV-1 are not responsible for the induction of apoptosis in infected human epithelial cells. Further support for this conclusion comes from the use of a virus possessing a temperature sensitive allele in the U_L36 gene (HFEM*ts*B7) (Sanfilippo *et al.*, 2004). At the nonpermissive temperature (39°C), the HFEM*ts*B7 mutant virus cannot inject its viral genome into the nucleus. HEp-2 cells infected with this virus at 39°C failed to undergo apoptosis as measured by membrane blebbing, chromatin condensation, and PARP cleavage. In other words, an HSV-1 infection in which the viral

life cycle is stalled prior to IE gene transcription did not end in apoptosis in these cells.

To directly test the role of viral IE gene expression in HSV's apoptosis induction, the HSV-1 recombinant virus *d*109, which contains deletions in all five immediate early genes (Samaniego *et al.*, 1998), was used (Sanfilippo *et al.*, 2004). While cells infected with vBSΔ27 displayed classic features of apoptosis, including death factor processing and apoptotic morphologies (Aubert and Blaho, 1999), *d*109-infected cells did not (Sanfilippo *et al.*, 2004). These data indicated that *d*109 does not induce apoptosis in HEp-2 cells due to the absence of immediate early gene expression.

The next goal in this line of research was to identify the IE gene(s) required for HSV-1 to trigger apoptosis. This was accomplished in a study in our laboratory (Sanfilippo and Blaho, 2006), which utilized recombinant viruses singly deleted for immediate early viral genes. Recombinant viruses lacking ICP27 (Soliman *et al.*, 1997), ICP4 (Johnson *et al.*, 1992), or ICP22 (Ogle and Roizman, 1999) were found to be capable of triggering apoptosis as well as wild-type HSV-1. However, deleting ICP0 (Cai and Schaffer, 1992) drastically reduced HSV-1's ability to trigger apoptosis. Conversely, a recombinant virus that solely expressed ICP0, *d*106 (Samaniego *et al.*, 1998), induced apoptosis at a level similar to wild-type HSV-1. Furthermore, it was found that ICP0 expression from a plasmid was capable of causing apoptotic cell death in HEp-2 cells. These findings indicated that ICP0 expression is necessary and sufficient to trigger HSV-1-dependent apoptosis. Additionally, a virus in which full-length ICP0 protein translation, but not transcription, was abrogated via the introduction of a nonsense mutation in the ICP0 open reading frame (*n*212) (Cai and Schaffer, 1992) was shown to trigger apoptosis (Sanfilippo and Blaho, 2006). This result indicated that the ICP0 transcript alone possesses proapoptotic activity. The finding that ICP0 translation is not required for its proapoptotic activity is consistent with prior data showing that HSV-1 triggers apoptosis in the presence of CHX (Aubert and Blaho, 1999; Koyama and Adachi, 1997). This study represents the first description of apoptosis induction in infected cells triggered as a result of transcription of a single viral gene. Future research is needed to clarify this unique mechanism of RNA-mediated triggering of apoptosis.

2. Requirement for Caspase 3

Caspase 3 is an effector caspase that chiefly cleaves cellular substrates important for maintaining the structural and biochemical integrity of the cell. Although caspase 3 shares substrate specificity

with at least one other caspase, caspase 7, it does seem to be essential for certain apoptotic processes. For example, mice lacking caspase 3 expression die prematurely due to a failure of apoptosis essential for brain development (Kuida *et al.*, 1996; Woo *et al.*, 1998). Caspase activation occurs by cleavage of prodomains from their precursor forms. Procaspase 3 levels dramatically decrease during HSV-l-dependent apoptosis (Aubert *et al.*, 1999; Nguyen *et al.*, 2005). Paradoxically, cells infected with wild-type HSV-1 also exhibit a small level of procaspase 3 reduction even though these cells do not die by apoptotic cell death (Aubert *et al.*, 1999; Nguyen *et al.*, 2005). To clarify the role of caspase 3 activation in HSV-1-dependent apoptosis, we probed directly for the active form of caspase 3. More of the active, cleaved form of caspase 3 (17,000 molecular weight) was observed in HEp-2 cells infected with HSV-1 than mock-infected cells, showing that the decrease in procaspase levels during infection is a result of caspase 3 activation. In cells undergoing HSV-1-dependent apoptosis, there was even greater increase in the level of active caspase 3 (Kraft *et al.*, 2006). These results indicated that processed caspase 3 exists inside productively infected cells and suggests that if its activity is not regulated by infected cell proteins, apoptotic death by HSV-1-dependent apoptosis will likely ensue.

To determine whether caspase 3 activation was necessary for HSV-1-dependent apoptosis, the ability of tumor cells, which differ only in their caspase 3 status, to undergo HSV-1-dependent apoptosis was compared (Kraft *et al.*, 2006). vBSΔ27-infected MCF-7 cells that ectopically express caspase 3 underwent more efficient apoptosis than their caspase 3-null parental counterparts, demonstrating that caspase 3 contributes to HSV-1-dependent apoptosis. However, caspase 3 reconstitution did not make the MCF-7 cells as sensitive as HEp-2 cells to HSV-1-dependent apoptosis, indicating that other cellular factors are involved in conferring resistance to this process. Identification of these additional cellular factors should help to clarify the mechanism of HSV-1-dependent apoptosis.

C. Summary and Unanswered Questions

A number of key cellular and viral factors for controlling apoptosis have been identified since HSV-1's apoptotic modulatory activities were discovered. The two viral genes found to be absolutely essential for apoptosis prevention during HSV-1 infection (i.e., infected cells die by apoptosis if they are absent) are regulatory proteins needed for the expression of other viral genes (Aubert and Blaho, 1999; Leopardi and Roizman, 1996). This suggests that blocking apoptosis during HSV-1

infection occurs through the cooperation of multiple viral gene products. Two cellular factors have been implicated in the prevention of apoptosis and surely more have yet to be discovered. The viral proapoptotic trigger has been shown to involve transcription of the ICP0 gene. In all, this supports the model in which there is a delicate balance between the pro- and antiapoptotic factors during a wild-type HSV-1 infection, which is presumably used by the virus to delay the onset of apoptotic death until progeny virions are produced. If the antiapoptotic factors are not efficiently made, then the infected cells die prematurely by apoptosis. Figure 2 illustrates the proposed roles of viral and cellular factors that regulate apoptosis during HSV-1 infection. Now that a number of the apoptotic regulatory factors have been identified for HSV-1 infection, the next major challenge will be to determine the molecular mechanism whereby they act.

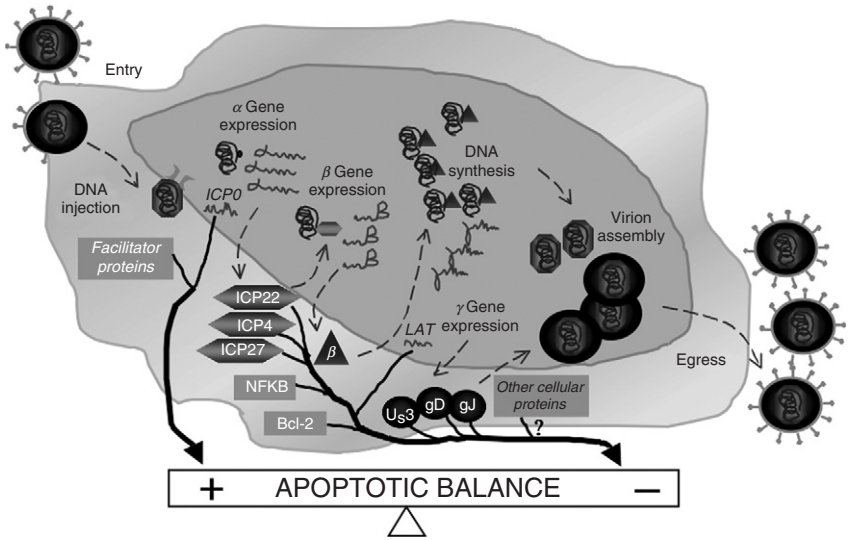

FIG 2. Model of apoptotic balance during HSV infection. The steps of productive HSV replication are outlined in the text. Apoptosis is initially triggered and subsequently blocked during a wild-type HSV-1 infection. Proapoptotic factors include the viral ICP0 gene transcript and as yet unknown viral or cellular facilitator proteins. Seven viral gene products and two cellular proteins have been proposed to act in the prevention of apoptosis during infection. Other, as yet unidentified, cellular and/or viral factors are also likely involved. Together, these signals set up an apoptotic balance, which presumably delays cell death until progeny virions are produced. If the preventers of apoptosis are not efficiently produced, as is the case when protein synthesis is blocked during HSV-1 infection, the infected cells die via apoptosis.

III. Cell-Type Differences

Much of the initial characterization of apoptosis modulation by HSV-1 utilized the HEp-2 strain of HeLa and SK-N-SH human cancer cells. When studies were expanded to include other cell types, it became apparent that a range of sensitivities to HSV-1-dependent apoptosis exists.

A. Oncoapoptosis

Human cancer cells display an exquisite sensitivity to apoptosis induced by HSV-1. This was initially realized in our laboratory when the responses of multiple human transformed cell lines and primary mouse and human fibroblasts to HSV-1-dependent apoptosis were compared (Aubert and Blaho, 2003). In this study, it was shown that the S3 and HEp-2 strains of HeLa cells and 143 osteogenic murine sarcoma virus transformed cells underwent apoptosis with similar efficiencies when infected with vBSΔ27 or CgalΔ3 viruses. Although primary human foreskin fibroblasts and mouse embryo fibroblasts supported HSV-1 infection, as measured by viral protein accumulation, they failed to display apoptotic morphologies or DNA laddering in response to infection with vBSΔ27 or CgalΔ3. We have recently found that several cell lines derived from breast, colon, and brain tumors are susceptible to HSV-1-dependent apoptosis (Nguyen *et al.*, unpublished results), suggesting that this extreme sensitivity may be a general characteristic of cancer cells and implicating a role for oncogenes in sensitizing cells to HSV-1-dependent apoptosis.

B. Immortalized Vs Transformed Cells

Vero cells are known among herpesvirologists as cells that are exemplary virus growers. They were derived from African green monkey kidney tissue and immortalized through serial passage in low serum. Although immortal, the Vero cells do not exhibit anchorage independent growth or the ability to form tumors in soft agar (Genari *et al.*, 1998; Zhang *et al.*, 2001). Therefore, they are considered nontransformed. In the initial report demonstrating vBSΔ27-induced apoptosis, Vero cells were found to be more resistant than the transformed HEp-2 and S3 strains of HeLa cells (Aubert and Blaho, 1999). As opposed to HeLa cells, the characteristic morphological changes of apoptosis, including cell shrinkage, loss of adherence to the monolayer, and classic oligosomal DNA laddering, were not apparent in vBSΔ27-infected Vero cells

at times prior to 24 hpi. Similarly, infection with HSV-1 in the presence of CHX to prevent viral protein synthesis, failed to yield apoptotic features in Vero cells (Aubert and Blaho, 1999).

We later examined the basis of these different apoptotic responses to HSV-1 (Nguyen *et al.*, 2005). Infected Vero cells were found to take substantially longer than HEp-2 cells to display membrane blebbing, chromatin condensation, DNA laddering, and PARP cleavage. In a later series of experiments, we found that unlike HEp-2 cells, where CHX treatment reveals HSV-1 dependent apoptosis, CHX treatment actually inhibited the apoptosis induced by vBSΔ27 in Vero cells. Moreover, Vero cells infected with wild-type virus in the presence of CHX did not undergo apoptosis up to 36 hpi. These results led to the conclusion that Vero but not HEp-2 cells require *de novo* protein synthesis to exhibit efficient HSV-1-dependent apoptosis. This implied that proapoptotic facilitator proteins were preexisting in HEp-2 cells but needed to be newly synthesized in Vero cells during infection. A time course of CHX addition was used to determine that these facilitators were produced prior to 3 hpi in Vero cells. This narrowed the candidates for facilitator proteins to immediate early viral proteins or cellular proteins produced during this time period. Vero cells infected with recombinant viruses devoid of the ICP27 and ICP4 proteins alone or both the ICP27 and ICP22 proteins were apoptotic, demonstrating that ICP27, ICP4, and ICP22 were not facilitating apoptosis in these cells. This leaves open the possibility that one or both of the other two immediate early viral proteins or some cellular factors act in apoptosis facilitation during HSV-1 infection.

C. Summary and Unanswered Questions

It is now clear that cells can exhibit different levels of sensitivity to HSV-1-dependent apoptosis. There seems to be at least three different phenotypes for HSV-1-dependent apoptosis. Cancer cells display an exquisite sensitivity to HSV-1-dependent apoptosis. HSV-1 apoptosis induction failed to elicit apoptotic phenotypes in the primary cells tested thus far. Immortalized but nontransformed cells have an intermediate phenotype as they do undergo HSV-1-dependent apoptosis and this requires protein synthesis. The association between oncogenic transformation status of the host cell and sensitivity to HSV-1-dependent apoptosis led us to propose a model in which molecular alterations required for tumorigenesis may mediate the sensitivity to HSV-1-dependent apoptosis (Nguyen *et al.*, 2005). This model raises an important question. Is the induction of apoptosis by HSV-1 restricted solely

to tumor cells? If so, exploiting this specificity may provide a novel strategy (viral oncoapoptosis) for HSV-1-based cancer therapy. In one study, infection with *Bovine herpesvirus 4*, which is also able to cause apoptosis in tumor cells, was shown to significantly reduce the growth of human cancer xenograft in nude mice (Gillet *et al.*, 2005). This supports the concept of viral oncoapoptosis therapy.

Yet, if HSV-1 triggered apoptosis is solely restricted to tumor cells, then it puts into question its relevance to the natural course of human HSV-1 disease. Since there are so many viral genes which act to prevent apoptosis, it is not unreasonable to hypothesize that apoptosis triggering occurs during the natural course of HSV-1 infection. Supporting this idea is the fact that apoptotic cells can be found in herpetic lesions in animal models and humans as will be discussed in Section III. It is possible, as previously suggested (Blaho, 2004; Goodkin *et al.*, 2004), that the early stages of apoptosis are actually beneficial for virus propagation in nontumor cells. However, due to the limited number of normal cells tested at this point, the possibility that HSV-1 causes apoptosis in a subset of normal cells cannot be ruled out. It also may be that certain cell states (e.g., certain phases of development) share properties with cancer cells that allow HSV-1 infections to induce apoptosis. Further investigation into the genes involved and molecular mechanisms of sensitizing cells to HSV-1-dependent apoptosis are needed to clarify these issues.

IV. Apoptosis and HSV-1-Associated Disease

Depending on the types of tissues affected, HSV-1 infection can lead to disease as minor as a cold sore or as devastating as blinding keratitis or fatal encephalitis (Whitley, 2001). In immune suppressed populations and neonates, HSV-1 infections commonly become disseminated to multiple organs, leading to life threatening disease. Traditionally, herpetic disease is thought to be caused by necrotic cell death caused by virus replication and immune-mediated cell death. However, recently reports have linked apoptosis with the severity of herpes-associated disease. The final section of this chapter will summarize the evidence for these links.

A. Ocular Disease

Ocular HSV-1 infections are the leading cause of infectious blindness in the United States (Liesegang *et al.*, 1989). Primary eye infections usually present as conjunctivitis, which may progress to epithelial

keratitis, takes weeks to heal. Ocular reactivation causes epithelial dendritic ulceration and in 20–25% of cases stromal alterations known as herpes stromal keratitis (HSK) (Liesegang, 2001). These HSK lesions reduce visual acuity and take months to fully resolve. With repeated reactivation, opacification of the cornea occurs, causing blindness. Although antiviral therapy reduces corneal recurrences by 40%, it, unfortunately, does little to expedite healing of existing lesions (Barron *et al.*, 1994).

Several lines of evidence support a role for apoptosis in limiting HSV replication in the eye, and thereby, protecting it from HSK. Ocular HSV-1 infection causes apoptosis in the eyes of mice (Qian and Atherton, 2003). HSV-1 infection of rabbit corneal epithelial cells induced apoptosis in the underlying keratinocytes (Wilson *et al.*, 1997). Human corneal epithelial cells from patients with ocular HSV-1 infections displayed increased apoptosis as measured by Annexin V staining (Miles *et al.*, 2003). Therefore, the results from both animal models and human infections indicate that HSV-1 infection leads to apoptosis. HSV-1-infected mice lacking the IFN responsive RNase L gene exhibited more severe HSV-1 keratitis and less apoptosis than their wild-type littermates (Zheng *et al.*, 2001), presumably because, in this case, the apoptosis balance was shifted to favor viral replication. Miles *et al.* (2004) reported a similar reverse correlation between apoptosis and HSK severity in human corneas. Together, these findings suggest that during HSV-1 infection, apoptosis reduces the severity of HSK.

B. Encephalitis

In an estimated 1 out of every 200,000 people per year, HSV-1 spreads to the central nervous system to cause herpes simplex encephalitis (HSE) (Whitley, 2001). Although HSE is a rare outcome of HSV-1 infection, it is the most common cause of sporadic, infectious encephalitis in the United States (Skoldenberg, 1991). Even with antiviral therapy, HSE has a mortality rate of over 30% and commonly leads to lifelong neurological sequelae (Skoldenberg, 1996; Whitley and Kimberlin, 1997). Recent reports have shown HSE contains an apoptotic component. Tissue sections from patients with HSV-associated acute focal encephalitis were found to contain neurons with TUNEL-positive staining, active caspase 3, and cleaved PARP (DeBiasi *et al.*, 2002; Perkins *et al.*, 2003). HSV-1 infection also increased the levels of these apoptotic markers in rat hippocampal cultures (Perkins *et al.*, 2003). Small molecule inhibitors of c-Jun N-terminal kinase (JNK) reduced apoptosis

in these cultures, suggesting that signaling through JNK may facilitate apoptosis. Additionally, the soluble Fas (sFas) apoptotic marker was detected in the cerebrospinal fluid (CSF) of patients with HSE, but not in the CSF of healthy individuals (Sabri *et al.*, 2006). Interestingly, increased levels of sFas in the CSF correlated with more severe disease (Sabri *et al.*, 2006). Additionally, IFN-γ-null mice infected with HSV displayed increased apoptosis and encephalitis while wild-type littermates did not (Geiger *et al.*, 1997). These results suggest that, as opposed to the protective role that apoptosis plays in HSK, apoptosis may facilitate herpetic brain disease.

C. Summary and Unanswered Questions

Mounting evidence suggests that apoptosis likely occurs in infected tissues of HSV-1-associated diseases; however, there is limited knowledge of the impact that apoptosis has on disease progression. The studies described above seem to indicate that apoptosis has opposite effects on the severity of HSK and HSE. However, data collected thus far has been largely correlative. Experiments aimed to directly alter apoptosis during the development of herpetic disease should clarify this issue. Another point that warrants further investigation is whether the apoptosis detected in diseased tissues is induced by the virus itself (through the triggering mechanism detailed in Section II.B) or mediated by interactions with other uninfected cells such as cytotoxic T cells.

V. Conclusions and Perspectives

An intricate balance between pro- and antiapoptotic signals is established during an HSV-1 infection. This regulation is no doubt complex, as the number of factors involved in the process continues to grow. Recent studies imply that there is cell-to-cell variation in the amount of "force" required to tip the apoptotic balance in favor of death. Interestingly, the variations in apoptotic sensitivity seem to involve genes that mediate tumorigenesis. Future research should provide insight into the specific molecular pathways shared by these distinct processes. The discovery that HSV-1 modulates apoptosis has revealed an important facet of the complex interaction between the virus and its host cells. Exploiting this interaction may allow for novel treatments for viral infections and cancer.

Acknowledgments

We wish to thank Rachel Kraft, Kristen Peña, and Elisabeth Gennis for their assistance in generating data that served as the basis of this chapter. M.L.N. was supported in part by US Public Health Service Institutional Research Training Awards (AI07647 and CA088796). These studies were supported in part by additional grants from the US Public Health Service (AI38873 and AI48582 to J.A.B.), and the American Cancer Society (JFRA 634 to J.A.B.). J.A.B. also thanks the Lucille P. Markey Charitable Trust and the National Foundation for Infectious Diseases for their support.

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ADVANCES IN VIRUS RESEARCH, VOL 69

THE BACULOVIRUSES OCCLUSION-DERIVED VIRUS: VIRION STRUCTURE AND FUNCTION

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0065-3527/07 $35.00
DOI: 10.1016/S0065-3527(06)69003-9

I. Introduction

A. Baculoviruses

Baculoviruses are a family of arthropod-specific viruses found ubiquitously in the environment and have been isolated from more than 600 host insect species including the orders Lepidoptera, Hymenoptera, Diptera, Orthoptera, Coleoptera, Neuroptera, Thysanera, and Trichoptera (Adams and McClintock, 1991; Herniou *et al.*, 2003; Larsson, 1984; Martignoni and Iwai, 1986; Murphy *et al.*, 1995; Tinsley and Kelly, 1985). Most baculovirus species have been isolated from Lepidoptera and the majority of nonlepidopteran isolates have not been well characterized. They are DNA viruses with closed, circular, double-stranded DNA genomes ranging from 80 to 180 kbp in size. The genomes are packaged in bacillus-shaped nucleocapsids, and the name "baculovirus" is in reference to the nucleocapsid shape. Presently, the genomes from 29 baculovirus species have been sequenced providing a database of more than 4000 genes (Hiscock and Upton, 2000).

Baculoviruses play an important ecological role regulating the size of insect populations (Evans, 1986; Odindo, 1983). For many decades, baculoviruses have been applied as targeted biocontrol agents against forestry and agriculture pests. Baculovirus insecticides have been effective against insect pests such as velvetbean caterpillar (*Anticarsia gemmatalis*) (Moscardi, 1999), cotton bollworm (*Helicoverpa zea*) (Zhang, 1994), and gypsy moth (*Lymantria dispar*) (Cook *et al.*, 2003). Baculovirus-based biocontrol applications have been restricted to lepidopteran and hymenopteran (sawflies) pests. Mosquito-specific baculoviruses have been characterized (Andreadis *et al.*, 2003; Moser *et al.*, 2001) with the potential to develop them for biocontrol of mosquitoes.

Baculoviruses are transmitted to insects by the oral route mediated by the occlusion-derived virus (ODV). This is reference to the occlusion of orally infectious baculoviruses in protein crystals called occlusion bodies (OBs). It is important to study the structure and function of ODVs and OBs because it expands the horizon for the application of baculovirus as insecticides. The ODV is also specialized to exploit the insect midgut which is one of the most extreme biological environments where the viruses are subject to caustic pH and digestive proteases. Understanding the molecular biology of the ODV should reveal new frontiers in protein chemistry. Finally, ODVs establish infection in insect gut tissues that are virtually nonsupportive to virus replication and which are continuously sloughed away. ODVs carry with them a battery of proteins that enable them to rapidly exploit and harness these unstable cells for virus replication. Learning about these proteins will have implications in biocontrol and biotechnology.

In the following sections, we will overview the basic biology of ODVs and OBs and will thoroughly examine the proteins within the ODV. Reviews on the ODV structure, function, and molecular biology have been lacking and, therefore, the goal of this chapter is to provide a more comprehensive picture and to stimulate future research.

An extensive analysis of the protein composition of the *Ac*MNPV ODV virion was done in 2003 (Braunagel *et al.*, 2003). That study produced a list of 44 ODV-associated viral proteins. We use this landmark study as the framework for much of this chapter. Among these 44 *Ac*MNPV-specific ODV proteins, 21 are conserved among all baculovirus genomes (Table I).

B. Origin of Baculoviruses

Fossil records tell us that terrestrial organisms originated from the sea and one of the earliest groups to venture out on to land was the arthropods, the predecessors of today's insects. Insects have been evolving and diversifying for the past 400 million years (Labanderia and Sepkoski, 1993) and so must have their viruses. Insect viruses of today may be related to the viruses brought onto land by ancient arthropod ancestors. The *Baculoviridae* is a family of insect viruses with complexity in form and function would suggest a long evolutionary lineage. Baculoviruses are genetically and morphologically distinct from other virus families and have an unusually stringent specificity for arthropod hosts. It has been suggested that baculovirus coevolved with Lepidoptera (Zanotto *et al.*, 1993) given that most baculoviruses are isolated from this order and that there is high species specificity among lepidopteran baculovirus isolates. Baculoviruses isolated from other orders such as Diptera and Hymenoptera are also comparably less complex than the lepidopteran baculoviruses (Afonso *et al.*, 2001; Garcia-Maruniak *et al.*, 2004; Herniou *et al.*, 2003; Lauzon *et al.*, 2004). The Lepidoptera were one of the last insect orders to experience significant species divergence, which occurred in the later half of the Cenozoic age (65 million years ago) (Labanderia and Sepkoski, 1993). If baculoviruses are as old as Lepidoptera, their survival as a virus family today can be attributed to the evolution of the ability of baculoviruses to occlude virions in protein crystals called OBs.

C. Baculovirus Occlusion

The baculovirus OB is a distinctive structure in virology. The OBs of baculoviruses are large enough to be observed by light microscopy and range from 500 to 2000 nm in diameter (Adams and McClintock, 1991;

TABLE I

SUMMARY OF OCCLUSION-DERIVED VIRION PROTEINS

AcMNPV ORF #	Names	Predicted mw (kDa)	*pI*	Motifs	Group I NPVs (7)	Group II NPVs (12)	Hymen NPV (2)	Diptera NPV (1)	GVs (7)	Total (29)	Proposed ODV localization
77	VLF-1	44.4	9.2	Nuclear	7	12	2	1	7	29	Nucleosome
100	Basic Protein (P6.9, VP12)	6.9	12.3	Nuclear	7	12	2	1	7	29	Nucleosome
14	LEF-1	30.8	8.8	DNA bind	7	12	2	1	7	29	Nucleosome
65	DNApol	114.3	8.6	DNA bind	7	12	2	1	7	29	Nucleosome
95	Helicase	143.2	8.4	DNA bind	7	12	2	1	7	29	Nucleosome
133	AN (Alk Exo)	48.3	8.9	DNA bind	7	12	2	1	7	29	Nucleosome
92	P33	30.9	8.2	P53 binding	7	12	2	1	7	29	Nucleosome
66	Desmoplakin (93 kDa)	93.9	5.3	Topoisomerase	7	24	4	2	18	55	Nucleosome
58	6.8 kDa	6.8	9.8	Nuclear	2	0	0	0	0	2	Nucleosome
86	PNK/PNL	80.8	8.8	NTPbind	2	0	0	0	0	2	Nucleosome
70	HCF-1	34.4	9.2	Zincfing	2	0	0	0	0	2	Nucleosome
49	PCNA	28.6	5.2	DNA bind	4	2	0	0	0	6	Nucleosome
147	IE-1	66.9	6.1	DNA bind	7	12	2	0	7	28	Nucleosome
67	LEF-3	44.6	5.1	DNA bind	7	12	0	0	7	26	Nucleosome
88	CG30	30.1	8.8	LeucineZip	7	8	0	0	0	15	Nucleosome
89	VP39 (capsid)	38.9	6.2		7	12	2	1	7	29	Capsid
129	P24 (capsid protein)	22.1	5.8		7	11	0	0	7	25	Capsid
9	p78/83 (ORF1629, 83/87)	60.7	5.8	LeucineZip	7	12	0	0	0	19	Capsid Base
54	VP1054	42.1	8.2		7	15	2	1	7	32	Nucleocapsid
109	44.8 kDa	44.8	8.5		7	12	2	1	7	29	Nucleocapsid
142	49 kDa	55.4	8.7		7	12	2	1	7	29	Nucleocapsid
101	BV/ODV-C42 (P40)	41.5	8.2		7	12	2	0	7	28	Nucleocapsid
102	13.1 kDa	13.3	5.3		7	12	0	0	7	26	Nucleocapsid
59	8.2 kDa	8.2	9.4		7	12	0	0	0	19	Nucleocapsid
104	VP80 (P87)	79.9	5.4		7	12	0	0	0	19	Nucleocapsid
79	12.1 kDa	12.2	10.3		7	6	0	0	0	13	Nucleocapsid
74	30.5 kDa	30.6	5.0		7	5	0	0	0	12	Nucleocapsid
5	12.4 kDa	12.4	9.1		7	0	0	0	0	7	Nucleocapsid
30	54.6 kDa	54.7	7.6		7	0	0	0	0	7	Nucleocapsid
114	49 kDa	49.3	6.9		7	0	0	0	0	7	Nucleocapsid
132	25 kDa	25.1	8.5		7	0	0	0	0	7	Nucleocapsid
39	P43	43.5	9.8		3	1	0	0	0	4	Nucleocapsid
80	GP41	45.4	8.2	O-glyc	7	12	2	1	7	29	Tegument
144	ODV-EC27	33.5	6.0	Cyclin	7	12	2	1	7	29	Tegument
138	P74 (PIF-0)	73.9	4.9	Transmembrane	7	12	2	1	7	29	Envelope
119	PIF-1	59.8	5.6	Transmembrane	7	12	2	1	7	29	Envelope
22	PIF-2	43.8	5.1	Transmembrane	7	12	2	1	7	29	Envelope
115	PIF-3	23.0	4.9	Transmembrane	7	12	2	1	7	29	Envelope
83	VP91 (P95, P91, P96)	96.2	4.6	Transmembrane	7	12	2	1	7	29	Envelope
94	ODV-E25	25.5	5.9	Transmembrane	7	12	2	1	7	29	Envelope
143	ODV-E18	6.6	12.0	Transmembrane	7	12	2	1	7	29	Envelope
148	ODV-E56	40.9	5.1	Transmembrane	7	12	2	1	7	29	Envelope
150/145	11K proteins (PIF-4)	11	4.6	Transmembrane	12	22	2	0	19	55	Envelope
23	F protein (ENV)	79.9	5.3	Transmembrane	7	12	0	1	7	27	Envelope
46	ODV-E66	79.0	6.3	Transmembrane	7	15	0	0	0	22	Envelope
61	FP25K	22.2	9.1		7	12	0	0	7	26	Envelope
16	BV/ODV-E26	25.9	10.1		7	0	0	0	0	7	Envelope

Bilimoria, 1991; Boucias and Pendland, 1998) depending on the virus. This feature led to baculoviruses being the earliest described virus particles. Occlusion is an adaptation to permit baculoviruses to remain in a dormant but viable state in the environment for decades and perhaps even centuries (Bergold, 1963b). Baculovirus virions have lipid bilayer envelopes which normally make viruses susceptible to desiccation and loss of viability outside the host (Cox, 1989). The survival strategy of many enveloped viruses is to persist in living hosts as latent viruses or to infect a reservoir host species. For example, herpesviruses remain latent for decades in human hosts (Efstathiou and Preston, 2005) and the SARS *Coronavirus* was shown to persist in bats and civets (Li *et al.*, 2005a). Insect viruses cannot rely on latency or reservoir strategies for several reasons. Insects are short lived compared to other animal classes thus limiting the chances for transmission of latent viruses between insect hosts. The dramatic physiological changes that occur in insects as they go from egg to adult also create complications at a cellular level that are not conducive to virus persistence. Probably the most important factor is that insect populations are seasonal and cyclic. There are long periods of time when insect species simply are not around in significant densities to transmit viruses. Insect viruses must persist for long periods in the environment waiting for population surges of their hosts.

To persist in the environment, baculoviruses evolved to surround their enveloped virions in the protective protein layers. In essence, they have acquired the hardier characteristics of nonenveloped viruses without significantly altering the biology of virion entry and attachment to host cells. Occluded viruses do not infect any species outside of invertebrates suggesting that persistence in the environment is critical for insect viruses. The occlusion strategy has evolved in parallel with some other insect viruses such as entomopoxviruses (*Poxviridae*) and cytoplasmic polyhedroviruses (*Reoviridae*) (Adams and Bonami, 1991; Rohrmann, 1986). However, baculoviruses have evolved occlusion to several orders of complexity above that of other occluded viruses. First, baculovirus OBs may contain multiple numbers of virions and second, the virions may contain multiple nucleocapsids. A single

The table summarizes the 44 ODV-associated proteins identified by Braunagel *et al.*, (2003), and also includes PIF-1, -2, -3, and 11K proteins. The sequences were analyzed for baculovirus homologues on the Viral Bioinformatics Resources Center (Hiscock and Upton, 2000). These resources searched baculovirus genomes of 7 group I NPVs, 12 group II NPVs, 2 hymenopteran NPVs, 1 dipteran NPV, and 7 GVs. The completely conserved proteins are shaded.

OB of some baculoviruses species may deliver dozens of virions to a tissue and infect a cell with multiple copies of the viral genome. In the next few sections, we will discuss the advantages of this strategy in baculovirus biology.

II. The Baculovirus Life Cycle

A. Virus Entry into the Midgut

The transmission and replication of baculoviruses occur exclusively in the larval stages of insects. Transmission occurs by the oral or "*per os*" route when insects inadvertently consume OB-contaminated food. OBs and food particles travel through the foregut and enter the midgut (Fig. 1). Lepidopterans have alkaline midguts (pH 10–11) (Terra and Ferreira, 1994) and baculoviruses have evolved to tolerate and exploit this extreme microenvironment. The alkalinity of the insect midgut triggers the dissolution of OBs and the release of occluded virions into the midgut lumen. These released virus particles are, therefore, precisely defined as ODVs. Occluded ODVs are released from OBs within 12 min post entry into the insect midgut (Adams and McClintock, 1991) and once released into the midgut, ODVs must breach the peritrophic membrane (PM).

The PM is a net or lattice of chitin and protein that is produced along the length of the midgut such that it forms a hollow tube protecting the delicate midgut epithelium from direct contact with food particles. The PM is the insect equivalent of mucous, providing lubrication for food particle passage. The PM lattice has pore sizes ranging from 21 to 36 nm in diameter (Barbehenn and Martin, 1995). Small particles, such as digestive enzymes, can pass freely through the lattice into the endoperitrophic lumen to liberate peptides, sugars, and nutrients. The PM provides an immune defense by restricting the passage of larger particles such as bacteria, fungi, and viruses. To gain access to midgut tissues, ODVs must breach the PM through chance encounter of lesions or by releasing the PM-compromising viral protease called enhancins (Greenspan *et al.*, 1991; Hashimoto *et al.*, 1991; Hotchkin, 1981; Lepore *et al.*, 1996; Ohba and Tanada, 1983; Wang *et al.*, 1994). Enhancins are co-occluded with ODVs in the OB matrix (Greenspan *et al.*, 1991; Hashimoto *et al.*, 1991; Hotchkin, 1981; Lepore *et al.*, 1996; Ohba and Tanada, 1983; Wang *et al.*, 1994) or are present on ODV surfaces (Slavicek and Popham, 2005). Enhancins are metalloproteases, which cleave mucin-like proteins bridging chitin

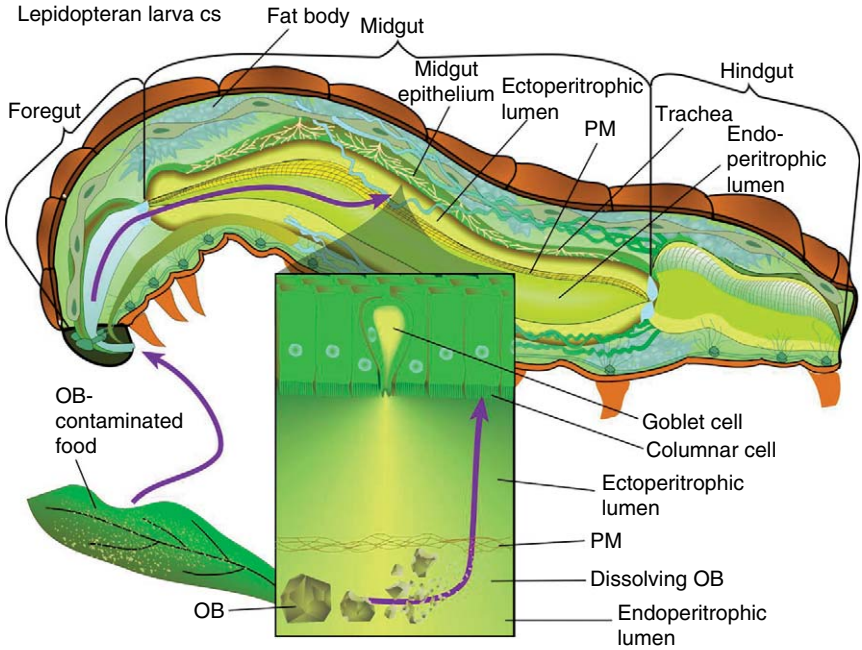

FIG 1. *Per os* infection by baculoviruses. A cross-sectional representation of the anatomy of an insect larva is depicted. A baculovirus OB enters by the *per os* route in contaminated food. OBs pass through the foregut and enter the midgut where they dissolve in the alkaline midgut lumen and release ODVs. The inset figure depicts the translocation of released ODVs past the peritrophic membrane (PM) to midgut columnar epithelial cells. The midgut region surrounded by the PM has been referred to as the endoperitrophic lumen and the region outside the PM has been referred to as the ectoperitrophic lumen.

strands in the PM lattice (Wang and Granados, 1997a,b). Even baculoviruses that do not encode an enhancin degrade the PM by other viral factors (Derksen and Granados, 1988). Enhancins are an example of specialized adaptation of baculoviruses to their insect hosts. The study of baculovirus enhancins is of great importance not only for viral biology but also for the fact that these proteins expose vulnerabilities in the insect immune system.

B. The Structure of OBs

Early on in the study of the *Baculoviridae* family, it was recognized that, based on OB morphology, there were two major divisions or genera; the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs).

The OB morphologies are illustrated in Fig. 2. The virions of both of these baculovirus groups were occluded in proteinaceous OBs that could be seen under compound light microscope. The OBs of NPVs are most easily seen due to their larger size and their light refractory polyhedral (multisided crystal) structure. The average diameter of NPVs is within the range of 800–2000 nm (Bilimoria, 1991). The OBs of GVs appear as dark granules and are comparatively more difficult to resolve on the light microscope. They are ovoid shaped and about 500-nm long and 200-nm wide (Boucias and Pendland, 1998). The OBs of NPVs are called polyhedra and the OBs of GVs are called granula. Following this nomenclature, the major protein component of polyhedra is polyhedrin and the major protein component granula is granulin. Polyhedrins and granulins are ~30 kDa in molecular weight. Both polyhedrin and granulin form a crystalline lattice that occludes virions. Dozens of virions are occluded in a polyhedron, while only a single virion is occluded in a granulum (Fig. 2). Although crystals are the natural state of polyhedrin and granulin, the atomic structures of neither of these proteins have been determined. The major reason for this is that polyhedra and granules form only inside the nuclei of insect cells. Abundant amounts of both of these proteins can be easily acquired but they have not been successfully solubilized and recrystallized into forms suitable for NMR or X-ray crystallography. X-ray diffraction has been done on OBs themselves (Bergold, 1963a; Harrap, 1972a) and these data along with electron microscopy (EM) studies suggest polyhedrin proteins contact each other at six points as nodular spheres. Despite the uniformity of the polyhedrin crystal, ODVs are randomly oriented within OBs. In addition, the interface between the ODV virion lipid bilayer envelope and the polyhedrin matrix contains a fibrous network of which the composition is unknown (Adams and McClintock, 1991). It has been suggested that virion envelopes themselves catalyze polyhedrin polymerization (Wood, 1980). The biology behind the occlusion of ODV virions in polyhedrin or granulin is not well understood but there are likely unique interactions among virion envelope proteins and the protein matrix of the OB.

Surrounding the OBs of NPVs and GVs is a glycoprotein multilayered lattice termed the calyx (Carstens *et al.*, 1992; Whitt and Manning, 1988), or the polyhedron membrane (Adams and McClintock, 1991) or the polyhedral envelope (PE) (Gross and Rohrmann, 1993; Gross *et al.*, 1994; Russell and Rohrmann, 1990). The PE is porous and does not resemble a conventional lipid bilayer virus envelope (Adams and McClintock, 1991; Harrap, 1972b; Robertson *et al.*, 1974). The PE is a protein/carbohydrate matrix that forms a lattice or net with hexagonal

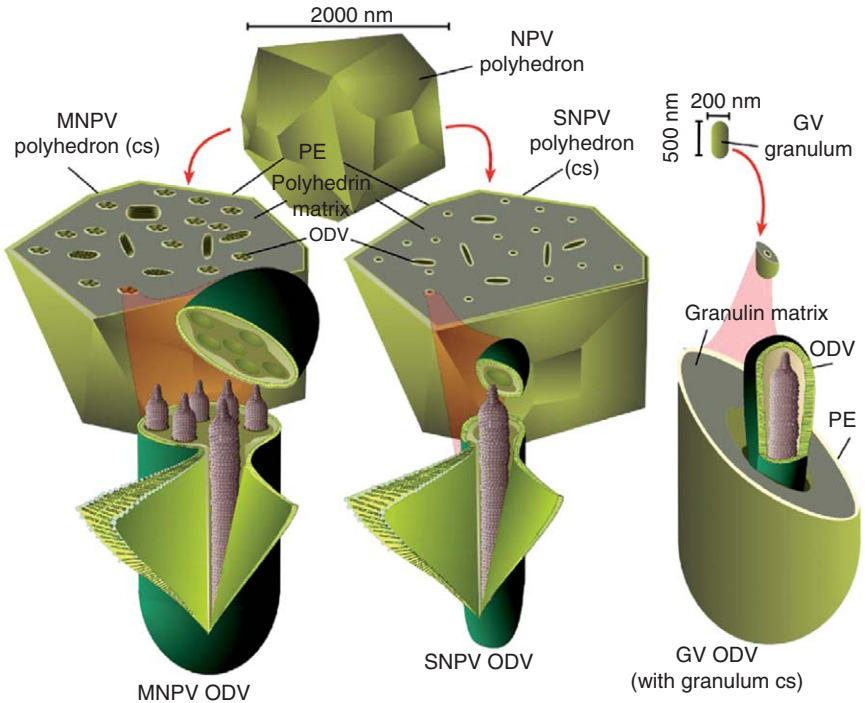

FIG 2. Major occlusion-derived virion forms. Three major OB phenotypes are illustrated in the background. The nucleopolyhedrovirus (NPV) OBs are larger than the *Granulovirus* (GV) OBs due to the fact that they contain multiple numbers of ODV virions. The OBs of GVs are capsule shaped and contain only single virions. The OBs of NPVs are multisided crystals or polyhedra. Some species of NPVs produce cuboidal OBs. The NPVs are further divided into the multiple nucleopolyhedroviruses (MNPVs) and single nucleopolyhedroviruses (SNPVs). The multiple (M) and single (S) designations are in reference to the number of nucleocapsids that are found in each virion. The ODVs of MNPVs, SNPVs, and GVs are depicted in the foreground. The ODVs are illustrated in dissected views and the GV ODV is illustrated as partially encapsulated.

pores ranging from 6 to 15 nm in diameter (Adams and McClintock, 1991; Harrap, 1972b). The primary PE carbohydrate sugars are hexoses (60%), pentoses (29%), uronic acids, and hexosamines (Minion *et al.*, 1979). The primary PE protein is a phosphoprotein called polyhedral envelope protein (PEP) or PP34. PEP is covalently linked to carbohydrates by disulfide bonds (Gombart *et al.*, 1989; Whitt and Manning, 1988). Early PE structures form in the nucleus as concentric rings (Adams *et al.*, 1977; Goldberg *et al.*, 2002). The PEs surround OBs during their formation and they are the last structure that physically separates the OB from its environment and, therefore, serves to protect the integrity of OBs during and after their release to the environment. The OBs of many baculoviruses are released in a bath of a viral protease. One of the more interesting modes of baculovirus transmission is through predators that consume virus-infected prey (Abbas and Boucias, 1984). The OBs survive predator digestive proteases and glycosidases such that viable OBs are dispersed in predator's feces. Presumably, the PE helps the OB to resist digestion from viral and nonhost enzymes. The PE is an elegantly designed structure for ensuring the protection and integrity of OBs until they are consumed by an insect host. The PE lattice is fine enough to restrict access of large enzymes to the OB matrix but would permit rapid permeation by anions from the alkaline host midgut. This would presumably lead to rapid decrystallization of polyhedrin or granulin, rupturing of the PE, and the release of ODV into the midgut. The complete release of ODVs from OB proteins may be further enhanced by OB-associated alkaline proteases that have been reported to be present in OBs from larvae (Eppstein and Thoma, 1975; Eppstein *et al.*, 1975; Langridge and Balter, 1981; Maeda *et al.*, 1983).

C. Infection of the Midgut Epithelium

On entering the ectoperitrophic space, ODVs diffuse to the midgut epithelium. The lepidopteran midgut epithelium is primarily composed of goblet and columnar cells. Goblet cells are involved in potassium ion transport from the hemolymph into the midgut and are not infected by baculoviruses (Adams and McClintock, 1991). ODVs primarily infect the predominant columnar cells, which are involved in secreting digestive enzymes and absorbing nutrients. On the luminal side, columnar cell surfaces are covered in brush border microvilli. ODVs have the specificity to bind to the apical ends of microvilli (Adams and McClintock, 1991). ODVs have lipid bilayer envelopes that fuse directly with midgut cell membranes (Haas-Stapleton *et al.*, 2004; Horton and Burand, 1993)

resulting in the release of nucleocapsids into the cytosol (Fig. 3). These nucleocapsids migrate to the nucleus where they are unpackaged and the viral DNA genome is released. Virus genes are expressed, products of structural genes are synthesized, DNA replication ensues, and new progeny viruses are assembled and released. In the primitive hymenopteran baculoviruses, new progeny virions are occluded and released after cell lysis back into the midgut lumen (Ackermann and Smirnoff, 1983; Garcia-Maruniak *et al.*, 2004; Lauzon *et al.*, 2004; Podgwaite *et al.*, 1984).

The insect midgut is a poor tissue for viruses to infect. Midgut cells are sloughed off regularly (Engelhard and Volkman, 1995) and are prone to apoptosis (Uwo *et al.*, 2002) and, it becomes a race against time for the baculoviruses to establish infection in these cells. In addition, midgut cells are in a cell cycle arrest that is not conducive for virus DNA replication. Tissues beyond the midgut columnar epithelium have actively dividing cells that are more suitable for virus replication. However, the ODV is narrowly specialized to infect midgut epithelial cells in an extreme alkaline environment and has been rarely observed to infect other cell types. Because insect viruses rely on chance encounter infection, maximal progenies are needed to ensure survival. It is a significant advantage for baculoviruses to infect tissues beyond the midgut as many more viral progeny can be produced in a given host. This advantage has driven baculoviruses to evolve a second, radically different, budded virus (BV) phenotype (Fig. 4). BVs are broadly specialized to infect many internal tissues of the host including tracheoblasts, hemocytes, and fat body. BV entry occurs by receptor-mediated, absorptive endocytosis (Volkman and Goldsmith, 1985), and acid-triggered fusion of BV envelopes with endosomal membranes (Blissard and Wenz, 1992; Leikina *et al.*, 1992). The existence of two virion phenotypes in one virus is a major distinction of the *Baculoviridae* family. In later sections, we will describe these virion phenotypes in more detail.

D. Infection of Tissues Beyond the Midgut

After ODVs infect midgut cells, BVs that are produced which bud from the basement membrane, penetrate the basal lamina and infect tissues of the hemocoel. Penetration of the basal lamina by BV is not well understood. Often EM images of this process show a clearing zone around BV as they traverse the basal lamina. The basal lamina is composed of protein, and it is possible that proteases produced by columnar epithelial cells are being directed by the virus to be released

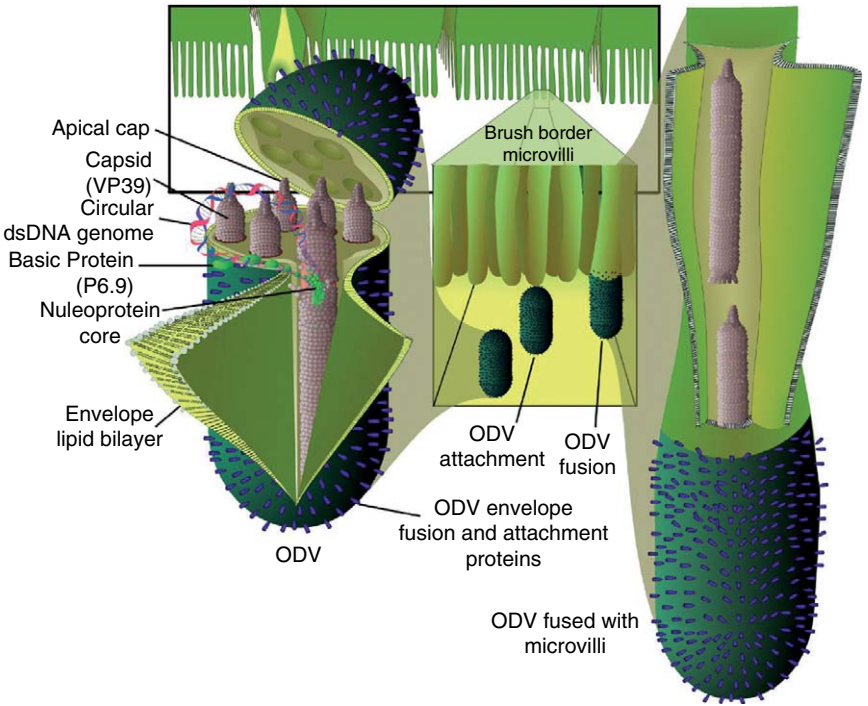

FIG 3. The ODV phenotype. The illustration on the left side represents a dissected view of the structure of the ODV. The DNA genome is shown expanding from the nucleocapsid to emphasize the presence of one viral genome in each nucleocapsid. This illustration is done in the context of an MNPV ODV. In the background and expanded into the center are representations of the insect midgut epithelium. The ODV-specific processes of attachment and viral envelope fusion with membranes of the brush border microvilli are shown. Expanded on the right hand side is resulting the release of ODV-nucleocapsids which are translocated up the microvillus into the midgut cell.

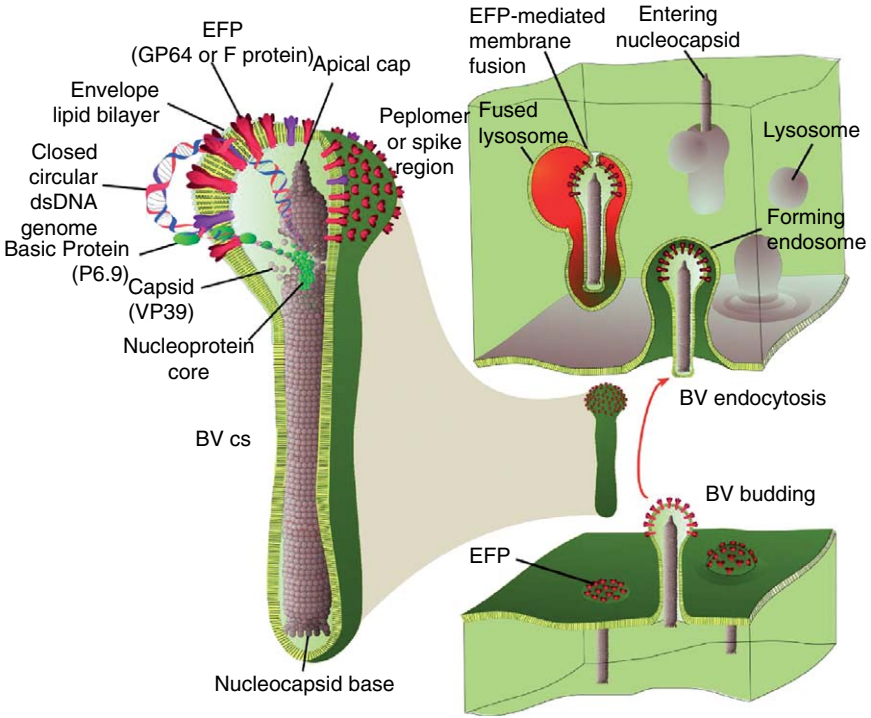

FIG 4. The BV phenotype. The illustration on the left side represents a dissected view of the structure of the BV. The DNA genome is shown expanding from the nucleocapsid in order to emphasize its presence in the nucleocapsid. The major BV envelope fusion proteins (EFPs), GP64 and F protein are shown at the upper peplomer end of the virion. This illustration is in the context of a group I NPV baculovirus. The right hand side illustrates the processes of BV egress from an infected cell (lower right) and BV infection of a new cell (upper right). Nucleocapsids bud out of the infected cell membrane where viral EFPs have concentrated. In budding, the virion acquires EFPs and the cell membrane as its virion envelope. The BV diffuses across to a new cell where it is taken into the cell by receptor-mediated endocytosis. The BV-containing endosome fuses with an acidifying lysosome. This pH shift triggers EFP-mediated envelope fusion with the endosomal membrane and release the BV nucleocapsid into the cytosol. The nucleocapsid then translocates to the nucleus.

at the basal lamina. In addition, a viral-encoded cathepsin (V-CATH) has been copurified with BV (Lanier and Volkman, 1998). In cancer metastasis, malignant tumor cells become invasive to tissues by producing cathepsins on their surfaces that degrade the extracellular matrix (Nomura and Katunuma, 2005; Yamaguchi *et al.*, 1990). V-CATH may have an analogous function enabling BV to be projected through the protein matrix of the basal lamina.

For most baculoviruses, the midgut epithelium does not serve as a major tissue of viral replication but those infected cells serve to produce the initial supply of BV needed to infect other susceptible cells and tissues in larvae (Fig. 5). Often baculoviruses completely bypass replication in midgut cells in a process which will be discussed in later sections. As a defense mechanism against viruses, the infected midgut cell is sloughed off and new cells are generated to aid in gut recovery. Permitting the midgut epithelium to recover allows the host to continue to eat and grow and, in turn, this allows for the virus to replicate and maximize production of progeny.

The major conduit for virus spread from the midgut is the tracheal system (Engelhard *et al.*, 1994; Kirkpatrick *et al.*, 1994; Washburn *et al.*, 1995). The tracheal system is the respiratory system of insects which begins on the outside as openings called spiracles along the lateral sides of insects. Tracheal tubes branch extensively as they lead from spiracles into the hemocoel. These branches transition abruptly into a network of very small tubes called tracheoles. Tracheoles are lined by tracheolar cells which can be 200–400 nm in length (Wigglesworth, 1984). Tracheoles impregnate nearly every tissue including the midgut epithelium. A midgut cell-derived BV infecting a tracheolar cell can bypass the basal lamina and its BV progeny will have access deep into the tissues of the hemocoel. To a lesser degree, midgut regenerative cells that lie basolateral to the midgut epithelium also have been shown to be sites of midgut cell-derived BV virus infection (Flipsen *et al.*, 1995). Once in the hemocoel, BV is further dispersed by infecting hemocytes. These cells circulate throughout the open circulatory system. Hemocytes are mostly involved in immune response and infection of these cells has the added effect of reducing the ability of the host to combat the virus infection.

An important tissue of baculovirus replication is the fat body that acts as the insect liver (Dean *et al.*, 1985) and is responsible for the storage and metabolism of lipids and sugars. It also produces vitellogenin, the primary egg protein. In the lepidopteran larva, the fat body is an amorphous and protuberant organ running throughout the insect. The tissue is highly accessible to BV and the energy-rich cells of

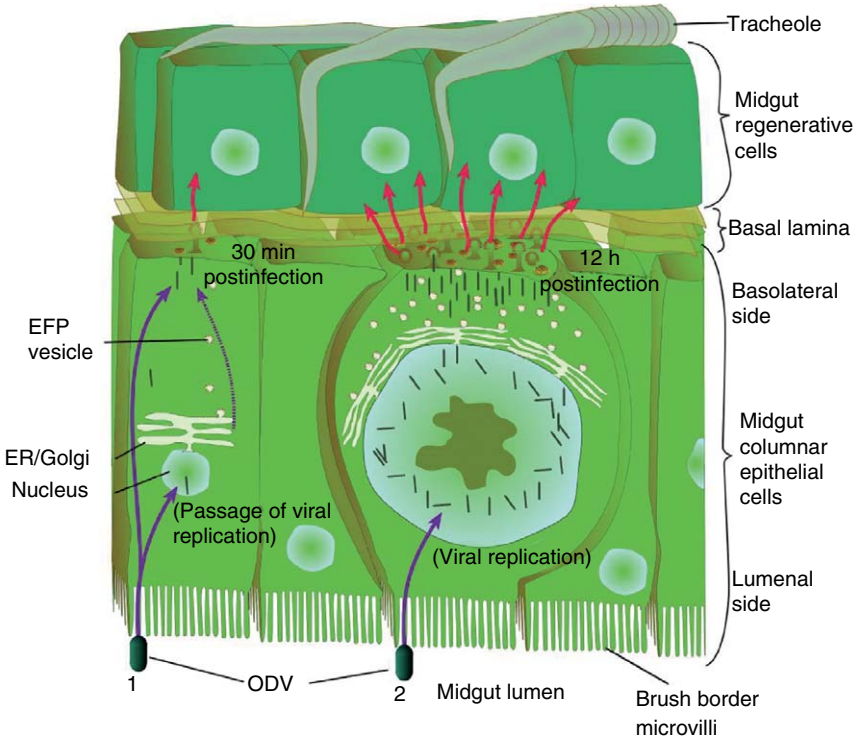

FIG 5. Two modes of infection in the midgut. Most baculoviruses use the midgut only to produce the first generation of BV progeny that bud out from the basal lateral side of the midgut cell to infect other tissues. ODVs that enter midgut cells may (1) bypass virus replication in the midgut cell or (2) initiate virus replication. When MNPVs bypass replication, most nucleocapsids translocate to the basolateral side of the midgut cell. One or a few nucleocapsids enter the nucleus, unpackaged, and expresses envelope fusion protein (efp) genes. EFP proteins are translocated via the endoplasmic reticulum (ER) and Golgi to the basal lateral side of the cell where they meet up with nucleocapsids which then bud out. This is the passage effect. When MNPVs replicate in the midgut cell significantly greater numbers of BV are produced. However, this mode of infection requires 8–12 h vs 30 min when the virus bypasses viral replication.

the fat body are ideal for producing abundant virus progeny. The fat body often becomes engorged with OBs such that the insect takes on an opalescent white, puffy appearance prior to death.

Exploiting the inner tissues of the insect host gives the virus the advantage of being able to produce enormous numbers of progeny. However, this virus is trapped inside the host, which is not necessarily a problem given that predators will often target slow moving, sick

larvae and disperse OBs after feeding (Abbas and Boucias, 1984). Cannibalism among some insect species may also contribute to horizontal transmission. These modes of transmission are not efficient and most baculoviruses release OBs from the host by virus-induced tissue liquefaction and the cuticle rupture after death. These processes are facilitated by the synergistic interaction of the viral protease, V-CATH (Ohkawa *et al.*, 1994; Slack *et al.*, 1995), and the viral chitinase, ChiA (Hawtin *et al.*, 1997). As the liquefied remains ooze out from the dead host, OBs are broadly dispersed along food surfaces that are eaten by a new host.

III. Cytopathology and Virion Phenotypes

A. The Early Phases

With the exception of some cell types, the baculovirus replication cycle includes a nonlytic phase of BV production, followed by ODV production and ending with the lytic release of OBs. These phases have been illustrated in Fig. 6. The baculovirus infection cycle stages occur at predictable time intervals. The length of those time intervals is virus species specific and many baculoviruses require longer replication times than the model *Ac*MNPV virus. The baculovirus replication cycle begins almost immediately after the nucleocapsid delivers the viral genome into host cell nucleus. Viral immediate early genes are expressed within 30 minutes postinfection (Chisholm and Henner, 1988) and their protein products along with virion-associated proteins begin to manipulate the host cell to become competent for DNA replication. The structure of the nucleus is modified resulting in its expansion or nuclear hypertrophy. For *Ac*MNPV, this process can be observed in the first 6 h. An electron dense, irregular-shaped, granular region begins to form in the center of the nucleus (Harrap, 1972a; Young *et al.*, 1993). This region is called the virogenic stroma and it is the site of viral RNA transcription, DNA replication, and nucleocapsid assembly. Concomitant with the appearance of the virogenic stroma is the movement of cellular heterochromatin to the edges of the nucleus along the inner nuclear membrane (INM) (Williams and Faulkner, 1997). Heterochromatin is highly condensed, histone-associated host genomic DNA. The rearrangement of the nucleus has been shown to be mediated by viral interaction with tubulin (Volkman and Zaal, 1990). The virus infection causes the nucleus to partition itself into two major regions, the virogenic stroma and the peristromal space or nuclear ring zone.

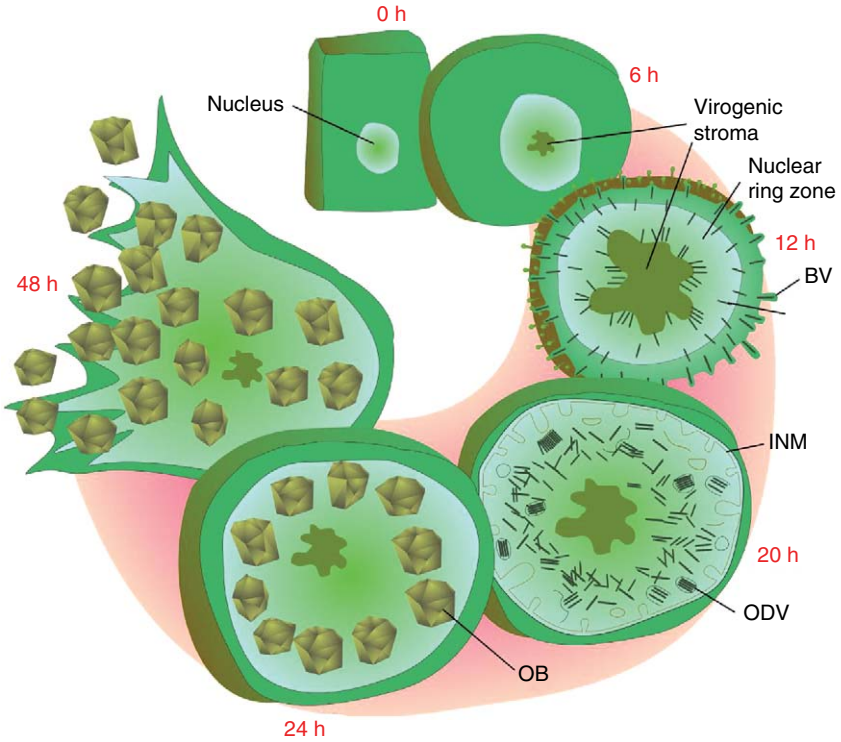

FIG 6. Baculovirus infection cycle. Several phases of virus replication are illustrated beginning with the rounding of newly infected cells and finishing with the lytic release of OBs. Indicated times are relative to the infection cycle of *Ac*MNPV. The purpose of the figure is to illustrate the progression of phases from BV production to ODV production. Nucleocapsids are initially translocated to the cell membrane for BV production and later become retained in the nuclear ring zone for ODV production. INM is in reference to the inner nuclear membrane (INM) which provides the ODV envelope.

The heterochromatin on the outer edges of the nuclear ring zone gradually disappears over time (Williams and Faulkner, 1997). As the virogenic stroma expands, cavities or lacunae form inside it and nucleocapsids are assembled along the edges inside these cavities (Young *et al*., 1993). The stroma is composed mostly of RNA with DNA and a protein scaffolding that supports RNA and DNA complexes (Young *et al*., 1993). At about 12 hours postinfection (hpi), the virogenic stroma expands to fill most of the nucleus. Between 12 and 20 hpi, the BV virion phenotype is produced. The earliest made nucleocapsids in

the baculovirus infection migrate out of the virogenic stroma, across the ring zone and to the nuclear membrane. Nucleocapsids are transported through the nuclear membrane and migrate across the cytosol to the cell membrane where they bud out. The process involved in translocation of nucleocapsids is not well understood although there is growing evidence of cellular actin involvement (Beniya *et al*., 1998; Braunagel *et al.*, 2001; Lanier and Volkman, 1998; Lanier *et al*., 1996; Lu *et al*., 2002, 2004). In the process of budding, nucleocapsid are enveloped in the host membrane and acquire virus-encoded proteins in their envelopes. In midgut columnar epithelial cells, BV nucleocapsids show basal polarity (Keddie *et al*., 1989).

B. The Budded Virus

BV virions only contain single nucleocapsids and their envelopes appear loose such that cytoplasmic proteins may be present in the space between the nucleocapsid and the envelope. A computer illustrated structure of BV is presented in Fig. 4. The BV envelope contains the major viral glycoproteins, GP64/GP67 (Whitford *et al.*, 1989), and fusion protein or F protein (Lung *et al.*, 2002; Pearson *et al*., 2000, 2001; Westenberg *et al.*, 2002, 2004; WF *et al.*, 2000). These proteins have also been called ENV proteins as they mediate budding, attachment, and entry of BVs (Blissard and Wenz, 1992; Hefferon *et al.*, 1999; Oomens and Blissard, 1999). In transmission EM preparations, BV envelopes are bulbous at one end and the surface is serrated (Adams *et al*., 1977). The serrations or notches are called peplomers and are thought to contain GP64 and F protein given that antibodies specific for BV envelope proteins localize in the peplomer region (Volkman *et al.*, 1984). BV envelope proteins are *N*-glycosylated and are sorted in the endoplasmic reticulum (ER) in order to be transported to the cell membrane. The GP64 protein is only present among group I NPVs and the F protein of these viruses has apparently lost its ENV functions (Whitford *et al.*, 1989). The group II NPVs and GVs all encode for F proteins (Bulach *et al.*, 1999; Hashimoto *et al.*, 2000; Hayakawa *et al.*, 1999; Herniou *et al.*, 2001; Lange and Jehle, 2003; Luque *et al.*, 2001; Slack *et al*., 2004; Wormleaton *et al*., 2003; Zanotto *et al*., 1993). F protein differs from GP64 in that it requires proteolytic cleavage before it can be functional (Westenberg *et al.*, 2002). The only baculoviruses that do not have F protein homologues are the hymenopteran baculoviruses *Nele*NPV (Lauzon *et al.*, 2004) and *Nese*NPV

(Garcia-Maruniak *et al.*, 2004). These baculoviruses likely do not to produce BVs.

C. The Occlusion-Derived Virus

Beginning at 20 hpi by *Ac*MNPV, the virogenic stroma recedes and the infection shifts to favor the production of ODVs. A computer illustrated ODV structure is presented in Fig. 3. The virogenic stroma becomes more condensed and the nuclear ring zone expands to accommodate accumulating nucleocapsids. Nucleocapsids in the nuclear ring zone become ODVs as they acquire lipid bilayer envelopes. The retention and formation of enveloped virions in the nucleus is a unique biological phenomenon. The ODV envelope is a lipid bilayer that resembles, but is not identical to, the INM in composition (Braunagel and Summers, 1994). The ODV envelope is more rigid than the BV envelope due to the presence of more saturated fatty acid phospholipids. The ODV envelope also contains phosphatidylcholine instead of phosphatidylserine which is found in BV envelopes.

There has been some controversy as to whether the ODV envelope is synthesized *de novo* or whether it is acquired from the INM. The time of ODV morphogenesis coincides with the appearance of membranous structures which are believed to be the precursors of ODV envelopes (Adams and McClintock, 1991; Fraser, 1986; Hong *et al.*, 1994). Considerable evidence has been generated to favor an INM source which invaginates as microvesicles into the ring zone late in infection (Braunagel *et al.*, 1996a; Hong *et al.*, 1994). It should be pointed out that even by this mechanism, the virus would have to induce "*de novo*" INM synthesis to ensure enough material for nucleocapsid envelopment. The ODV envelope contains a number of integral and associated viral proteins. These proteins are more diverse than BV envelope proteins. They are mostly nonglycosylated and they are transported to the ODV envelope by unique mechanisms. The ODV envelope proteins will be described more in detail in later sections of this chapter. Unlike BV nucleocapsids, ODV nucleocapsids are also packaged in a proteinaceous virus derived tegument prior to envelopment. After occlusion, this tegument becomes more condensed around nucleocapsids (Knudsen and Harrap, 1976).

Soon after ODVs begin to appear in the ring zone, occlusion begins to occur and by 24 hpi, fully formed OBs are present. By 48 hpi, OBs are liberated from the nuclei of infected cells by lytic release. The viral protein V-CATH that has been previously mentioned to be

important for liquefaction of the host also plays a role in releasing OBs from infected cells. The OBs of V-CATH deletion mutant *Ac*MNPV viruses are not released from infected cells in cell culture. V-CATH has been linked with appearance of free OBs in the hemolymph of *Bm*NPV-infected insects (Suzuki *et al.*, 1997). P10, which forms fibrillar bodies in the nucleus and cytoplasm, has been shown to interact with host cell microtubules (Patmanidi *et al.*, 2003) and also to be involved in cell lysis (Williams *et al.*, 1989).

D. Multiple Occlusion and Bypass of Midgut Cell Replication

The NPVs are further divided into single and multiple occlusion "subphenotypes" (SNPV and MNPV). The virions of SNPV contain single nucleocapsids and the virions of MNPV contain multiple nucleocapsids. MNPV virions can contain more than 40 nucleocapsids (Kawamoto and Asayama, 1975). The MNPVs have, however, only been observed in lepidopteran baculovirus isolates (Rohrmann, 1986). It could be that there are not enough examples of nonlepidopteran baculoviruses. While it has been somehow debatable whether to retain the "S" and "M" designations in baculovirus nomenclature, single and multiple capsid subphenotypes have been suggested to be anomalous and with no genetic basis or biological importance (Herniou *et al.*, 2003). The more compelling argument for the distinction of SNPV and MNPV are biological studies that demonstrate the advantage of the MNPV over the SNPV in oral infectivity (Washburn *et al.*, 1999, 2003b). These studies are difficult to do as the genes for multiple and single occlusion are not yet known and comparisons must be made between related but not identical MNPV and SNPV baculoviruses.

One of the earliest proposed theories for multiple occlusions was that it was a way for baculoviruses to bypass replication in the midgut and to deliver virions to the hemocoel via the tracheoles or other cell types (Keddie *et al.*, 1989). This has been termed the "passage effect" (Granados and Lawler, 1981) (Fig. 5). It was demonstrated that when MNPV ODVs infect midgut cells, some nucleocapsids bypass the nucleus, migrate to the basal lamina side (basement membrane) of the midgut cell, and bud through to infect other cell types (Adams *et al.*, 1977; Granados and Lawler, 1981). This can occur because other nucleocapsids from the MNPV ODV virion enter the nucleus, uncoat, and express early genes including the BV envelope fusion protein gene, *gp64*. The *gp64* gene has a bipartite early and late promoter and the GP64 protein is produced by 6 hpi by *Ac*MNPV (Blissard

and Rohrmann, 1989). The GP64 protein accumulates on the basement membrane of midgut cells (Keddie *et al.*, 1989) and enables ODV-derived nucleocapsids to bud as infectious BV. This theory has been supported by the demonstration that the elimination of the *gp64* early promoter reduces the oral infectivity of *Ac*MNPV (Washburn *et al.*, 2003a). F proteins such as Ld130 of *Ld*MNPV are produced at late times postinfection (Pearson *et al.*, 2001). However, the promoter structures of the *f protein* genes of *Ld*MNPV and *Spodoptera exigua* multiple nucleopolyhedrovirus (*Se*MNPV) contain early and late virus transcriptional motifs (IJkel *et al.*, 1999; Kuzio *et al.*, 1999).

Group I MNPVs such as *Ac*MNPV retain F protein homologues (*Ac23*) that are unable to perform any fusion function in the BV (Monsma *et al.*, 1996) but which have been shown to enhance baculovirus oral infectivity (Lung *et al.*, 2003). This is intriguing given that the *Ac*MNPV F protein present in ODV envelopes (Braunagel *et al.*, 2003). The presence of F protein in the ODV and its enhancement of oral infectivity points to F protein being an important ODV protein. ODVs enter midgut cells by direct fusion of the virion envelope with the midgut cell membrane (Horton and Burand, 1993) and the simplest explanation is that F protein is involved in the fusion of ODV envelopes with the midgut cell membrane. Presumably the F protein is C-terminally anchored in the ODV envelope with its N-terminus exposed.

Another possible role of F protein may involve the passage effect. After fusion of the ODV with the midgut cell membrane, there would be opportunity for F protein transfer into midgut cell membranes. F protein could be absorbed by pinocytosis and subsequently translocated along side nucleocapsids to the basement membrane. This would be a rapid mechanism for ODV virions to pass through the midgut cell cytoplasm and to form BV at the basement membrane. GVs such as *Epap*GV appear only to infect midgut cells but completely evade midgut replication and infect fat body, epidermis, and tracheolar tissues (Goldberg *et al.*, 2002). GVs have a single nucleocapsid in each OB and the early ENV protein expression strategy would not work. F protein membrane transfer and translocation may offer these viruses a mechanism of passage through the midgut. The F proteins of group II NPVs and GVs have not yet been identified in the ODV. It has been noted that the nonfunctional *Ac*MNPV F protein homologue has low identity to the functional F protein homologues of group II NPVs and GVs (Lung *et al.*, 2002). However, F protein homologues of group I NPVs, group II NPVs, and GVs have common structural features (Lung *et al.*, 2002, 2003) and thus would be expected to be also localized in the ODV envelope.

It has been postulated that *gp64* was acquired during baculovirus evolution (Pearson *et al*., 2000) and it is established that GP64 is not present in ODV envelopes (Hohmann and Faulkner, 1983; Keddie *et al*., 1989; Wang and Kelly, 1985). The *gp64* gene has a common origin with tick-transmitted mammalian arboviruses (Morse *et al*., 1992) while the *f protein* gene has an insect origin (Lung and Blissard, 2005). The different origins of these proteins would influence how they function in the virus.

There are other explanations of the evolution of multiple occlusions. For example, ODV nucleocapsids may be delivering associated proteins that augment virus replication. Multiple occluded nucleocapsids would effectively deliver more of these factors. In addition, delivering several copies of viral genomes decreases the chances that mutation will compromise infection. After waiting for long periods of time in the environment, mutations that occur would be random and multiple genome copies could complement each other.

IV. ODV Nucleocapsid Proteins

A. The Nucleocapsid

The nucleocapsids of the ODV and BV have many similarities as they both contain complete viral genomes and have major proteins in common. The ODV nucleocapsid is illustrated in Fig. 7. Baculovirus nucleocapsids are 40–70 nm in diameter and 250–400 nm in length (Boucias and Pendland, 1998). The size of the viral genome determines the length of the nucleocapsid. Nucleocapsids are polar, with a claw or base on one end and a nipple or apical cap on the other end (Federici, 1986; Fraser, 1986). The apical cap is oriented toward the virogenic stroma during nucleocapsid assembly (Fraser, 1986). It is also found associated with the forming envelopes of the ODV and is oriented on the peplomer end of BV (Adams and McClintock, 1991; Fraser, 1986). The nucleocapsid is composed of an outer protein capsid surrounding a nucleoprotein core (Arif, 1986). The most abundant structural protein of the nucleocapsid is VP39 (Blissard *et al.*, 1989; Guarino and Smith, 1990; Pearson *et al.*, 1988; Thiem and Miller, 1989b) with monomers arranged in stacked rings around the nucleoprotein core (Federici, 1986).

In addition to the major VP39 capsid protein, there are number of other minor but important capsid-associated proteins. The PP78/83 (ORF1629) protein is a phosphoprotein that was first identified in viral

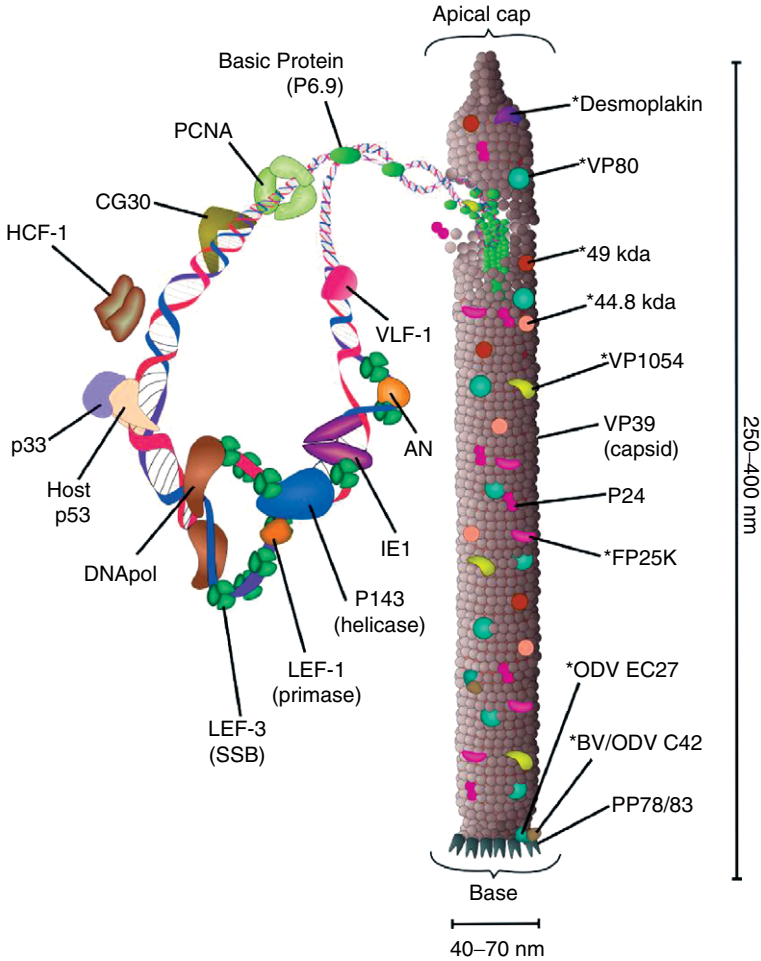

FIG 7. Proteins of the ODV nucleocapsid. Some of the proteins found associated with the ODV nucleocapsid are illustrated. The figure is in the context of *Ac*MNPV. The "*" indicates that the proteins for which the specific location on the nucleocapsids has not been confirmed. We have also placed nucleocapsid-associated proteins which have DNA-binding activities or potential transcriptional activation activities on the viral genome. The intent of the figure is to reveal the nucleocapsid as a complex structure of multiple protein types instead of simply protein sheath surrounding Basic Protein and the viral genome.

fractionation studies as a BV/ODV envelope protein and/or ODV tegument protein (Pham *et al.*, 1993). EM studies later revealed that PP78/83 is associated with the nucleocapsid base (Russell *et al.*, 1997; Vialard

and Richardson, 1993). Discovery that PP78/83 nucleates actin polymerization has lead to the suggestion that this protein is involved in nucleocapsid translocation into the nucleus after infection (Lanier and Volkman, 1998). Only 19 PP78/83 homologues are found among 29 baculovirus genomes. PP78/83 appears to play a critical and a multifunctional role (Kitts and Possee, 1993). The study of a number of baculovirus proteins often reveals multifunctional roles. The name PP78/83 is in reference to the unphosphorylated and phosphorylated state of this protein (Vialard and Richardson, 1993). PP78/83 has also been shown to be a component of the virus-encoded RNA polymerase complex (Iorio *et al.*, 1998). It is difficult to find a linkage between transcription and virion translocation; however, PP78/83 would be translocated to the nucleus to participate in the transcriptome.

BV/ODV-C42 is a capsid-associated protein that was shown by yeast two-hybrid analysis to interact directly with PP78/83 (Braunagel *et al.*, 2001). Homologues to the *bv/odv-c42* gene are present in all sequenced baculoviruses except *Cuni*NPV. BV/ODV-C42 has a conserved nuclear localization signal and localizes along with PP78/83 in the DNA-rich virogenic stroma. BV/ODV-C42 has not been specifically localized to the capsid base. However, we consider this likely given its association with PP78/83. BV/ODV-C42 interacts directly with another highly conserved capsid-associated viral protein called ODV-EC27 (Braunagel *et al.*, 2001). ODV-EC27, which is also associated with the ODV envelope, is involved in regulating the cell cycle and will be covered in detail in following sections. It is possible that BV/ODV-C42, ODV-EC27, and PP68/83 are found associated together as complex at the nucleocapsid base.

The VP80 protein is a capsid-associated structural protein that was first identified as P87 in *Op*MNPV (Muller *et al.*, 1990). Other homologues were characterized in *Ac*MNPV (VP80) (Lu and Carstens, 1992) and *Cf*MNPV (P82) (Li *et al.*, 1997). Gene homologues to the *vp80* gene are only found in NPV genomes. The *vp80* gene is transcribed late in infection and the protein localizes in the nucleocapsids of BV and ODV (Li *et al.*, 1997; Muller *et al.*, 1990). The VP80 homologue of *Cf*MNPV has 72 and 82 kDa molecular weight protein forms (Li *et al.*, 1997) and only the 82-kDa protein is associated with ODV nucleocapsids. The 82-kDa form is the result of posttranslational modification given that this protein has a predicted mass of 71.2 kDa. *N*-Glycosylation was eliminated as a possibility (Li *et al.*, 1997) and phosphorylation is an alternative explanation given that this protein has 52 potential phosphorylation sites. Phosphorylation also is prevalent with other capsid-associated proteins such as PP78/83.

The capsid-associated protein P24 is conserved among some but not all NPVs and GVs. The *p24* gene is an auxiliary gene as it is absent from variants of *Ld*MNPV (Slavicek and Hayes-Plazolles, 2003). It was confirmed by EM and virus fractionation immunoanalysis that P24 was a component of the nucleocapsids of BV and ODV (Wolgamot *et al.*, 1993). The P24 homologue of *Splt*NPV is present only in the ODV nucleocapsids and migrates in SDS-PAGE at significantly higher molecular weight than expected (79 kDa instead of 36 kDa) (Li *et al.*, 2005c). It was suggested that this protein is a homodimer in ODV given that the 36-kDa monomeric form was detected in infected cell lysates.

B. Nucleoprotein Core

Within the nucleocapsid is a nucleoprotein core composed of supercoiled viral DNA and viral protein. The predominant protein in the nucleoprotein core is a small peptide with a p*I* value of 12 that is aptly named, Basic Protein. It is has been suggested that the positively charged arginine residues of Basic Protein interact with the negatively charged DNA backbone to enable its condensation in the nucleocapsid (Kelly *et al.*, 1983). Basic Protein is protamine-like (Tweeten *et al.*, 1980) and has been called P6.9 or VP12 depending on the baculovirus (Wilson and Price, 1988; Wilson *et al.*, 1987). Basic Protein is also a phosphoprotein and its phosphorylation is inhibited by the presence of Zn^{2+} (Funk and Consigli, 1992, 1993; Wilson and Consigli, 1985). When nucleocapsids are unpackaged in a new cell, cellular chelators remove Zn^{2+} which promotes Basic Protein to be phosphorylated by capsid-associated kinases (Funk and Consigli, 1993; Miller *et al.*, 1983; Wilson and Consigli, 1985). The resulting change in charge dissociates Basic Protein from viral DNA which then unpackages from the nucleoprotein core (Funk and Consigli, 1993; Wilson and Consigli, 1985). This unpackaging may occur at nuclear pores or in the nucleus. Ultimately, the viral genome is delivered into the nucleus where it is bound up by host histones (Wilson and Miller, 1986).

On the basis of pH and salt-dissociation properties, it was suggested that there is only one major viral protein that is associated with packaged viral DNA (Young *et al.*, 1993). However, a significant number of ODV-associated proteins have DNA-binding activity and are thus likely minor constituents of the packaged nucleosome.

The *Ac*MNPV ORF59 gene is predicted to encode for an 8.2-kDa unnamed protein that is associated with ODV nucleocapsids (Braunagel *et al.*, 2003). This protein bears strong homology to the *Escherichia coli*

protein, ChaB, and we suggest naming this protein, V-ChaB. There are 20 V-ChaB homologues among the group I and group II NPVs. The structure of the *E. coli* ChaB has been solved and it has been suggested that ChaB is involved in the regulation of divalent cation transport (Osborne *et al.*, 2004). The most important divalent cation in the nucleocapsid is Zn^{2+} (Funk and Consigli, 1992, 1993; Wilson and Consigli, 1985) and perhaps V-ChaB protein is involved in the transport of this cation during the condensation of the viral genome. The observed condensation of the tegument after ODV occlusion (Knudsen and Harrap, 1976) may be facilitated by the removal of ions from the ODV.

C. Nucleocapsid Proteins Involved in DNA Packaging

There is evidence that some capsid-associated proteins are involved in nucleocapsid assembly and DNA packaging. VP1054 is an essential 42-kDa baculovirus protein that is required for capsid assembly and the arrangement of VP39 monomers (Olszewski and Miller, 1997a). Temperature sensitive (Ts) mutants of the VP1054 protein produce spherical capsids in the virogenic stroma instead of tubular capsids (Olszewski and Miller, 1997a). Association of this protein with nucleocapsids (Braunagel *et al.*, 2003) may suggest interaction with VP39 during the formation or stacking of VP39 oligomer rings. In one model of nucleocapsid assembly, the capsid base and main body are preassembled in specific locations in the virogenic stroma (Fraser, 1986). The nucleoprotein passes through the apical cap and is supercoiled as it is packaged into empty capsid tubes. The supercoiling of viral DNA into the capsid has not been characterized. The nucleocapsid-associated protein, desmoplakin (*Ac*MNPV ORF66), has some homology to type I topoisomerases and could be involved in mediating the supercoiling of DNA. There are 55 homologues of baculovirus desmoplakins shared among 29 baculovirus genomes. Type I topoisomerases are found associated with the nucleocapsids of *Vaccinia virus* and it has been shown that these proteins accelerate the expression of early viral genes (Da Fonseca and Moss, 2003).

Several nucleocapsid-associated proteins have been characterized to have activities suggestive of involvement in viral DNA packaging. Alkaline nuclease (AN or Alk Exo) is a highly conserved and essential nucleocapsid-associated protein that may be involved in the processing of the viral genome into nucleocapsids by removing aberrant DNA branched structures from condensing DNA (Li and Rohrmann, 2000; Okano *et al.*, 2004).

Very late factor-1 (VLF-1) is a protein that binds to burst elements on delayed late gene promoters and is associated with enhancement of delayed late gene expression (McLachlin and Miller, 1994; Mistretta and Guarino, 2005; Yang and Miller, 1998b). VLF-1 is essential for virus replication (Li *et al.*, 2005b; Vanarsdall *et al.*, 2004; Yang and Miller, 1998a) and it is associated with BV and ODV nucleocapsids (Yang and Miller, 1998b). The VLF-1 protein binds to cruciform DNA structures and may have a role in processing of the viral genome during nucleocapsid assembly (Mikhailov and Rohrmann, 2002b). On the basis of the DNA-binding activities, VLF-1 and AN would be expected to be associated with the viral genome in the nucleoprotein core region. The persistence of VLF-1 and AN with ODV nucleocapsids may point to additional functions for these proteins. Perhaps VLF-1 binding to burst elements blocks early transcription of very late promoters so that essential early promoters are favored.

D. DNA Replication Proteins in the Nucleocapsid

As has been discussed earlier, baculoviruses have evolved to minimize or bypass viral replication in columnar epithelial cells. One of the reasons for this is that columnar epithelial cells are in an arrested state. Many of the host DNA replication factors are not present in arrested cells and this is a challenge for replication of a large genome DNA virus. The nucleocapsids of ODVs have consequentially evolved to carry with them viral DNA replication factors along with the viral genome. The six core baculovirus DNA replication proteins are immediate early 1 (IE-1), DNA polymerase (DNApol), P143 (helicase), LEF-1 (primase), LEF-2 (primase cofactor), and LEF-3 (single-stranded DNA-binding protein or SSB) (Kool *et al.*, 1994; Lu and Miller, 1995b). ODV nucleocapsids contain all of these proteins but for LEF-2 (Braunagel *et al.*, 2003). The absence of LEF-2 is surprising given its oligomerization with LEF-1 (Evans *et al.*, 1997; Hefferon and Miller, 2002) and its stabilization of LEF-1 binding to DNA (Mikhailov and Rohrmann, 2002a). BV nucleocapsids do not contain IE-1 or P143 (Braunagel *et al.*, 2003).

All of the core DNA replication factors bind to DNA and the binding by DNApol and P143 is inferred by their functions in DNA synthesis and in strand separation, respectively. IE-1 binds to replication origin DNA sequences called homologous repeats (hrs) (Kovacs *et al.*, 1992; Pearson *et al.*, 1992). LEF-3 binds to single-stranded DNA (Hang *et al.*, 1995) and binds to P143 (Wu and Carstens, 1998) and to IE-1 (Hefferon and Miller, 2002). The baculovirus exonuclease AN has been shown to bind to DNA and to complex with LEF-3 (Mikhailov *et al.*, 2003).

The location of DNA replication factors in the ODV nucleocapsid has not been determined. However, their DNA-binding activities and co-interactions would lead to the conclusion that these factors are bound to the viral genome in the nucleoprotein core of the nucleocapsid.

Other ODV capsid-associated proteins with potential involvement in DNA replication include host cell factor HCF-1 (Braunagel *et al.*, 2003) and viral proliferating cell nuclear antigen (PCNA) (O'Reilly *et al.*, 1989). HCF-1 is required for *Ac*MNPV DNA replication in *Trichoplusia ni* cell lines (Lu and Miller, 1995a) and it augments the ability of this virus to orally infect *T. ni* larvae (Lu and Miller, 1996).

PCNA, which functions as a DNA replication factor has strong DNA-binding tendencies and is probably part of the nucleosome. Eukaryotic PCNAs have been correlated with DNA synthesis, DNA repair, and cell cycle regulation (Bravo, 1986; Kurki *et al.*, 1986; Madsen and Celis, 1985; Solomon *et al.*, 2004). The viral PCNA is localized in viral DNA replication complexes (Iwahori *et al.*, 2002) and is not essential (Iwahori *et al.*, 2004) or stimulatory (Kool *et al.*, 1994) for viral DNA replication in insect cell lines. In cell culture, the host PCNA appears to compensate for absence of the viral PCNA (Iwahori *et al.*, 2004). Supplementing the ODV virion with viral PCNA may be advantageous to the virus as insect midgut cells produce low levels of cellular PCNA (Zudaire *et al.*, 2004).

E. DNA Repair Proteins of the Nucleocapsid

One of the major antagonists to the viability of baculoviruses is exposure to light and when absorbed by the virions, it changes the energetic state of proteins and DNA. This leads to uncontrolled covalent bond breakages and functional inactivation. In one study examining the effects of time, temperature and light, the latter was the major factor influencing viability (Jarvis and Garcia, 1994). Ultraviolet (UV) light only represents 5% of sunlight energy but is the form of energy that most damages baculovirus viability (Shapiro *et al.*, 2002). Baculovirus OBs persist in the environment for long periods of time and are frequently exposed to UV light. Exposure of OBs to UV light results in dramatic decrease of oral infectivity (Shapiro *et al.*, 2002). Natural reservoirs of viable baculovirus OBs are most prevalent on plant stems and nonlight exposed surfaces (Raymond *et al.*, 2005).

When insect cells are exposed to UV radiation and subsequently are infected with UV-inactivated baculoviruses, these cells are able to recover viral viability (Witt, 1984). It was hypothesized that UV induces synthesis of host enzymes that enable repair of UV-damaged

viral genomes. Some baculoviruses may have acquired the host DNA repair genes such as viral-three prime repair exonuclease (V-TREX) and photolyase. Homologues to the *v-trex* gene have only been identified in *Ag*MNPV (Slack and Shapiro, 2004; Slack *et al.*, 2004), *Cf*MNPV (Yang *et al.*, 2004), and *Cf*DEFNPV (Lauzon *et al.*, 2005). The *v-trex* gene may have been acquired from the host given its homology to insect genes (Slack *et al.*, 2004). Although there is no direct evidence of virion localization, V-TREX overproduction leads to significantly increased levels of associated 3′ to 5′ exonuclease activity in BV (Slack and Shapiro, 2004). The astounding success of *Ag*MNPV as viral insecticide may be partially attributed to the resistance of this virus to UV damage in the field. Another example of a candidate baculovirus DNA repair enzyme is a photolyase that has been identified in the genomes of *Tn*SNPV (Willis *et al.*, 2005) and *Chch*NPV (van Oers *et al.*, 2005). The baculovirus photolyase and V-TREX proteins point to a selective pressure for baculoviruses to acquire DNA repair enzyme genes.

It would be advantageous for ODV virions to carry DNA repair proteins so that lethal mutations can be repaired immediately after the genome is released into a new host cell. ODV virions carry with them a number of proteins that could be involved in DNA repair. For example, damaged DNA strands could be removed by the 5′ to 3′ activities of the exonuclease AN. The DNApol complex proteins could also play a role in the resynthesis of damaged DNA.

It is possible that the viral PCNA is involved in DNA repair for viral genomes after infection. In some systems, PCNA has been shown to be specifically involved in the repair of UV-damaged DNA (Aboussekhra and Wood, 1995; Li *et al.*, 1996).

The ODV-associated peptide product of *Ac*MNPV, ORF79, has 20 other baculovirus homologues and is very similar to a family of UvrC intron-encoded nucleases (URI) (Aravind *et al.*, 1999). This family of enzymes is involved in bacterial DNA repair defenses after UV damage (Aravind *et al.*, 1999). The URI nucleases enable repair of damaged DNA by making strand incisions on both sides of the DNA lesion. In bacterial systems, they function as part of a complex with ABC-type UvrA ATPases and UvrB helicases (Lin and Sancar, 1992; Lin *et al.*, 1992). It is likely that the *Ac*MNPV ORF79 gene was acquired from bacteria or another insect virus. The entomopathogenic nematode bacteria, *Photorhabdus luminescens* and the insect iridovirus type 6, both encode ORF79 homologues.

Some researchers have engineered DNA repair genes into baculoviruses such as the DNA glycosylase, cv-PDG (Petrik *et al.*, 2003). This resulted in increased tolerance of the BV form of baculoviruses to UV-C radiation but had only negligible effects on the ability of ODVs to

tolerate UV light. Failure of this strategy may have been due to poor engineering of the protein to be a component of the ODV nucleocapsid. This kind of approach attempting to engineer baculovirus tolerance to UV light will likely become more important in the future. Given the complexity of the ODV, it may be simplest to evaluate some of less common baculovirus auxiliary UV protection gene candidates such as *v-trex* or *photolyase* before venturing into other systems.

F. Cell Cycle Regulator Proteins in the Nucleocapsid

As has been mentioned previously, midgut cells are in a state that metabolically will not support viral DNA replication. In addition to DNA replication factors, baculovirus ODV virions carry proteins that promote midgut cell rejuvenation. Differentiated midgut cells are in a nondividing, Gap 0 (G_0) cell cycle phase (Garcia *et al.*, 2001; Loeb *et al.*, 2003). Dividing cells have four possible phases; G_1 phase (gap 1), S phase (DNA synthesis), G_2 phase (gap 2), and M phase (mitosis). ODV nucleocapsids carry with them proteins that appear designed to shift the cell cycle out of the G_0 phase and into the S phase or DNA synthesis phase.

ODV-EC27, which is conserved among all baculovirus genomes, is the first ODV protein to be described with involvement in the cell cycle (Belyavskyi *et al.*, 1998). This protein has the "EC" designation due to localization in the envelopes and nucleocapsids of ODVs (Braunagel *et al.*, 1996b). When isolated from ODVs, this protein migrates at 27 kDa in reducing SDS-PAGE gels which is less than the expected size of 33 kDa (Braunagel *et al.*, 1996b). Other larger sizes of ODV-EC27 were also detected in ODVs and BVs, which may be homodimers and homotrimers (Belyavskyi *et al.*, 1998; Braunagel *et al.*, 1996b).

One of the more important avenues of investigation of ODV-EC27 centered around a cyclin box motif that was identified in residues 80–110 of *Ac*MNPV ODV-EC27 (Belyavskyi *et al.*, 1998). Cyclins are proteins that mediate shifting of the cell cycle through interaction with cyclin-dependent kinases (CDKs). ODV-EC27 was shown to bear significant similarity to *Bombyx mori* cyclin B (Baluchamy and Gopinathan, 2005). Notably, the authors of this study refer to the *Bm*NPV ODV-EC27 homologue as "viral cyclin" or V-CYC.

Immunoprecipitation experiments demonstrated that the ODV-EC27 coprecipitates with CDKs and that these complexes will phosphorylate the known CDK substrates, histone H1 and retinoblastoma (pRB) (Belyavskyi *et al.*, 1998). Immunoprecipitation experiments

ODV-EC27 binding specificity to cell division cycle kinase 2 (Cdc2) (Belyavskyi *et al*., 1998). Cdc2 forms complexes with cyclin B to trigger cells to enter mitosis and divide (Nurse, 1990). In other words, cyclin B/Cdc2 complexes induce the G_2/M phase transition. At late times in baculovirus infection, cyclin B is rapidly degraded and cells enter G_2/M phase transition arrest (Braunagel *et al.*, 1998). This loss of cyclin B coincides with formation of ODV-EC27/Cdc2 complexes (Belyavskyi *et al*., 1998). ODV-EC27 lacks a cyclin-like destruction box motif thus providing mechanism to maintain ODV-EC27/Cdc2 complexes and G_2/M phase arrest. It has been suggested that maintaining G_2/M phase arrest at late times in infection is very important from the prospective of the ODV assembly (Belyavskyi *et al*., 1998). During M phase, the nuclear envelope breaks down to permit chromosomal segregation and cell division. Arresting the cells before the onset of M phase may be required for ODV assembly given that they acquire their envelopes from the INM, which would be lost if cells were permitted to proceed through M phase. The inner nuclear envelope may also have physical characteristics at the end of the G_2 phase which may make it more easily acquired to form ODV envelopes. It should be noted however, that the nuclear membrane completely disintegrates prior to the assembly of GV ODVs (Goldberg *et al.*, 2002).

Why is ODV-EC27 a structural protein of the ODV given its involvement in G_2/M phase arrest at late times in infection? ODV-EC27 also coimmunoprecipitates with cyclin-dependent kinase 6 (Cdk6) (Belyavskyi *et al*., 1998). Cdk6 normally binds to cyclin D to promote cells to proceed from quiescent G_0 and G_1 phases to the DNA synthesis S phase (for review see Ewen, 2000). DNA viruses require cells to enter the S phase in order that nucleotides and other cellular resources are made available for virus DNA replication. Emphasis on S phase transition early in baculovirus infection is supported by studies of the baculovirus immediate early gene, IE-2, which independently causes cells to enter S phase arrest (Prikhod'ko *et al.*, 1999a). Curiously, IE-2 has not been detected in the ODV.

ODV-EC27 complexes with cellular PCNA and possibly viral PCNA in nucleocapsids of BV and ODV (Belyavskyi *et al*., 1998). In addition to being involved in DNA synthesis and repair, PCNA has also been shown in mammalian models (Xiong *et al*., 1993; Zhang *et al*., 1993) and insect models (Kisielewska *et al*., 2005) to form complexes with cyclin D and CDKs to promote cell cycle shifts into S phase (for review see Maga and Hubscher, 2003). The viral PCNA is nonessential for baculovirus replication but appears to enhance infectivity (Iwahori *et al*., 2002, 2004). Providing the PCNA cell cycle factors may provide

an advantage for ODVs as cellular PCNA is present in low abundance in insect midgut cells (Zudaire *et al.*, 2004).

Yeast two-hybrid experiments revealed that ODV-EC27 binds directly with nucleocapsid proteins BV/ODV-C42 and PP78/83 (Braunagel *et al.*, 2001). As mentioned earlier, PP78/83 is a nucleocapsid base-associated structural protein. BV/ODV-C42 was identified to contain the canonical pRB-binding motif (LXCXE) (Braunagel *et al.*, 2001; Forng and Atreya, 1999). This may be significant for association of ODV-EC27 with pRB (Belyavskyi *et al.*, 1998). The LXCXE motif is however only conserved among three BV/ODV-C42 homologues (*Ac*MNPV, *Bm*NPV, and *Ro*MNPV).

There are likely more ODV-associated protein involved in shifting the cell cycle that have not been characterized. The 13.3-kDa predicted product of *Ac*MNPV ORF102 is may be one of those proteins. A BLAST search of this protein's sequence identified some similarity to NIMA kinases. NIMA is an acronym for Never In Mitosis gene A which is a type of protein involved in regulation of the cell cycle (Noguchi *et al.*, 2002, 2004).

G. Apoptosis Inhibitor Proteins in the Nucleocapsid

One of the primary strategies that cells use to evade virus infection is a self-destructive response called programmed cell death or apoptosis. This altruistic behavior sacrifices the virus-infected cell to mitigate virus replication and spread throughout the organism. Insect midgut epithelial cells are easily triggered to undergo apoptosis not only from response to viral infections but also from their routine turnover in the midgut.

Baculoviruses produce two classes of proteins that can prevent cells from undergoing apoptosis, P35 (Clem *et al.*, 1991) and inhibitors of apoptosis (IAP) type (Thiem and Chejanovsky, 2004) (for review see Clem, 1995). Baculoviruses frequently encode both classes of *p35* and *iap* genes and even multiple copies. The P35 and IAP proteins are synthesized abundantly at the onset of infection due to strong early promoters. Given the obvious emphasis baculoviruses place on apoptosis inhibitors, it is surprising that P35 and IAP have not been localized in ODV or BV. This may have something to do with localization of P35 and IAP in the cytoplasm and assembly of nucleocapsids in the nucleus.

The nucleocapsid-associated baculovirus protein P33 may be involved in apoptosis given that this protein binds to murine or human

p53 (Prikhod'ko *et al.*, 1999b). The cellular p53 protein is involved in apoptosis and cell cycle regulation (for review see Bai *et al.*, 2005; Collot-Teixeira *et al.*, 2004). Cosynthesis of P33 with the cellular protein p53 in insect cells leads to apoptosis (Prikhod'ko *et al.*, 1999b). The P33 protein appears essential for baculovirus infection (Prikhod'ko *et al.*, 1999b), and existence of an insect homologue to p53 (Jin *et al.*, 2000) leads to the possibility that P33 is involved in mechanisms which target p53 in order to inhibit apoptosis or permit cell cycle progression. P53 is a DNA-binding protein and it is possible that P33 associates with host P53 proteins on the viral genome in nucleocapsids. The apoptosis observed with the coexpression of p33 and p53 would be inhibitory to virus infection and thus the virus likely retains p33 to interact with additional proteins.

It was hypothesized that baculovirus ring finger proteins like CG30, IE-2, PE38, *Bm*NPV ORF35 (*Ac*MNPV ORF44), IAP1, and IAP2 were ubiquitin ligases that may catalyze the ubiquitination and destruction of cellular p53 (Imai *et al.*, 2003). Herpesviruses and adenoviruses shift the cell cycle for DNA replication by employing virus-encoded ring finger/ubiquitin ligases to targeting cellular P53 for ubiquitination by viral (Boutell and Everett, 2003; Weger *et al.*, 2002). Only IAP2, IE-2, and PE38 had ubiquitin ligase activities and such activities were inconclusive for CG30 (Imai *et al.*, 2003). CG30 is the only protein among these that have been localized to ODVs.

H. Nucleocapsid-Associated Proteins Involved in Transcriptional Activation

The IE-1 protein is not only involved in DNA replication but is also a highly conserved transcriptional activator of viral genes (Choi and Guarino, 1995a,b,c; Kremer and Knebel-Morsdorf, 1998; Passarelli and Miller, 1993) and is also a required component of viral DNA replication complex (Kool *et al.*, 1994; Lu and Miller, 1995b). IE-1 predominantly localizes in the virogenic stroma where DNA replication occurs (Kawasaki *et al.*, 2004). IE-1 also colocalizes with VP39 in the virogenic stroma (Kawasaki *et al.*, 2004) and thus is present in regions of capsid assembly. The *ie-1* gene is strongly expressed early in infection and its transcripts can be detected within 20 minutes postinfection (Kovacs *et al.*, 1991). One the potential roles of nucleocapsid-associated IE-1 may be to transcriptionally activate the expression of early viral genes immediately after the genome has been unpackaged in the nucleus of a new host cell.

The nucleocapsid-associated protein PNK/PNL (polynucleotide kinase/polynucleotide ligase) is an 80-kDa protein that was first identified as a unique gene in the genome of *Ac*MNPV (Ayres *et al.*, 1994). The *pnk*/*pnl* gene is a nonconserved auxiliary gene and is not present in many baculoviruses including variants of *Ac*MNPV such as *Ac*MNPV 1.2, *Gm*MNPV, and *Bm*NPV (Yanase *et al.*, 2000). The *pnk*/*pnl* gene is expressed early in infection and is nonessential for virus replication (Durantel *et al.*, 1998). *Ro*MNPV is the only other baculovirus known to encode for this 80-kDa peptide. It was shown that PNK/PNL has three functionally active domains with kinase, phosphatase, and RNA ligase activities, respectively (Martins and Shuman, 2004). Speculation was made that PKN/PNL enabled the virus to evade an RNA damage-based host defense.

PNK/PNL could be involved in the RNA splicing and repair activities that occur with the expression of the immediate early transcription factor gene *ie0*. The *ie0* gene is the only known early baculovirus gene to be spliced (Kovacs *et al.*, 1991; Pearson and Rohrmann, 1997; Theilmann *et al.*, 2001) and it plays an essential role establishing baculovirus infection (Pearson and Rohrmann, 1997; Stewart *et al.*, 2005). The *ie0* gene results from the splicing of a small exon onto the 5′ end of the *ie-1* gene (Chisholm and Henner, 1988). Expression of *ie0* occurs very rapidly in the first few hours of infection and then diminishes while *ie-1* expression continues late into the virus replication cycle (Kovacs *et al.*, 1991). At the beginning of infection, there is likely an immediate need for robust RNA-splicing activities to ensure translation of IE0. The capsid-associated PNK/PNL protein may be present to augment these cellular splicing activities. This may be necessary in some cell types that the virus infects. The ratios of IE0 and IE-1 also affect baculovirus host specificity (Lu and Iatrou, 1996; Pearson and Rohrmann, 1997; Stewart *et al.*, 2005) and thus PNK/PNL may be playing a role in specificity through RNA splicing.

The CG30 protein is a nucleocapsid-associated protein with a very distinctive C-terminal C_3HC_4 zinc ring finger motif, an N-terminal leucine zipper motif (Thiem and Miller, 1989a). The leucine zipper and zinc finger are considered DNA-binding motifs; it is likely that CG30 is binding to viral genomic DNA within nucleocapsids.

The CG30 protein is nonessential for virus replication (Passarelli and Miller, 1994) and was suggested to be involved in transcriptional activation due to the presence of a central acidic domain (Passarelli and Miller, 1994; Thiem and Miller, 1989a). Oddly, the deletion mutant of CG30 was slightly more orally infectious and produced more BV (Passarelli and Miller, 1994). Given its immediate cost on the virus,

cg30 must be an auxiliary gene with long-term evolutionary benefits such as host range. Homologues to the *cg30* gene are present only in NPVs and in all cases, the *cg30* gene is downstream and in the same orientation as the major capsid protein encoding gene *vp39*. The *cg30* gene is transcribed from an early promoter and late *vp39* transcripts are bicistronic such that they include the *cg30* ORF in addition to the *vp39* ORF (Blissard *et al.*, 1989; Lu and Iatrou, 1996; Thiem and Miller, 1989a). There is no direct evidence that CG30 is translated from this late transcript; however, only 2 bp separate the end of the *vp39* ORF from the beginning of the *cg30* ORF and from what is known of baculovirus bicistronic translation (Chang and Blissard, 1997), CG30 is likely synthesized in lesser amounts but at the same time as VP39. This timing of production would provide CG30 proteins for inclusion with nucleocapsids.

V. ODV Envelope and Tegument Proteins

A. ODV Proteins Interacting with the Tegument

Tegument proteins are defined by viral fractionation techniques involving several detergents. In these techniques, ODVs are treated with detergents such as Nonidet P-40 (NP-40) and the soluble proteins are defined as envelope proteins and the remaining insoluble proteins are assumed to be nucleocapsid proteins. Tegument proteins are solublized by NP-40 due to loss of viral envelopes retaining them within the virions. Discriminating ODV integral envelope proteins from tegument proteins can be done using phase separation and the detergent Triton X-114 (TX-114) (Bordier, 1981). The tegument protein GP41 is released from ODVs by NP-40 detergent (Pan *et al.*, 2005; Whitford and Faulkner, 1992), and some have suggested GP41 is an integral ODV envelope protein (Pan *et al.*, 2005). However, GP41 partitions entirely into the aqueous phase in TX-114 detergent separation experiments (Whitford and Faulkner, 1992). Hidden Markov analysis (Krogh *et al.*, 2001) of all GP41 homologues found no predicted transmembrane domains.

The GP41 protein is also an *O*-glycosylated protein (Jain and Das, 2004; Pan *et al.*, 2005; Whitford and Faulkner, 1992) and to our knowledge is the only *O*-glycosylated baculovirus protein. The significance of this to the function of GP41 is unknown. The only functional studies of GP41 suggest this ODV structural protein affects BV production. A Ts mutant of GP41 blocked nucleocapsid exit from the nucleus at Ts conditions (33°C) and prevented formation of BV (Olszewski and

Miller, 1997b). It was suggested that presence of GP41 in the ODV tegument was "adventitious" and was the result of the proteins presence in the nucleoplasm. This may be a premature interpretation of the role of GP41. This protein is conserved among all baculovirus genomes sequenced so far. The hymenopteran *Nese*NPV and *Nele*NPV *gp41* gene homologues (Garcia-Maruniak *et al.*, 2004; Lauzon *et al.*, 2004) and these viruses appear to produce only ODV. Earlier observations could have been the result of a Ts mutant GP41 inappropriately interacting with nucleocapsids exiting the nucleus. ODVs are not occluded in wt *Ac*MNPV at 33°C (Lee and Miller, 1979) and thus the role of GP41 in the ODV could not be examined for GP41 Ts mutants.

ODV-EC43 was first characterized in the baculovirus *Ha*SNPV (Fang *et al.*, 2003) and was found to be localized both in the ODV nucleocapsid and in the envelope. Some homologues of this protein contain transmembrane domain motifs, although the majority do not. ODV-EC43 is conserved among all baculovirus genomes and is the predicted product of *Ac*MNPV ORF109. ODV-EC43 was also detected in the ODVs of *Ac*MNPV (Braunagel *et al.*, 2003).

P91 is a highly conserved baculovirus protein that may be in the ODV tegument cross linking the virion envelope with nucleocapsids (Russell and Rohrmann, 1997). P91 is present in the *Op*MNPV ODV as 91- and 102-kDa molecular weight forms. P91 is not specific to the ODV and is also found in the BV as single 91-kDa form. The homologues to this protein consistently contain highly hydrophobic N-terminal transmembrane motifs similar to ones present in ODV other envelope proteins (Hong *et al.*, 1997; Slack *et al.*, 2001). The localization pattern of P91 is in the nuclear ring zone (Kawasaki *et al.*, 2004; Russell and Rohrmann, 1997) similar to what is observed for ODV envelope proteins such as ODV-E66 (Hong *et al.*, 1994) and P74 (Slack *et al.*, 2001). Immuno-EM images showed association of P91 with envelopes and with nucleocapsids (Russell and Rohrmann, 1997). P91 could not be dissociated completely from ODV virions by NP-40 detergents or by disulfide reducing agents. This is thought to be due to unknown covalent bonds between P91 and nucleocapsid proteins (Russell and Rohrmann, 1997).

B. ODV Envelope Proteins

ODV envelope proteins are more diverse in composition compared to those of BV envelopes. ODV envelope proteins are playing biological roles in ODV occlusion and interaction with the midgut. ODV envelope

proteins include a group of proteins called *per os* infectivity factors (PIFs). The core conserved ODV envelope proteins are ODV-E18, ODV-E56, P74, PIF-1, PIF-2, Ac115 (PIF-3), and Ac150 (PIF-4). These proteins are shown as components of the ODV envelope in Fig. 8. ODV-E18 is listed as not to be present in the mosquito baculovirus *Cuni*NPV (Afonso *et al.*, 2001). However, *Cuni*NPV ORF31 appears to be a distant homologue to *odv-e18*. It is located upstream of *odv-ec27*, and its protein product is predicted to have a hydrophobicity profile similar to ODV-E18. In addition to the universally conserved ODV envelope proteins, ODV envelope proteins ODV-E66 and ODV-E25 are in all lepidopteran baculoviruses.

C. ODV-E18, ODV-E35, and ODV-EC27

As covered earlier, ODV-EC27 is a nucleocapsid-associated protein involved in altering the host cell replication cycle. The *Ac*MNPV homologue of ODV-EC27 lacks a transmembrane domain motif and we are reluctant to classify ODV-EC27 as an envelope protein. In the NPVs, the *odv-e18* gene is found immediately upstream and in the same orientation as the *odv-ec27* gene. ODV-E18 is an integral ODV envelope protein and all homologues have N-terminal transmembrane motif regions. ODV-E18 and ODV-EC27 were first characterized as products from *Ac*MNPV (strain E2) (Braunagel *et al.*, 1996b). For this virus, the *odv-e18* gene is 269 bp long and encodes for a predicted 9.5-kDa protein product. However, the ODV-E18 protein migrates at an estimated size of 18 kDa in reducing SDS-PAGE gels (Braunagel *et al.*, 1996b). Post secondary modification has been ruled out and it has been proposed that ODV-E18 is a strongly associated homodimer (Braunagel *et al.*, 1996b). ODV-EC27 is translated from the 872-bp ORF, 15 bp downstream of the ODV-E18 ORF. The ODV-EC27 gene product has a predicted mass of 33 kDa but migrates at 27 kDa in SDS-PAGE.

Immuno-based experiments suggest that ODV-E18 and ODV-EC27 produce a chimeric fusion protein or a heterodimer called ODV-E35 (Braunagel *et al.*, 1996b). In *Ac*MNPV, ODV-E18, and ODV-EC27, ORFs are only separated by 15 bp and they are transcribed as a bicistron mRNA. The ODV-E18/ODV-EC27 bicistron is conserved among the following baculoviruses (the distance between ORFs is indicated in brackets); *Ac*MNPV (15 bp), *Adho*NPV (31 bp), *Bm*NPV *Cf* DEFNPV (14 bp), *Hear*SNPV (14 bp), *Hz*SNPV (14 bp), *Eppo*NPV (14 bp), *Nele*NPV (14 bp), *Nese*NPV (25 bp), *Op*MNPV (26 bp),

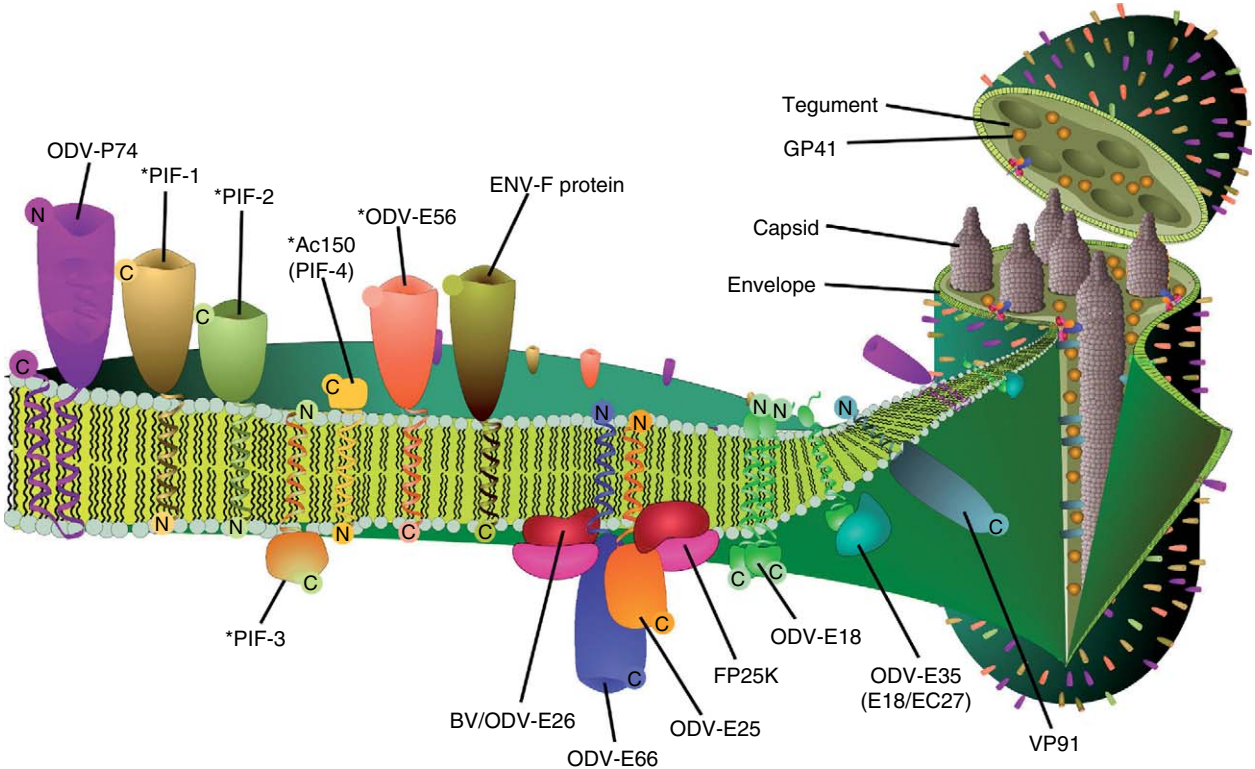

FIG 8. Proteins of the ODV envelope and tegument. The illustration represents a dissected view of the structure of the ODV with emphasis on the envelope. The figure is in the context of *Ac*MNPV. The major ODV-associated proteins with predicted transmembrane domains are shown in the ODV envelope. The "*" indicates that there is no data to suggest the orientation of these proteins in the ODV envelope. FP25K, which is capsid associated, also appears on the ODV envelope due to its apparent interaction with the transmembrane domains of ODV-E66 and ODV-E25. Tegument proteins are also illustrated in the main virion on the right side.

*Ld*MNPV (17 bp), *Cf*MNPV (28 bp), *Chch*NPV (36 bp), *Maco*NPV-A (38 bp), *Maco*NPV-B (38 bp), *Raou*NPV (16 bp), *Se*MNPV (54 bp), *Splt*NPV (31 bp), and *Tn*SNPV (40 bp).

The genetic linkage of ODV-E18 and ODV-EC27 is not present in GV baculoviruses. In these viruses, the genes are separated by great distances: *Agse*GV (NC_005839) (77,419 bp), *Ador*GV (NC_005038) (57,419 bp), *Cyle*GV (65,249 bp), *Cypo*GV (69,143 bp), *Plxy*GV (54,987 bp), *Phop*GV (69,183 bp), and *Xecn*GV (96,723 bp). We noted that the ODV-EC27 homologues of these viruses all contain strong transmembrane motifs in their C-terminal regions that are not present in the NPV homologues. It is possible that ODV-EC27 localizes exclusively in the ODV envelopes of GVs.

Braunagel *et al.* (1996a,b) suggested that ODV-E35 is produced from translational frameshifting between ODV-E18 and ODV-EC27. Translational frame shifting in the context of baculovirus infection has been observed before (Williams *et al.*, 1989). The combined molecular weights of 9-kDa monomeric E18 and 27-kDa EC27 would produce a protein migrating at 36 kDa.

ODV-E35 is an integral ODV envelope protein and we speculate that ODV-E35 may be employed by the virus to localize ODV-EC27 peptide domains on the tegument side of the envelope. As mentioned earlier, ODV-EC27 binds to a number of nucleocapsid-associated proteins including BV/ODV-C42 and PP78/83 (Braunagel *et al.*, 2001). ODV-E35 may be stabilizing the ODV virion structure by cross-linking the envelope with nucleocapsid bound proteins.

D. ODV-E66 and ODV-E25

ODV-E25 is an integral ODV envelope protein that is N-terminally anchored in the envelope (Hong *et al.*, 1997; Russell and Rohrmann, 1993). The single N-terminal transmembrane motif of ODV-E25 is conserved among homologues. ODV-E25 is also found in BV but is comparatively more abundant in ODV (Russell and Rohrmann, 1993).

ODV-E66 is an integral ODV envelope protein that like ODV-E25 is N-terminally anchored in envelope (Hong *et al.*, 1997; Russell and Rohrmann, 1993). ODV-E66 is however exclusively found in the envelope (Hong *et al.*, 1994). In addition to a conserved N-terminal transmembrane motif, some ODV-E66 homologues have internal transmembrane motifs. However, they are not conserved in position and do not have as high transmembrane probability scores. Yeast two-hybrid and immunoprecipitation experiments showed that the exposed C-terminal regions of ODV-E66 and ODV-E25 interact (Braunagel *et al.*, 1999). This would

argue that ODV-E66 and ODV-E25 have common orientation in the ODV envelope. Experiments with membrane-trafficking proteins also show that ODV-E25 and ODV-E66 have common orientation in the ER membrane (Saksena *et al.*, 2004).

Data has been published addressing the orientation of ODV-E66 and ODV-E25 in the ODV envelope. In a study where the transmembrane region of ODV-E66 was fused to the N-terminus of GFP, the resulting protein chimera was exposed on microvesicle surfaces (Braunagel *et al.*, 2004). This data was extrapolated to suggest that ODV-E66 is exposed on the cytoplasmic side of the ER and the nucleoplasmic side of the INM. Several indirect lines of evidence point to ODV-E66 and ODV-E25 being oriented on the inner side of the ODV envelope. First, ODV-E25 is not completely released from ODV by NP-40 detergent and remains residually associated with the nucleocapsid (Russell and Rohrmann, 1997). Second, yeast two-hybrid and immunoprecipitation show that ODV-E66 binds to the major capsid protein VP39 (Braunagel *et al.*, 1999). From this we would suggest that ODV-E66 and ODV-E25 are involved in ODV capsid envelopment. A caveat to our prediction of ODV-E66 orientation is that the ODV-E66 of *Ac*MNPV may be cleaved by trypsins in ODV preparations. This would indicate surface exposure of ODV-E66. When this 66-kDa protein was purified from OBs, a smaller 60-kDa molecular weight form appeared (Hong *et al.*, 1994). N-terminal sequencing suggested that this was a tryptic digest product of ODV-E66 (Hong *et al.*, 1994). The trypsin cleavage site is not conserved among ODV-E66 homologues and the authors of this study did not speculate on the origin of the trypsin activity present in ODV preparations.

Deletion mutants of ODV-E66 demonstrate that this protein is not required for BV production (Russell and Rohrmann, 1997). Insect oral bioassay data has not been published on these viruses so it is not known if ODV-E66 is an essential PIF. Deletion mutants have not been made for ODV-E25. If we assume orientation inside the virion, ODV-E66 and ODV-E25 are not likely to be involved in ODV attachment or fusion to the midgut.

E. Trafficking of ODV Envelope Proteins, FP25K and BV/ODV-E26

Studying ODV envelope proteins is made more difficult by the complexity of the translocation of viral proteins into the ODV envelope. The ER, outer nuclear membrane (ONM), INM, and nuclear pore complex are a continuous network of membranes exploited by baculoviruses to deliver proteins to the ODV envelope (Braunagel *et al.*, 2004). The INM

is presumed to be the source of ODV envelopes (Hong *et al.*, 1994, 1997). ODV envelope proteins which begin in the ER are targeted to the INM which blebs off as ODV envelope precursor microvesicles in the nuclear ring zone (Hong *et al.*, 1994, 1997).

The major ODV envelope proteins do not have N-terminal membrane insertion signal peptides and ODV envelope proteins are almost never *N*-glycosylated (Braunagel *et al.*, 2004). One exception is the *Spodoptera litura* MNPV ORF137 ODV envelope protein that has been shown to be *N*-glycosylated (Yin *et al.*, 2003). There are few other viral homologues to this protein. F protein is an *N*-glycosylated protein with an N-terminal signal peptide and although it is associated in the ODV, F protein is considered specific for the BV. The primary ODV envelope proteins have prominent hydrophobic transmembrane motifs that are involved in membrane insertion, anchoring, and localization in the ODV envelope (Hong *et al.*, 1994, 1997; Slack *et al.*, 2001). ODV envelope proteins exhibit the ability to spontaneously insert themselves into membranes (Hong *et al.*, 1997; Slack *et al.*, 2001; Yao *et al.*, 2004). These hydrophobic transmembrane motifs have been termed INM sorting motifs (Braunagel *et al.*, 2004).

The proteins FP25K and BV/ODV-E26 (Beniya *et al.*, 1998; Braunagel *et al.*, 1999) may play an important role in the trafficking of ODV envelope proteins. FP25K associates with BV and ODV nucleocapsids (Braunagel *et al.*, 1999) and BV/ODV-E26 associates with BV and ODV envelopes (Beniya *et al.*, 1998). BV/ODV-E26 has been localized on the plasma membrane, BV envelopes, INM microvesicles, and ODV envelopes (Beniya *et al.*, 1998). BV/ODV-E26 lacks transmembrane domain motifs and is released from ODVs by NP-40 detergent treatment (Beniya *et al.*, 1998). Like the tegument protein GP41 (Whitford and Faulkner, 1992), BV/ODV-E26 fractionates into the aqueous phase of TX-114 detergent phase partition assays (Beniya *et al.*, 1998). BV/ODV-E26 is thus a peripheral membrane protein and interaction with FP25K would orient BV/ODV-E26 to inner side of the ODV envelope. A small proportion of BV/ODV-E26 protein remains with purified BV and ODV nucleocapsids (Beniya *et al.*, 1998). Presumably this is due to association with FP25K.

FP25K studies began with a few polyhedra (FP) mutant baculovirus phenotypes that occurred during high titer, serial passage in cell culture (Fraser and Hink, 1982; Fraser *et al.*, 1983; Stairs *et al.*, 1981). Many FP mutant baculovirus phenotypes resulted from the disruption of the *fp25k* gene (Beames and Summers, 1989), thus the nomenclature "few polyhedra 25K" or FP25K. The lack of polyhedra in FP25K mutants was first correlated with the inappropriate localization of

polyhedrin (Jarvis *et al.*, 1992) and decreased *polyhedrin* gene transcription (Harrison *et al.*, 1996). FP25K mutants failed to translocate ODV-E66 to INM vesicles (Rosas-Acosta *et al.*, 2001) and ODV-E66 peptide became significantly less abundant while the mRNA transcripts of *odv-e66* were unchanged (Braunagel *et al.*, 1999; Rosas-Acosta *et al.*, 2001). Immunoprecipitation and yeast two-hybrid analysis revealed interaction of FP25K with ODV envelope proteins, ODV-E25 in addition to ODV-E66 (Braunagel *et al.*, 1999; Katsuma *et al.*, 1999).

Despite being localized in BV nucleocapsids, FP25K is not required for BV production. Absence of FP25K results in increased abundance of GP64 (Braunagel *et al.*, 1999) and increased production of BV (Harrison and Summers, 1995; Wu *et al.*, 2005). BV budding is driven by GP64 accumulation on the cell membrane (Oomens and Blissard, 1999). Coinciding with GP64 accumulation, FP25K deletion mutants also produce more BV/ODV-E26 (Braunagel *et al.*, 1999). Homologues to BV/ODV-E26 are exclusive to group I NPVs that use GP64 as their primary ENV protein. However, BV/ODV-E26 has not been shown to interact with GP64 and paradoxically, FP25K coimmunoprecipitates GP64 (Braunagel *et al.*, 1999).

It is interesting that FP25K deletion mutants also affect the transport of the viral protein V-CATH (Katsuma *et al.*, 1999). V-CATH is cathepsin-like proteinase involved in the lytic release of OBs from virus-infected cells and from host tissues (Ohkawa *et al.*, 1994; Slack *et al.*, 1995; Suzuki *et al.*, 1997). Both V-CATH and GP64 are *N*-glycoproteins with membrane insertion signal peptides and are likely sharing common pathways in the ER. Normally, V-CATH copurifies with BVs (Lanier *et al.*, 1996) but is apparently not being secreted in FP25K mutants.

It has been suggested that FP25K mutant effects may be "stoichiometric" in nature (Braunagel *et al.*, 1999) and not directly caused by FP25K mutation. FP25K is definitively directly involved in the transport of membrane proteins. Covalent cross-linking experiments revealed that FP25K and also BV/ODV-E26 bind to the transmembrane region sorting motifs of ODV-E66 and ODV-E25 (Braunagel *et al.*, 2004; Saksena *et al.*, 2004). Interaction between BV/ODV-E26 and FP25K may determine the trafficking of BV bound envelope proteins (GP64) and ODV bound envelope proteins (ODV-E25, ODV-E66). The mechanism by which FP25K and BV/ODV-E26 traffic proteins may involve actin or cystoskeleton filaments. FP25K has been identified to contain three myosin-like regions and one actin-binding motif (Beniya *et al.*, 1998; Braunagel *et al.*, 1999). BV/ODV-E26 also has a tropomysosin motif. The nucleocapsids of FP25K deletion mutants were not efficiently enveloped (Katsuma *et al.*, 1999).

Given the role FP25K plays in ODV-E66 translocation, this indirectly supports the premise that ODV-E66 is involved in ODV capsid envelopment.

The mosquito baculovirus *Cuni*NPV (Afonso *et al.*, 2001) and the sawfly baculoviruses *Nele*NPV (Lauzon *et al.*, 2004) and *Nese*NPV (Garcia-Maruniak *et al.*, 2004) lack ODV-E25 and ODV-E66 and yet the major ODV envelope proteins ODV-E56, PIF-1, PIF-2, and P74 are present in these viruses. This would imply that FP25K is specifically involved in translocation of ODV-E66 and ODV-E25 and not required for transport of the other ODV envelope peptides.

F. ODV-E56

ODV-E56 is another protein that is exclusively associated with the envelope (Braunagel *et al.*, 1996a; Theilmann *et al.*, 1996). The *Op*MNPV ODV-E56 homologue migrated in SDS-PAGE as 46- and 43-kDa proteins (Theilmann *et al.*, 1996) and the *Ac*MNPV ODV-E56 homologue migrated as a 67- and 56-kDa proteins (Braunagel *et al.*, 1996a). Both homologues are posttranslationally modified as the *Op*MNPV and *Ac*MNPV homologues should migrate at 40 to 41 kDa, respectively. *N*-Gycosylation was ruled out (Braunagel *et al.*, 1996a) and proteolytic cleavage has proposed to the potential source of multiple forms (Theilmann *et al.*, 1996).

ODV-E56 has a strong transmembrane motif on its C-terminus that is required for translocation into the nucleus and localization into ODV envelopes (Braunagel *et al.*, 1996a). It is not required for BV production, ODV assembly, or occlusion (Braunagel *et al.*, 1996a). Bioassay data have not been presented on ODV-E56 deletion mutant viruses. The transmembrane domain prediction profile of ODV-E56 is similar to P74. ODV-E56 also has a central region that contains a membrane insertion signal peptide motif and a single C-terminal transmembrane domain. It is not established if ODV-E56 is exposed on the ODV surface. In our model, we placed it to be exposed on the surface of the ODV and have suggested that only the C-terminal transmembrane domain is utilized by this protein for envelope insertion.

G. P74 (PIF-0)

P74 has been shown to be an ODV envelope protein by viral fractionation (Faulkner *et al.*, 1997) and by EM (Rashidan *et al.*, 2005). P74 was the first ODV envelope protein to be demonstrated essential for oral infection (Kuzio *et al.*, 1989; Yao *et al.*, 2004; Zhou *et al.*, 2005).

Perhaps P74 should be renamed PIF-0 or P74-PIF-0 as it was the first PIF to be discovered. The P74 protein is C-terminally anchored in the ODV envelope (Slack *et al.*, 2001) and is N-terminally exposed on the ODV surface (Faulkner *et al.*, 1997). P74 has an internal transmembrane domain and a C-terminal double transmembrane domain. The internal transmembrane domain has characteristics of an internal transmembrane insertion signal peptide with positively charged amino acids on one side and negatively charged amino acids on other side (Alberts *et al.*, 1994). Experimental data suggest that the central transmembrane motif is not inserted into membranes and remains buried in the protein. A chimeric P74-GFP protein lacking only the P74 C-terminal double transmembrane domain is soluble and not associated with membranes (Slack *et al.*, 2001). The double transmembrane domain motif on the C-terminus of P74 is odd because of the closeness of these domains and suggests a "hairpin" or folded transmembrane domain that may spontaneously insert into membranes. It was demonstrated that bacterially expressed and purified P74 proteins could spontaneously insert into ODVs and rescue oral infectivity of a P74 null virus (Yao *et al.*, 2004). This work also indicates that P74 does not require post secondary modification to be functional.

Evidence from several studies suggests P74 is an ODV attachment protein that binds virions to midgut cells (Haas-Stapleton *et al.*, 2004; Yao *et al.*, 2004). It has been known for over a decade that ODVs bind to midgut protein receptors (Horton and Burand, 1993) and data have shown that P74 binds to a 30-kDa insect midgut receptor protein (Yao *et al.*, 2004).

It was shown that P74 was proteolytically cleaved on its N-terminus by insect midgut trypsins (Slack and Lawrence, 2005). Earlier studies did not observe this phenomenon because the antisera were specific for the N-terminal cleaved region of P74 (Faulkner *et al.*, 1997; Slack and Lawrence, 2005). Among all P74 homologues including the "pseudobaculovirus" Hz-1, there is one conserved trypsin cleavage site corresponding to R156 in *Ac*MNPV. The position of this site correlates with the size of the P74 cleavage product produced after incubation in the midgut trypsins. The P74 is the most complex of the ODV envelope proteins. We speculate that that the central transmembrane domain region may be involved in membrane fusion and is perhaps exposed by protease cleavage.

H. Proteases, P74, and Oral Infectivity

Data suggesting that ODV envelope peptide P74 may be cleaved by insect midgut trypsins leads to questions about the role of trypsins in oral infection. The role that insect midgut proteases play in baculovirus

infection is not been well investigated. The lepidopteran midgut is rich in trypsin and chymotrypsin (Johnston *et al.*, 1991; Terra and Ferreira, 1994) and it is likely that baculoviruses have evolved to tolerate and exploit these proteases just as they have for midgut alkalinity. Our work suggests that supplementation of baculoviruses with trypsin enhances oral infectivity and addition of soybean trypsin inhibitors decreases oral infectivity. Tryptic cleavage activation is documented for other viruses including *Coronavirus* (Frana *et al.*, 1985), *Rotavirus* (Vonderfecht *et al.*, 1988), *Sendai virus* (Muramatsu and Homma, 1980), and *Vaccinia virus* (Ichihashi and Oie, 1982). Tryptic activation occurs in other entomopathogens as *Bacillus thuringiensis* (Bt). The midgut binding Bt toxin protein requires trypsin cleavage to be activated (Miranda *et al.*, 2001).

Occlusion is a complex viral evolutionary development and it can be assumed that baculovirus ancestors were not occluded. These nonoccluded ancestors may have required alkaline proteolytic activation to infect midgut tissues. Midgut alkalinity is the primary trigger for the release of ODVs from OBs. Why might baculoviruses retain the need for trypsin activation? One possible reason is that viral attachment protein receptor-binding domains may need to be shielded from the OB matrix proteins. Another possibility is that hydrophobic membrane fusion elements must be exposed during ODV assembly. When N-terminally truncated forms of the ODV envelope protein P74 are synthesized in insect cells, the protein becomes trapped nonspecifically in regions of the cell (Slack *et al.*, 2001).

Baculovirus OB-associated alkaline serine proteases (Eppstein and Thoma, 1975; Eppstein *et al.*, 1975; Langridge and Balter, 1981; Maeda *et al.*, 1983) may be playing a synergistic role in infection by ensuring activation of released ODVs. The OB-associated proteases are of host origin and are not present in tissue culture derived OBs (McCarthy and Dicapua, 1979). It is notable that the hymenopteran baculoviruses *Nele*NPV (Lauzon *et al.*, 2004) and *Nese*NPV (Garcia-Maruniak *et al.*, 2004) encode for serine protease genes. These "ancestral" baculoviruses do not appear to have the BV phenotype and their replication is restricted to the midgut. As the infection ensues, the midgut physiology is likely affected and protease production may be compromised. Perhaps these viruses are supplementing insect midgut trypsins with viral trypsin in order to infect more tissues of the midgut in secondary rounds of infection.

I. PIF-1 and PIF-2

In the past, ODV-associated proteins were discovered by chance as the viral genes were methodically characterized. A *per os* bioassay functional genomic approach has been used to identify PIF proteins

that are essential for oral infection (Kikhno *et al.*, 2002; Pijlman *et al.*, 2003). The first PIFs to be identified this way were PIF-1 (originally PIF) and PIF-2 (Kikhno *et al.*, 2002; Pijlman *et al.*, 2003). PIF-1 was characterized as the ORF7 gene product of the *Spodoptera littoralis nucleopolyhedrovirus* (*Spli*NPV) (Kikhno *et al.*, 2002). PIF-2 was characterized as the ORF35 gene product of the *Se*MNPV (Pijlman *et al.*, 2003). The *pif-1* and *pif-2* gene homologues are ORF119 and ORF22 of *Ac*MNPV, respectively. The 60-kDa PIF-1 protein and the 44-kDa PIF-2 protein contain prominent N-terminal transmembrane motifs that is likely to serve as transmembrane anchors. When we analyzed all homologues of these two proteins for transmembrane motifs (Krogh *et al.*, 2001), we identified only a single N-terminal transmembrane motif for both of these proteins. These hydrophobic N-terminal transmembrane motifs are similar to the INM sorting motifs present on ODV-E66 and ODV-E25 (Braunagel *et al.*, 2004; Hong *et al.*, 1997).

PIF-1 was confirmed to be an integral membrane protein of the ODV envelope (Kikhno *et al.*, 2002) but the exposure of the C-terminal portion of PIF-1 on the ODV surface has not been shown. There are presently no data published to show that *Se*MNPV PIF-2 is a component of the ODV; however, the *Ac*MNPV homologue was detected in ODVs (Braunagel *et al.*, 2003). Interestingly, the *Ac*MNPV ORF119 PIF-1 protein product was not identified in this study as an ODV component. PIF-1 is produced in low abundance in *Spli*NPV (Gutierrez *et al.*, 2004) and may not be easily detected in *Ac*MNPV ODV preparations. PIF-1 and PIF-2 have been shown to be involved in ODV binding to midgut cells (Ohkawa *et al.*, 2005). This would suggest that these proteins are exposed on the surface of the ODV along with P74.

J. PIF-3 and 11K Proteins

As has been emphasized throughout this review that the ODV is a highly complex virus phenotype. The number of PIF protein types associated with the ODV continues to grow. PIF-3 is a 23-kDa protein encoded by *Ac*MNPV ORF *Ac115* (Ohkawa *et al.*, 2005). The protein is conserved among all baculoviruses and is essential for oral infection. It has a good N-terminal transmembrane domain and could be an integral ODV envelope protein. However, PIF-3 does not affect ODV binding or envelope fusion with midgut cells (Ohkawa *et al.*, 2005). It was noted by Ohkawa *et al.* (2005) that ODV infection involves processes beyond attachment and fusion and that PIF-3 may play a role in the translocation of ODV capsids along the microvillus. Perhaps

PIF-3 is exposed to the inside of the ODV envelope where it can interact with cytoskeletal elements.

Another group of proteins that play an enhancing role in oral infection are the 11K proteins (Lapointe *et al.*, 2004; Zhang *et al.*, 2005). Like the PIFs, these proteins are conserved among many baculovirus genomes and in addition, homologues are found in entomopoxviruses (Dall *et al.*, 2001). The 11K protein homologues of *Ac*MNPV are encoded by ORFs *Ac145* and *Ac150* (Lapointe *et al.*, 2004). Their distinguishing feature is not so much amino acid sequence identity but the presence of C_6 chitin-binding motifs or peritrophin-C domains that are common among mucins, peritrophins, and chitinases (Tellam *et al.*, 1999). This pointed to the possibility that 11K proteins interact with and compromise the PM similar to enhancins (Lapointe *et al.*, 2004). The experimental evidence suggests that the 11K protein enhancement of oral infection does not involve the PM (Zhang *et al.*, 2005). In some circumstances, the Ac145 protein acts synergistically with the Ac150 protein (Lapointe *et al.*, 2004) while in other cases Ac150 alone acts as an enhancer of oral infection (Zhang *et al.*, 2005). Ac150 contains a prominent N-terminal transmembrane domain and it is associated with BV and OBs (Lapointe *et al.*, 2004). Immuno-EM data showed that Ac150 could be an ODV envelope protein (Lapointe *et al.*, 2004) and possibly a PIF-4 given that it has been classified as a PIF (Zhang *et al.*, 2005). However, the oral infectivity enhancement effects of Ac150 only occur with OBs and not with alkali liberated ODVs (Zhang *et al.*, 2005). Perhaps Ac150 is exposed on the ODV surface and is involved in the timely release of ODVs from the OB protein matrix.

As in the case of PIF-1, the proteins of PIF-3 and Ac150 were not identified as components of the ODV when ODVs were analyzed directly for protein composition (Braunagel *et al.*, 2003), which provides a baseline for studying the ODV, but it appears that functional genomics is a critical tool for discovering the proteins that are needed for oral infection.

K. Summary of ODV Envelope Proteins

Of the 44 proteins identified as components of the *Ac*MNPV ODV (Braunagel *et al.*, 2003), 8 have transmembrane domain motifs. Included among these are VP91, P74, ODV-E56, ODV-E66, ODV-E25, ODV-E18, PIF-2, and F protein. PIF-1, PIF-3, and Ac150 (PIF-4) would bring the number of potential ODV "integral" envelope proteins to 11. Deletion mutants or gene fusion disruption mutants have been generated for P74 (Kuzio *et al.*, 1989), PIF-1 (Kikhno *et al.*, 2002), PIF-2

(Pijlman *et al.*, 2003), PIF-3 (Ohkawa *et al.*, 2005), Ac150 (Lapointe *et al.*, 2004; Zhang *et al.*, 2005), ODV-E66 (Hong *et al.*, 1997), and ODV-E56 (Braunagel *et al.*, 1996a). Relevant biological data relating to the requirement of ODV-E66 and ODV-E56 for oral infection have not been generated, but it may be predicted that these proteins could also be PIFs. P74 is the only ODV envelope protein that has been experimentally demonstrated to be exposed on the ODV surface (Faulkner *et al.*, 1997). Our illustration of ODV envelope proteins (Fig. 8) is thus speculative and much work remains to be done to determine the orientation of the other ODV envelope proteins.

Considerable progress has been made using ODV envelope proteins to understand the morphogenesis of the ODV envelope from the INM (Pijlman *et al.*, 2003). However, a model for how INM vesicles form the ODV envelope after entry into the nuclear ring zone has not been presented. We suggest that this process may be similar to the process that occurs with BV formation (Fig. 9). The lumen of the ER is continuous with the lumen between the ONM and INM. For BV formation, ER secretory vesicles containing GP64 and F protein are translocated (through the Golgi complex) to the cell membrane where they fuse. In these ER-originating vesicles, GP64 and F protein are oriented toward the lumen to result in their orientation on the outside of the cell membrane. BV nucleocapsids then bud out acquiring these proteins as BV envelope proteins. For ODV formation, INM vesicles containing ODV envelope proteins are released into the nucleus. ODV nucleocapsids attach to these INM vesicles just as BV nucleocapsids attach to the cell membrane. It has been suggested that ODV-E66 is exposed on the surfaces of INM vesicles (Pijlman *et al.*, 2003). We propose that ODV-E66 interacts directly with the nucleocapsids as they bind to INM vesicles. The ODV nucleocapsids essentially bud into INM microvesicles and are enveloped. Membrane proteins oriented on the luminal side of INM vesicles are consequently exposed on the surfaces of ODVs. Thus, F protein and P74 would be expected to be present on the luminal side INM vesicles prior to their becoming ODV envelope proteins. This is presently only a hypothesis and much work is still needed to understand the morphogenesis of the ODV.

VI. Conclusions

Most baculovirus research has been directed at studying the BV, which has been the direct path for several reasons. Experiments involving BV function can be done in cell culture while ODV studies are

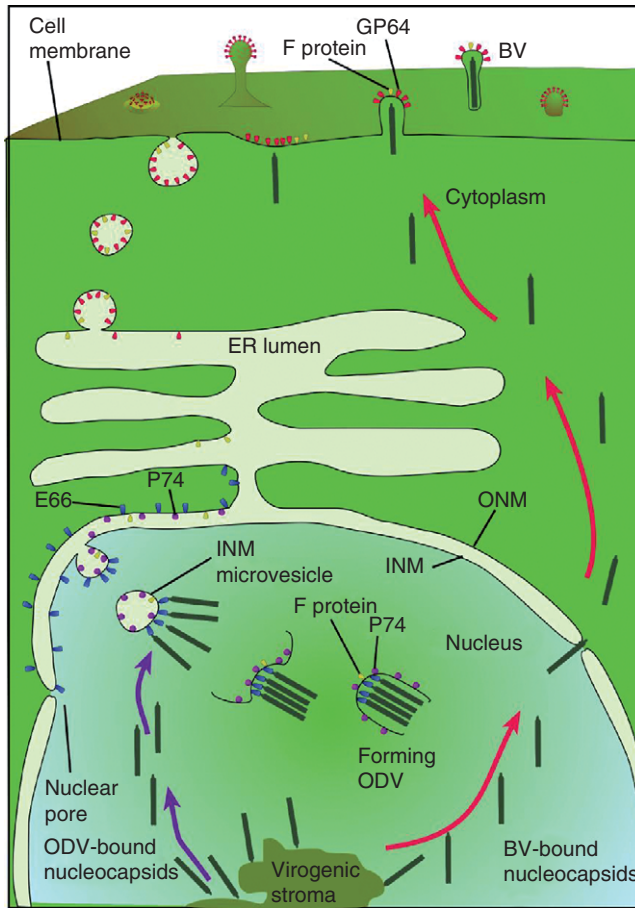

FIG 9. A simple model for baculovirus virion envelopment and envelope protein translocation. The BV and ODV forms in distinctly different locations in the cell. BV nucleocapsid must translocate through nuclear pores out of the nucleus, across the cytosol, and to the cell membrane. ODV nucleocapsids are retained within the nuclear ring zone. The formation of enveloped virions in the nucleus is unusual for viruses. However, there may be a common thread between the ODV and the BV morphogenesis. Cell surface proteins such as BV integral envelope fusion proteins (EFPs) are synthesized in the ER such that they are exposed in the ER lumen. EFPs are translocated to the cell surface in ER vesicles, which after sorting in the Golgi complex, fuse with the host cell membrane. This permits EFPs to be presented on the outside of BVs as they bud out of the cell. ODV virions may acquire their envelopes by "budding" into INM vesicles. The double nuclear membrane is continuous with the ER. In this model, ODV envelope proteins such as P74 and F protein would be displayed on the luminal side of the ER and nuclear double membrane. Envelope proteins such as E66 that may interact with nucleocapsids would be displayed on the nucleoplasmic side of INM vesicles.

best when they include feeding bioassays with insects. For most laboratories, rearing insects is messy and the quality of animals can vary from even the best equipped facilities. Consequently, many ODV studies have been lacking due to the omission of an animal model. The media environment of cell culture is well defined while the animal model and particularly the midgut are infinitely more complex and have an extreme microenvironment for which the biology is still not fully understood. Finally, the ODV is a far more complex structure than the BV. Multiple capsids per virion, occlusion, more diverse viral protein composition, and the tegument all contribute to the complexity of ODV virions. The ODV-associated proteins with functions involving viral DNA packaging, capsid translocation, cell cycle disruption, DNA replication, and DNA repair have been the easiest proteins to study given that their activities can be evaluated in insect cell culture.

ODV proteins that interact with the OB matrix are yet to be described. Occlusion is a unique biological phenomenon. Understanding the mechanism behind this could have important applications beyond baculovirus biology. It may be possible to engineer baculoviruses to occlude vaccines or biopharmaceutical products. The OB would be an ideal package of preservation as contained particles can be rapidly released with just a change in pH. Preliminary studies hint that it may be possible to alter or control the shape of OBs (Lin *et al.*, 2000). This has the potential to lead to applications in nanobiotechnology.

ODV proteins that interact with the insect midgut have been more difficult to investigate. Simple experiments such as protease digest determination of the surface exposure of ODV envelope proteins have not been done despite the fact that many of these proteins have single transmembrane anchor regions. Significant progress in this area is anticipated. The availability of insect genomes and midgut cDNA/EST libraries will expedite the discovery of ODV receptors on the midgut. Indeed, may be through these studies that the ODV attachment protein(s) become characterized. No doubt, baculoviruses have evolved to target highly conserved midgut receptor proteins. Identifying these receptors will have implications in baculovirus biology, insect biology, and will potentially lead to new biocontrol technologies.

Acknowledgments

Part of this research was supported by a grant from Genome Canada through the Ontario Genomics Institute, the Biotechnology Strategy Fund of Canada, and the Biocontrol Network of Canada.

The authors would like to thank Hilary Lauzon for helpful discussions and Chris Upton for introducing the Viral Bioinformatics Resource Center, University of Victoria.

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ADVANCES IN VIRUS RESEARCH, VOL 69

COUPLING OF ROTAVIRUS GENOME REPLICATION AND CAPSID ASSEMBLY

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ABSTRACT

The *Reoviridae* family represents a diverse collection of viruses with segmented double-stranded (ds)RNA genomes, including some that are significant causes of disease in humans, livestock, and plants. The genome segments of these viruses are never detected free in the infected cell but are transcribed and replicated within viral cores by RNA-dependent RNA polymerase (RdRP). Insight into the replication mechanism has been provided from studies on *Rotavirus*, a member of the *Reoviridae* whose RdRP can specifically recognize viral plus (+) strand RNAs and catalyze their replication to dsRNAs *in vitro*. These analyses have revealed that although the rotavirus RdRP can interact with recognition signals in (+) strand RNAs in the absence of

0065-3527/07 $35.00
DOI: 10.1016/S0065-3527(06)69004-0

other proteins, the conversion of this complex to one that can support initiation of dsRNA synthesis requires the presence and partial assembly of the core capsid protein. By this mechanism, the viral polymerase can carry out dsRNA synthesis only when capsid protein is available to package its newly made product. By preventing the accumulation of naked dsRNA within the cell, the virus avoids triggering dsRNA-dependent interferon signaling pathways that can induce expression and activation of antiviral host proteins.

I. Introduction

Viruses of the *Reoviridae* family have genomes consisting of 9, 10, 11, or 12 segments of double-stranded (ds)RNA contained within icosahedral capsids formed from 1, 2, or 3 layers of protein (Mertens, 2004). Rotaviruses represent the most medically significant genera within the family, infecting nearly all children by the age of 5 (Parashar *et al.*, 2003). Annually, such infections lead to ~500,000 deaths throughout the world. The importance of rotavirus disease has stimulated extensive efforts not only to develop rotavirus vaccines but also to define the basic tenets of its life cycle. These latter studies have shown that the viral genome segments only exist in a packaged form within the infected cell, and never as free naked dsRNA species. This characteristic is the consequence of interdependent replication and packaging processes mediated by the need of the viral RNA-dependent RNA polymerase (RdRP) to interact with an interior capsid protein before it gains catalytic activity (Patton *et al.*, 1997). By this mechanism, the viral polymerase can carry out dsRNA synthesis only when capsid protein is available to package its newly made product. By preventing the accumulation of naked dsRNA within the cell, the virus avoids triggering dsRNA-dependent interferon-signaling pathways that can induce expression and activation of antiviral host proteins.

II. General Features of Rotavirus

A. Virion Architecture

The rotavirion is an icosahedron that contains 11 segments of dsRNA surrounded by three layers of protein (Fig. 1) (Prasad *et al.*, 1988; Yeager *et al.*, 1990). The outermost layer of this triple-layered particle (TLP) is assembled from trimers of the glycoprotein, VP7,

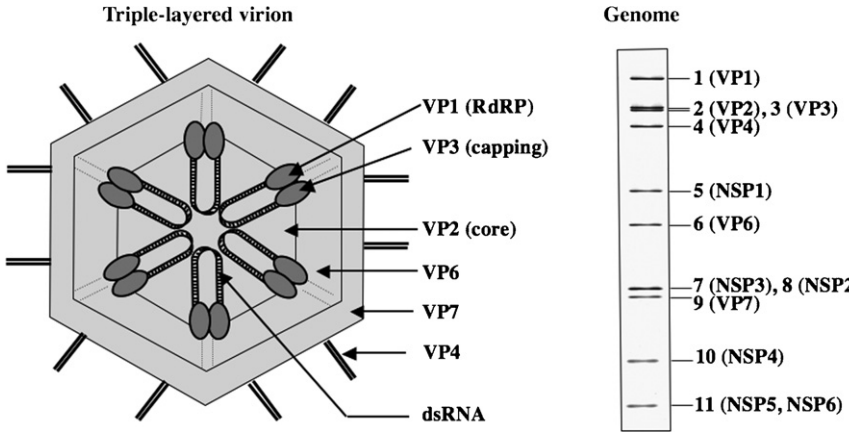

FIG 1. The rotavirus virion is a triple-layered icosahedron that contains 11 segments of dsRNA. The innermost protein layer is formed by the core lattice protein, VP2. Anchored at the vertices of the VP2 lattice are copies of the viral RdRP, VP1, and the multifunctional capping enzyme, VP3. DLPs represent cores surrounded by VP6, and TLPs represent DLPs surrounded by VP7 and VP4. The genome segments can be resolved by electrophoresis on polyacrylamide gels (right panel). Proteins encoded by the genome segments by the SA11 strain of rotavirus are indicated.

arranged with T = 13 symmetry. Projecting from the VP7 layer are multimeric spikes formed by the attachment protein VP4 (Prasad *et al.*, 1990). Exposure to trypsin-like proteases cleaves VP4 into VP5* and VP8* fragments, a process necessary for entry of rotavirus into the cell (Dormitzer *et al.*, 2001; Konno *et al.*, 1993). The intermediate protein layer of the virion consists of trimers of VP6, also organized with T = 13 symmetry. The innermost protein layer is formed by 60 dimers of the sequence-independent RNA-binding protein, VP2, organized as a T = 1 icosahedral lattice. VP2 is oriented in the lattice such that its N-terminal RNA-binding domain is situated toward the interior of the particle (Lawton *et al.*, 1997a,b). One copy each of the viral RdRP, VP1, and the mRNA-capping enzyme, VP3, are positioned at most if not all of the 12 vertices of the VP2 lattice (Liu *et al.*, 1992; Pizarro *et al.*, 1991; Valenzuela *et al.*, 1991). These enzymes project inwardly from the VP2 lattice toward the apex of the particle and are linked to the lattice through interactions requiring the N-terminus of VP2 (Lawton *et al.*, 1997b). Together VP1, VP2, VP3, and the genome segments form the core of the virion. The rotavirus genome segments are collectively organized in the core with dodecahedral symmetry,

with each face of the dodecahedron proposed to represent a contact site between a single VP1–VP3 complex and a single dsRNA segment (Prasad *et al.*, 1996).

In contrast to the rotavirus core, five copies of the viral mRNA-capping enzyme (λ2) and a single copy of the viral RdRP are present at each vertex of the orthoreovirus core (Reinisch *et al.*, 2000). The five λ2 molecules are organized such that they produce a turret-like structure, with a central channel, that projects outward from the corners of the reovirus core. This morphological characteristic has been used to separate members of the *Reoviridae* into turreted (*Orthoreovirus*, *Cypovirus*, and *Aquareovirus*) and nonturreted (*Rotavirus*, *Orbivirus*, and *Phytoreovirus*) subgroups (Hill *et al.*, 1999).

A classification scheme has been established for rotaviruses that is based on the reactivity of the inner and outer capsid components to reference antisera (Hoshino and Kapikian, 1996, 2000). Rotaviruses are assigned to one of seven groups (A to G) based on the antigenic epitopes contained within their VP6 component. Viruses within each group are further defined into G-type (G representing *g*lycoprotein) and P-type (P representing protease-sensitive protein), based on the recognition of their VP7 and VP4 components by specific neutralizing antibodies. The group A rotaviruses are the most important from the perspective of human morbidity and mortality, representing the agents responsible for the majority of cases of acute dehydrating diarrhea in infants and young children throughout the world (Parashar *et al.*, 2003). The group B rotaviruses have been associated with large infrequent outbreaks of severe and sometimes fatal diarrheal illness occurring in young adults in China (Su *et al.*, 1986; Wang *et al.*, 1985). The group C viruses have been associated with sporadic but limited outbreaks of gastroenteritis in children and adults in many parts of the world (Otsu, 1998; Sanchez-Fauquier *et al.*, 2003).

B. Genome Coding Potential

The genome segments of rotaviruses range in size from ~0.7 to 3.1 kB and are generally monocistronic, with a single functional open-reading frame (ORF) contained within each plus (+) strand. Several important exceptions are worth noting, however, that impact on the potential coding capacity of the viral genome: (1) Genome segment 11 of group A rotaviruses contains an ORF not only for the nonstructural protein 5 (NSP5) but usually for a second out-of-frame ORF that encodes NSP6 (Mitchell and Both, 1988). Evidence exists that both

proteins are made in infected cells, albeit at low levels in the case of NSP6 (Mattion *et al.*, 1991). (2) The (+)RNA of segment 11 of group C rotavirus contains a single ORF, that encoding NSP5 (Bremont *et al.*, 1993). In addition, a potential ORF has been noted in the minus (−) strand of group C segment 11, although whether a protein is expressed from this ORF is not known. (3) Although group C segment 7 contains only a single ORF, the expressed product undergoes proteolytic processing to produce two proteins, NSP3 and a small protein containing a dsRNA-binding motif typical of proteins like PKR that are involved in innate immune responses (Langland *et al.*, 1994). (4) The single ORF in segment 5 of group A and C rotaviruses encodes NSP1, a RING-finger protein known to antagonize the innate immune response (Barro and Patton, 2005). In contrast, group B segment 5 contains two [adult diarrhea rotavirus (ADRV)] or three [infectious diarrhea of infant rats strain (IDIR)] potential ORFs, none with similarity to group A or C NSP1. Segment 5 of the nongroup A, B, C novel adult diarrhea virus (NADRV) appears to encode a polyprotein, potentially cleaved into two proteins, neither with similarity to group A or C NSP1 (Yang *et al.*, 2004). Interestingly, the putative C-terminal cleavage product of NADRV segment 5 has a dsRNA-binding motif with similarity to the small protein product of group C segment. (5) The ORF in segment 4 encodes VP4, a protein that is cleaved by host proteins into VP5* and VP8*. For Banna virus, a nonturreted member of the *Reoviridae*, the VP5*-like and VP8*-like components are encoded on separate genome segments (Mohd Jaafar *et al.*, 2005).

C. Genome Sequences

The (+) strand RNA [(+)RNA] of each genome segment contains a 5′-methylated cap structure but lacks a 3′-poly(A) tail (Imai *et al.*, 1983a). The 5′-end of the (−)RNA is uncapped and lacks a γ-phosphate. The 5′ and 3′ untranslated regions (UTRs) of the 11 genome segments show considerable variation in length and sequence, perhaps not surprisingly given that each segment can be expected to contain its own unique set of packaging and translational signals (Mitchell and Both, 1990). In contrast, the UTRs of homologous segments are highly conserved among viruses belonging to the same group, in some cases more so than the sequences of the ORF.

The only similarity between all 11 genome segments is the presence of short highly conserved terminal sequences [5′ and 3′ consensus sequences (CS)] (Table I) (Mitchell and Both, 1990). In the case of the group A rotaviruses, the (+)RNAs of the genome segments typically end with the

TABLE I
TERMINI OF THE GENOME SEGMENTS OF GROUP A, B, AND C ROTAVIRUSES

Gene[a]	5′-Group A SA11–3′	5′-Group B ADRV-3′	5′-Group C Bristol-3′
1	GGcuauuaaa—UgUGaCC	gGcacuau—uauACCC	GGCuaaaAAa—UGUGGCU
2	GGcuauuaaa—UaUGaCC	gGcaauug—aaaACCC	GGCuuaaAAa—UGUGGCU
3	GGcuuuuaaa—UgUGgCC	gGcacuua—uauACCC	GGCuuaaAAa—UGUGGCU
4	GGcuauaaaa—UgUGaCC	gGcaauau—aaaACCC	GGCaaaaAAa—UGUGGCU
5	GGcuuuuuuu—UgUGaACC	gGuauaau—aaaACCC	GGCauuuAAa—UGUGGCU
6	GGcuuuuaaa—UgUGaCC	gGuuuaaa—aaaACCC	GGCuuuuAAa—UGUGGCU
7	GGcauuuaau—UgUGgCC	gGuauaau—aaaACCC	GGCuuuaAAa—UGUGGCU
8	GGcuuuuaaa—UgUGaCC	gGuagaaa—aaaACCC	GGCauuuAAa—UGUGGCU
9	GGcuuuaaaa—UgUGaCC	gGcaauaa—aaaACCC	GGCuuuuAAa—UGUGGCU
10	GGguuuuaaa—UgUGaCC	aGcaauua—aggACCC	GGCuuuaAAa—UGUGGCU
11	GGcuuuuaaa—UgUGaCC	gGuauaua—aaaACCC	GGCuuuaAAu—UGUGGCU

[a] Accession numbers for SA11: X16830, X16831, X16837, X14204, AF290883, AY187029, AY065843, L04532, K02028, AF087678, AF306493; ADRV: NV007548, M91433, NC007551, M91434, M91435, M55982, AJ867610, M91437, M33872, AY548957, M34380; Bristol: NC007547, NC007546, NC007572, NC007574, NC007570, NC007543, NC007544, NC007571, NC007545, NC007569, NC007573.

5′CS, 5′-GGC(U/A)$_7$-3′, and the 3′CS, UGUGACC. Infrequently, minor differences are noted between the terminal sequences of viruses in the same group. For instance, the 3′CS of segment 5 is UGUGACC for the bovine UK strain, but is UGUGAaCC (atypical residue in lower case) for the simian SA11, human KU, and canine K9 strains (Patton *et al.*, 2001). The atypical segment 5 3′CS is highly stable at least in the case of the SA11 strain, and so far is not known to be affected by virus passage conditions. Unlike SA11, serial passage of the rhesus rotavirus strain, RRV, at high multiplicity of infection (m.o.i.) in highly permissive cells results in extensive mutation of its segment 5 3′CS (Table II) (Kearney *et al.*, 2004). These mutations include base substitutions, deletions, or insertions. No other segment of RRV is known to undergo such mutation during serial passage, suggesting a unique tolerance of the segment 5 RNA 3′-end to variation that does not impact on virus replication under at least some passage conditions. Characterization of the atypical 3′-ends of RRV segment 5 and of the segments of other rotavirus strains has allowed the 3′-terminal sequence essential for replication [(+)RNA > dsRNA] of group A rotavirus (+)RNAs to be defined as URN$_{0–5}$CC (R = purine, N = any base) (Kearney *et al.*, 2004). Excluding the hypervariability noted for the gene 5 RNAs, the common 3′-terminal sequence

TABLE II
3′-TERMINAL SEQUENCE OF RRV SEGMENT 5[a]

RRV isolate	Gene 5 3′-end
RRV (wild type)	UGUGACC
RRVg5-u	UGUuCC
RRVg5-uu	UGUuuCC
RRVg5-auu	UGauuCC
RRVg5-cuu	UGcuuCC
RRVg5-ccuu	UGccuuCC

[a] Details given in Kearney *et al.* (2004).

becomes URUGRCC, a sequence showing a close similarity to the 3′CS (UGUGACC).

The 3′CS not only includes sequence information that promotes genome replication but also contains a determinant that is required for efficient translation of rotavirus (+)RNAs. Specifically, the last four to five nucleotides of the 3′CS [(U)GACC] are recognized by dimers of NSP3 (Poncet *et al.*, 1993, 1994), a viral NSP that has affinity for the cap-associated eukaryotic initiation factor, eIF4GI (Piron *et al.*, 1998, 1999). By binding simultaneously to the 3′CS and eIF4GI, NSP3 has been proposed to act as a translation enhancer for viral gene expression by promoting circularization of (+)RNAs in polysomes (Vende *et al.*, 2000). Because of the dual role of the 3′CS, its mutation to a derivative of $URN_{0–5}CC$, although not affecting genome replication, may have an effect on the translational efficiency of (+)RNAs. Indeed, mutation of the NSP3-binding site has been correlated with reductions in the translational efficiency of viral (+)RNAs or analogue reporter (+)RNAs both *in vitro* and *in vivo* (Chizhikov and Patton, 2000; Kearney *et al.*, 2004; Vende *et al.*, 2000).

For most rotavirus segments, the importance of the 3′CS in promoting both the replication and the translation of the (+)RNAs would presumably select against mutational drift away from the UGUGACC terminal sequence. In the case of the segment 5 RNA, its interferon-antagonist protein product NSP1 has an important role in promoting the successful cell-to-cell spread of the virus in cell types that have competent innate immune responses (Barro and Patton, 2005; Graff *et al.*, 2002). However, when rotavirus is passaged at high m.o.i., all cells in the culture are infected by the viral inoculum, thus precluding a need for NSP1 in promoting the cell-to-cell spread of the virus. As a consequence, the selective pressure on the segment 5 3′CS under high-m.o.i. passage conditions is altered to favor its mutation to a more

efficient promoter for (−) strand synthesis, and to a less efficient translation enhancer (Kearney *et al.*, 2004). This change in selective pressure likely accounts for the extensive mutation of the RRV segment 5 3′CS to derivative sequences fitting the motif $URN_{0–5}CC$ that occurs on high-m.o.i. serial passage of the virus. Why such extensive mutation of segment 5 3′-end is seen for RRV and not for some other rotavirus strains (e.g., SA11, UK) under similar passage conditions remains unexplained, but suggests that other factors influence either the rate in which mutations are introduced into the 3′-end or the selective pressures that allow the mutation to be retained within the virus population.

D. Genome Rearrangement

Although the segment 5 3′CS remains stable on high-m.o.i. serial passage of rotavirus strains SA11 and UK, the segment 5 RNAs of these strains and others will often undergo sequence rearrangement during these passage conditions, producing a radical change in the size of the RNA (Desselberger, 1996; Kojima *et al.*, 2000; Patton *et al.*, 2001; Taniguchi *et al.*, 1996b). The most typical rearrangement results from a head-to-tail sequence duplication which in some cases can increase the 1.6-kB size of the segment 5 dsRNA by as much as 1 kB. Less frequently, the rearrangement results from a sequence deletion, which can cause a reduction in the size of the segment 5 dsRNA of more than 0.5 kB. In most cases, rearrangements in the segment 5 RNA interrupt the NSP1 ORF such that the gene no longer encodes a functional form of the protein. The fact that viruses with such rearrangements remain viable, in at least some cell lines, has provided strong evidence of a nonessential role for NSP1 in virus replication (Hua and Patton, 1994; Taniguchi *et al.*, 1996a).

Viruses have also been isolated with sequence duplications in other genome segments, including those encoding VP6, NSP2, NSP3, NSP4, and NSP5 (Kojima *et al.*, 1996, 2000; Mendez *et al.*, 1992; Shen *et al.*, 1994). The sequence duplication for these segments invariably initiate near or at the junction of the ORF and the 3′UTR. Given this location, the coding capacity of the rearranged segment is usually not affected, allowing the production of a wild-type protein. However, there are examples where the sequence duplication begins within the 3′-end of the ORF, thereby causing minor alteration to the extreme C-terminus of the protein product (Gault *et al.*, 2001). For segments other than

gene 5, duplications that significantly disrupt the ORF of any segment are probably not tolerated because of the essential nature of the protein product of the ORF in virus replication.

Suggestive evidence exists that sequence rearrangements occur during transcription of the rotavirus genome. In one proposal, the viral RdRP and associated nascent RNA are thought to disengage from the RNA template during (+) strand synthesis, then reattach at an upstream or downstream location on the same template, where (+) strand synthesis reinitiates, thereby elongating the initial nascent RNA and generating a rearranged sequence (Desselberger, 1996; Kojima *et al.*, 1996). The appearance of rearranged segments in the viral genome would require packaging of the putative rearranged transcript and its utilization as a template for (−) strand synthesis. The possibility that the disengaged viral RdRP and its associated nascent RNA reattach not to its original template but to a second template has been supported by sequencing studies that have identified viruses with chimeric-like genome segments appearing to be formed by intragenic recombination (Matsui *et al.*, 1990; Parra *et al.*, 2004; Suzuki *et al.*, 1998). Interestingly, genome rearrangements have been detected only for the nonturreted viruses of the *Reoviridae*, suggesting that capsid structure is a factor affecting the potential of the genome segments to undergo rearrangement.

E. Genome Reassortment

Coinfection of cells with different rotavirus strains belonging to the same serogroup typically yields progeny with genome constellations that reflect the mixing of the genome segments of the parental strains (Ramig and Ward, 1991). The ability of human and nonhuman mammalian (e.g., RRV, UK) group A viruses to undergo such genetic recombination (reassortment) has been used to create rotavirus vaccine candidates that induce immunological protective responses in the host without causing disease (Clements-Mann *et al.*, 1999; Hoshino *et al.*, 1997; Midthun and Kapikian, 1996). In contrast to viruses belonging to the same group, viruses of different groups are not known to undergo reassortment. The basis for this reassortment restriction has not been defined, but one possibility includes a failure of these two groups of viruses to encode proteins with the necessary compatibility to support a joint replication process. Certainly, sequence analyses showing that proteins encoded by viruses of different groups have

limited homology reinforce this possibility. As an example, the RdRPs of groups A and C viruses have an amino acid identity of only ~20%. The reassortment restriction may also reflect the marked differences in the 5′CS and 3′CS that are present on the (+)RNAs of viruses belonging to different groups. This includes even the two terminal residues of the 3′CS, which seem invariant for viruses of the same group (e.g., CC for group A strains, CU for group C strains) (Table I) (Chen *et al.*, 2002; Mitchell and Both, 1990). Such differences raise the possibility that proteins encoded by viruses belonging to one group may be incapable of recognizing the critical *cis*-acting signals in the viral RNAs of other groups of viruses, and therefore, cannot form the protein–RNA complexes required for reassortment.

Even though rotavirus strains belonging to the same group have the capacity to reassort with one another, there is less evidence than might be expected that co-circulating strains in nature can generate, by reassortment, new epidemiologically predominant variants that can compete successfully with existing strains in infecting host populations (Gentsch *et al.*, 2005). The low frequency at which new reassortants become widely established within the circulating pool of viruses is probably not due to the failure of two different strains to routinely coinfect hosts. Indeed, epidemiological studies have indicated that such mixed infections can be fairly common, particularly in developing countries (Castello *et al.*, 2004; Gentsch *et al.*, 2005; Nielsen *et al.*, 2005). Instead, the infrequency at which new significant reassortants become established in nature suggests that the genome constellation of each virus strain provides a degree of genetic fitness that cannot be easily achieved by reassortants formed from two different strains. The observation that the homologous segments of viruses belonging to the same group can differ considerably in sequence is consistent with this hypothesis. For example, VP1 sequences of the group A simian SA11 and human HU strains have an identity of only 89%. Such sequence variation raises the possibility that the homologous protein encoded by two virus strains may have enough differences as to prevent one of the proteins from operating as ideally in the genetic background of a different virus as it does in its own background. Hence, coevolution of the genome constellation of any particular virus strain may have generated a viral-protein set that functions optimally in virus replication as long as the set is retained intact. Reassortment would bring together a set of viral proteins that have not evolved together, and therefore may be expected to work together less than optimally, imposing a selective disadvantage on the long-term viability of the reassortant.

III. Replication Cycle

The VP4–VP7 outer protein layer is lost during entry of the virion into the cell, yielding transcriptionally active double-layered particles (DLPs) that synthesize by a conservative mechanism 11 capped but nonpolyadenylated (+)RNAs (Fig. 2) (Benureau *et al.*, 2005; Cohen *et al.*, 1979). The nascent (+)RNAs are extruded from a DLP through channels present at each of the 12 vertices of the icosahedral (Lawton *et al.*, 2000; Prasad *et al.*, 1996). Purified DLPs will carry out (+)RNA synthesis *in vitro* at a high rate for several hours, suggesting that the 5′ and 3′ ends of the genome segments are linked together in the particle in such a way that the dsRNAs functionally act as circular templates during transcription (Cohen, 1977). For a typical group A rotavirus, translation of the (+)RNAs gives rise to six structural (VP) and six NSPs in the infected cell (Mitchell and Both, 1990). The NSPs have varied roles in the replication cycle, with at least two having only indirect roles in the genome replication or virion assembly. This includes NSP1 and NSP3, proteins that subvert the host innate

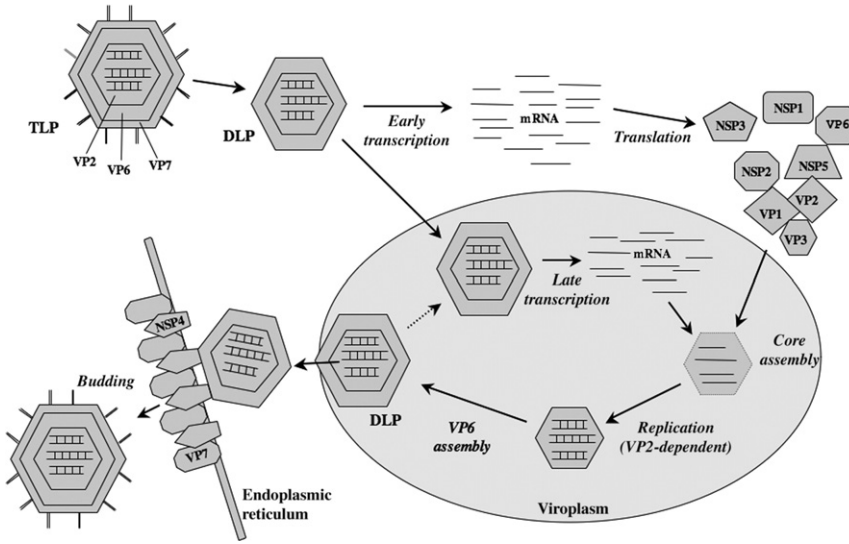

Fig 2. Schematic of the rotavirus replication cycle. Loss of the outer protein layer during entry generates DLPs that catalyze (+)RNA synthesis. Viral inclusion bodies (viroplasms) are viral factories that support transcription, genome packaging and replication, and the assembly of cores and DLPs. DLPs mature into TLPs by budding through the endoplasmic reticulum. Details are given in the text.

immune response and that enhance the translational efficiency of viral (+)RNAs (discussed above), respectively (Barro and Patton, 2005; Graff *et al.*, 2002; Vende *et al.*, 2000). The remaining NSPs (NSP2, NSP4, NSP5, NSP6) have more direct roles in genome replication and particle assembly. Notably, the interaction of two of these proteins, NSP2 and NSP5, induces the formation of cytoplasmic electron-dense inclusion bodies, termed viroplasms (Fig. 3) (Altenburg *et al.*, 1980; Fabbretti *et al.*, 1999). These inclusions serve as putative sites of viral transcription, genome packaging and replication, and core assembly (Fig. 2). The activities of the viroplasm are supported in part by the ability of NSP2 and NSP5 to recruit unassembled core proteins to these sites (Berois *et al.*, 2003; Kattoura *et al.*, 1994). Cores generated within viroplasms are converted to DLPs by interacting with VP6 that accumulates at the periphery of these inclusions. Such DLPs may amplify the replication cycle by supporting secondary rounds of (+) strand synthesis or may acquire VP4–VP7 outer shells by interacting with the endoplasmic reticulum (ER) (Gonzalez *et al.*, 2000).

The movement of DLPs to the ER is mediated by the affinity of VP6 for the cytosolic tail of the viral ER-transmembrane protein NSP4 (Taylor *et al.*, 1996). NSP4 forms complexes with VP7 in the ER membrane, creating target regions through which DLPs bud into the lumen of the ER (Maass and Atkinson, 1990; Tian *et al.*, 1996). A transient membrane envelope can be detected around newly budded DLPs but is subsequently lost leaving the VP7 outer shell (Suzuki *et al.*, 1984).

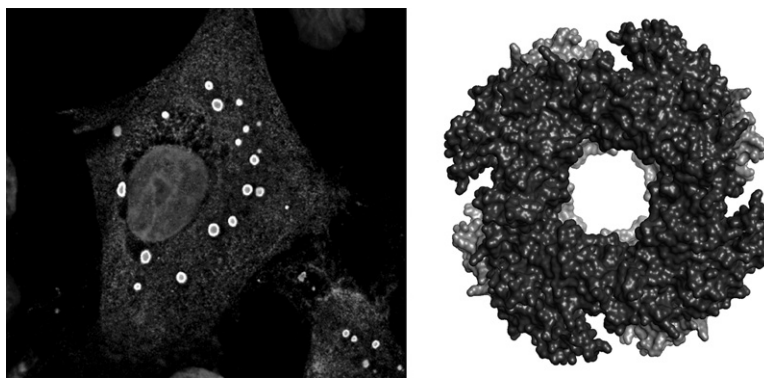

FIG 3. Viroplasms formation is dependent on the nonstructural proteins NSP2 and NSP5. Detection of viroplasms in the cytoplasm of infected cells by immunofluorescence staining with NSP2-specific antisera (left panel). The functional form of NSP2 is as an octamer generated by the tail-to-tail interaction of two tetramers (right panel).

Uncertainty exists as to when and where VP4 assembles onto the particle (Delmas *et al.*, 2004). However, there is evidence that VP4 is associated with NSP4–VP7 complexes contained in the ER membrane, suggesting that VP4 and VP7 are coassembled onto the DLP during the budding process (Maass and Atkinson, 1990).

IV. Viroplasms

Viroplasms mature from numerous small punctate structures early in the infection cycle to fewer larger structures at later stages (Altenburg *et al.*, 1980; Eichwald *et al.*, 2004b; Petrie *et al.*, 1984). This maturation likely reflects the continuous accumulation of NSPs (NSP2 and NSP5) and structural proteins (VP1, VP2, VP3, and VP6), (+)RNAs, and progeny particles in viroplasms, and the fusion of viroplasms with one another (Gonzalez *et al.*, 2000). The importance of NSP2 and NSP5 to viroplasm development has been illustrated by studies showing that the expression of NSP2 and NSP5 together is sufficient to induce formation of viroplasm-like structures (VLS) in uninfected cells (Fabbretti *et al.*, 1999). Knockdown of NSP2 and NSP5 expression in rotavirus-infected cells using small interfering RNAs (siRNAs) has likewise indicated that these two proteins are essential for viroplasm formation (Campagna *et al.*, 2005; Silvestri *et al.*, 2004). NSP2 and NSP5 are both RNA-binding proteins, and therefore, may contribute to the accumulation of the (+)RNAs needed for replication within the viroplasm (Kattoura, *et al.*, 1992; Vende *et al.*, 2002).

A. NSP2 and NSP5

NSP2 is a basic octameric protein with associated ATP-independent helix-unwinding activity (Fig. 3) (Jayaram *et al.*, 2002; Taraporewala and Patton, 2001). Like the single-stranded binding (ssb) proteins used in eukaryotic DNA replication, this unwinding activity may prepare and organize (+)RNAs for packaging and replication by removing interfering secondary structures and other RNA–RNA entanglements (Iftode *et al.*, 1999; Reddy *et al.*, 2000). NSP2 also has an Mg^{2+}-dependent hydrolysis activity that can cleave the γ-phosphate both from NTPs (NTPase) and from the 5′-end of RNAs (RTPase) *in vitro* (Taraporewala *et al.*, 1999; Vasquez-Del Carpio *et al.*, 2006). This activity is associated with a *HI*stidine-*T*riad (HIT)-like motif that is situated in a deep cleft that divides the NSP2 monomer into two

halves (Carpio *et al.*, 2004). Numerous cellular proteins have been identified that contain HIT motifs (Brenner, 2002). While many of these proteins are known to hydrolyze dinucleotide polyphosphates (pApA), by and large, their functions remain undefined. Mutation of conserved residues making up the HIT-like motif in NSP2 has shown that its hydrolytic activity is critical for genome replication but not for viroplasm formation (Carpio *et al.*, 2004; Taraporewala *et al.*, 2006). NSP2 exists in the infected cell as a doughnut-shaped octamer formed by the tail-to-tail interaction of two NSP2 tetramers (Jayaram *et al.*, 2002). This octamer undergoes a conformation shifts as it interacts with nucleotides, a phenomena that has led to the suggestion that the structure functions as a molecular motor that helps to translocate viral RNA during packaging (Schuck *et al.*, 2001). If this is the case, then NSP2 may represent the functional equivalent of the hexameric P4 protein of the *Cystoviridae*, a family of phages (ϕ6–ϕ14) with segmented dsRNA genomes. The P4 multimer constitutes an ATP-driven RNA-packaging motor that translocates (+)RNAs into preformed T = 1 capsids where the templates are replicated to dsRNAs by capsid-associated RdRPs (Mancini *et al.*, 2004).

NSP5 is an acidic dimeric O-linked glycoprotein that exists as multiple isoforms in the infected cell due to varying degrees of phosphorylation (Afrikanova *et al.*, 1996; Welch *et al.*, 1989). NSP2 stimulates the phosphorylation of NSP5 possibly by inducing a conformational shift that triggers an autokinase activity or that allows its phosphorylation by CKI- and CKII-like cellular kinases (Afrikanova *et al.*, 1998; Eichwald *et al.*, 2004a). The importance of phosphorylation on the function of NSP5 in the viral life cycle is not known, although the modification seems unconnected to viroplasm formation (Carpio *et al.*, 2004). Given the impact of phosphorylation on surface potential, the various isomers of NSP5 may differ in their capacity to interact with RNA and viral and host proteins.

Immunofluorescence analysis of cells expressing various combinations of rotavirus proteins has provided evidence that NSP2 and NSP5 are responsible for the accumulation of VP1 and VP2 in viroplasms (unpublished results). Such accumulation may be mediated by the affinity of NSP2 for VP1 and the affinity of NSP5 for VP2 (Berois *et al.*, 2003; Kattoura *et al.* 1994). A characteristic of rotavirus capsid proteins is that they can efficiently assemble into empty virus-like particles (VLPs) independently of RNA replication events. Indeed, cores, DLPs, and TLPs analogous to those made in the infected cell, but devoid of nucleic acid, can be formed by infecting insect cells with recombinant baculoviruses (rBVs) expressing the appropriate

combinations of rotavirus capsid proteins (Charpilienne *et al.*, 2001, 2002; Lawton *et al.*, 1997b; Zeng *et al.*, 1994). In rotavirus-infected cells, the intrinsic self-assembly tendencies of the core proteins may be suppressed through the interaction of VP1 and VP2 with NSP2 and NSP5, respectively, until appropriate packaging and replication events have occurred.

B. NSP4

Along with its role in supporting the budding of DLPs into the ER, studies using siRNAs have shown that NSP4 is connected to several upstream events in the viral life cycle (Lopez *et al.*, 2005; Silvestri *et al.*, 2005). For example, one notable effect of the knockdown of NSP4 expression was to cause a several-fold increase in (+)RNA synthesis during the replication cycle (Silvestri *et al.*, 2005). Presumably, this effect resulted from the lack of NSP4 necessary for recruiting transcriptionally active and potentially active DLPs from viroplasms to the ER, where the DLPs could be converted to quiescent virions. Despite the higher levels of (+)RNA synthesis that occurred due to NSP4 knockdown, no significant change was seen in the levels of viral protein synthesis (except for NSP4) or dsRNA synthesis. Thus, levels of genome replication in rotavirus-infected cell are probably not a direct reflection of the sizes of the available pools of (+)RNAs. Furthermore, levels of protein synthesis late in infection are probably at their maximum, as increases in levels of (+)RNAs during this time period was not found to further increase levels of protein synthesis.

NSP4 knockdown also caused a misdistribution of VP6 in the infected cell, such that the protein no longer accumulated efficiently around viroplasms (Lopez *et al.*, 2005). This defect in VP6 localization may be responsible for the decreased accumulation of DLPs and TLPs that occurs in these cells (Lopez *et al.*, 2005; Silvestri *et al.*, 2005). Results with the NSP4 siRNA raise the possibility that NSP4 interacts with unassembled VP6 and that this interaction guides VP6 to the periphery of viroplasms, where it can stimulate the conversion of cores to DLPs. This guidance activity may be discontinued when the number of DLPs released by any one viroplasm becomes great enough such that they tie up the available NSP4 that surrounds the inclusion. Perhaps due to the failure of VP6 to appropriately localize, viroplasms in NSP4 siRNA-treated cells are less well developed (based on size) than those in control infected cells. Despite their smaller size, viroplasms in the NSP4 siRNA-treated cells supported dsRNA synthesis to the same levels as control infected cells (Silvestri *et al.*, 2005).

Thus, the defect in viroplasm development in the NSP4-depleted cells appears more closely linked to a defect in core to DLP maturation than to a defect in RNA replication processes (i.e., RNA packaging and replication, core assembly).

Interestingly, while NSP2 and NSP5 localize almost exclusively to viroplasms in siRNA-free infected cells, a significant proportion of NSP2 and NSP5 failed to localize to these inclusions in NSP4 siRNA-treated cells (Silvestri *et al.*, 2005). This result indicates that while NSP2 and NSP5 can cooperate to form VLSs in the absence of other viral proteins, their accumulation in viroplasms in infected cells may be modulated by their interaction with other viral proteins or by viral-specific RNAs.

C. RNA Localization

Given the dual role of rotavirus (+)RNAs in genome replication and gene expression, it was anticipated that the introduction of siRNAs into infected cells that target any of the 11 (+)RNAs would prevent both the replication and the translation of that RNA. Surprisingly, this was found not to be true for (+)RNAs that encoded proteins involved in late stages of morphogenesis (VP4 and VP7) or in subverting the host immune response (NSP1) (Dector *et al.*, 2002; Lopez *et al.*, 2005; Silvestri *et al.*, 2004, 2005). Specifically, siRNAs targeting VP4, VP7, or NSP1 (+)RNAs were found to prevent the translation of these RNAs without affecting their replication. Northern blot analysis indicated that siRNAs induce the formation of nuclease complexes [RNA-induced silencing complex (RISC)], but that these complexes can degrade only those (+) RNAs located outside viroplasms (cytosolic) used in translation. In contrast, the analysis indicated that these nuclease complexes could not degrade (+)RNAs contained within viroplasms, serving as templates for (−) strand synthesis (Silvestri *et al.*, 2004). These results provided some of the first direct evidence of the existence of two functionally distinct pools of (+)RNAs in the infected cell, and point to viroplasms as safe houses in which the genetic material of the virus is protected from attack by the action of some antiviral pathways. Moreover, the results suggest that (+)RNAs contained within viroplasms do not originate from outside sources, as RISC-induced degradation of cytosolic (+)RNAs did not have the downstream effect of inhibiting dsRNA synthesis within the viroplasm. Instead, the analysis suggests that (+)RNAs used as templates for dsRNA synthesis are synthesized by transcriptionally active DLPs contained within viroplasms. Indeed, IF assays performed with antibodies to BrUTP

indicate that viroplasms are a primary site of (+)RNA synthesis in the infected cell (Silvestri *et al.*, 2004). Taken together, these findings support a hypothesis that there is no RNA-trafficking system in the infected cell that can translocate (+)RNAs from the cytosol to the viroplasm.

On the basis of evidence that viroplasms are sites of transcription and that (+)RNAs are not transported to viroplasms from the cytosol, it can be predicted that transcriptionally active DLPs originating from incoming infectious virions must serve as focal points for viroplasm formation in the infected cell. Viroplasm formation may be triggered by the recruitment of newly made RNA-binding proteins to the gradient of (+)RNAs likely to surround transcriptionally active virion-derived DLPs. Eventually, the expanding interaction of proteins with (+)RNAs would create an electron-dense RNA-protein inclusion that embeds DLPs. Transcription products not captured by the RNA-binding proteins can be anticipated to migrate to polysomal fields adjacent to these inclusions, where they may direct the synthesis of additional RNA-binding proteins that promote further maturation of viroplasms. Thus, viroplasm maturation may represent an autoregulatory process mediated by the amount of (+)RNA escaping from inclusions with transcriptionally active DLPs. In this scenario, the formation of reassortant viruses would require multiple transcriptionally active DLPs to become embedded within the same viroplasm.

V. Replication Intermediates

Attempts have been made to define steps in genome replication and packaging by analyzing replication intermediates (RIs) recovered from rotavirus-infected cells. Although far from definitive, the results suggest that an initial step involves the interaction of VP1 and VP3 with (+)RNAs to form precore RIs (Gallegos and Patton, 1989). Subsequently, VP2 is believed to interact with precore RIs to form core RIs that contain the full complement of 11 (+)RNAs. Enzymatic analysis has indicated that precore RIs lack associated replicase activity [(+)RNA → dsRNA] *in vitro*, despite the presence of the viral RdRP (VP1) and single-stranded (ss)RNA (Patton and Gallegos, 1990). In contrast, such analyses indicate that core RIs have replicase activity, providing evidence that the catalytic activity of VP1 is VP2 dependent. Subsequent studies performed with *tsB*, an SA11 mutant with a temperature-sensitive (*ts*) lesion in the VP2 gene, revealed that rotavirus-infected cells expressing an assembly defective form of VP2 were inefficient in assembling RIs with

replicase activity (Mansell and Patton, 1990). This too was interpreted as evidence of an important role for VP2 in the polymerase activity of the rotavirus VP1. Analysis of intermediates recovered from infected cells indicates that VP6 associates with core RIs to form double-layered RIs and that these latter intermediates also possess replicase activity (Gallegos and Patton, 1989; Patton and Gallegos, 1990). The presence of replicase activity with both core RIs and double-layered RIs indicates that VP6 is added to the core RI even as it synthesizes dsRNA, implying that replication and core → DLP morphogenesis can occur concurrently. Treatment of purified RIs with ssRNA-specific RNase destroys their ability to synthesize dsRNA. In contrast, the dsRNA product of RIs following *in vitro* replicase assay is resistant to degradation by dsRNA-specific RNase. These differences suggest that the (+)RNA template moves from the RNase-sensitive exterior to the RNase-resistant interior of RIs during dsRNA synthesis. Assays showing that RIs with replicase activity undergo a concurrent reduction in size as they synthesize dsRNA also supports the concept that (+)RNAs extend from newly formed RIs and are drawn into the core of the RI during RNA replication (Patton and Gallegos, 1990). This size reduction can be mimicked by treating newly formed RIs with ssRNA-specific RNase.

Taken together, the data indicate that packaging of (+)RNAs and their replication to dsRNAs are linked events dependent on the availability of the core lattice protein. This linkage assures that the dsRNA product is directly introduced into a capsid shell, thereby avoiding the generation of naked dsRNA which may induce dsRNA-dependent innate immune responses. Moreover, analysis of RIs indicates that (+)RNA packaging initiates prior to dsRNA synthesis and that packaging is not completed prior to the onset of dsRNA synthesis. It follows from this that the relevant packaging signals must be located in (+)RNAs, and not in dsRNAs. The replication process of rotavirus fundamentally differs from that of the segmented dsRNA bacteriophage ϕ6, as the (+)RNA templates of the phage are fully packaged into core equivalents prior to onset of dsRNA synthesis (Mindich, 1999). The rotavirus replication process restricts its (+)RNAs to only a single round of usage as templates for (−) strand synthesis.

NSP2 and NSP5 are components of RIs with replicase activity (Aponte *et al.*, 1996; Gallegos and Patton, 1989), an observation consistent with the idea that genome replication and core assembly occur in viroplasms. The presence of these proteins may be mediated by their RNA-binding activity or their affinity for the structural proteins of the RIs.

VI. Genome Replication

A. Replicase Activity

The single-layered capsid of the L-A dsRNA virus of *Saccharomyces cerevisiae* shows remarkable similarity with the rotavirus core, as both are T = 1 icosahedrons built from 60 protein dimers (Naitow *et al.*, 2002). Akin to the transcriptase activity of the rotavirus core, the L-A virus capsid contains an RdRP that synthesizes (+)RNA through a conservative mechanism (Fujimura *et al.*, 1986). Fujimura and Wickner (1988, 1989) determined that exposure of the L-A virus capsid to low-salt conditions disrupts the particle, leading to the release of its dsRNA genome. These investigators found that polymerase activity associated with disrupted L-A capsids can synthesize dsRNAs using exogenous L-A (+)RNAs as templates for (−) strand synthesis.

Protocols for preparing rotavirus cores by sequentially removing the outer and intermediate protein shells of the virion using chelating (EDTA) and chaotropic (Ca^{2+}) agents were developed by Cohen and colleagues (Bican *et al.*, 1982; Cohen *et al.*, 1979; Jashes *et al.*, 1986). Guided by the techniques used with L-A capsids, Chen *et al.* (1994) showed that exposure to low ionic conditions caused the disruption of rotavirus cores, leading to the release of their dsRNA genome. When incubated with exogenous viral (+)RNAs, the disrupted ("open") cores exhibited an Mg^{2+}-dependent replicase activity that directed the synthesis of dsRNA. The replicase activity was highly processive showing little evidence of premature termination of (−) strand synthesis, and continued efficiently for hours. Several studies have relied on the replicase activity of open cores as a tool for locating *cis*-acting replication signals in rotavirus (+)RNAs (see below).

Despite its usefulness in replicase assays, the open-core system does have limitations. For example, open cores fail to efficiently transcribe exogenous viral dsRNAs, thus preventing identification of *cis*-acting signals contributing to (+)RNA synthesis. Also, the dsRNA products made by open cores are not packaged, preventing application of the system to the identification of packaging signals in (+)RNAs. In the infected cell and in purified virions, the 11 rotavirus dsRNAs exist in equimolar levels indicating a coordination in the packaging of (+)RNAs into cores and their replication to dsRNAs (Patton, 1990). The fact that open cores can replicate any single rotavirus (+)RNA in the absence of the 10 other (+)RNAs suggests that the regulatory mechanism that drives the coordinated synthesis of the 11 dsRNAs in the viral life cycle is not active in the open-core system.

The disruption of rotavirus cores by incubation in low ionic strength buffers not only results in the release of the dsRNA genome but also destabilizes interactions between VP1 and VP2, especially in the presence of nonviral ssRNA (Patton, 1996). This effect of low ionic strength buffers has made it possible to separate VP1 from the VP2 and VP3 components of open cores by gradient sedimentation. Such purified VP1 showed no evidence of replicase activity when incubated in reaction mixtures containing (+)RNAs. However, when combined with fractions of open-core gradients containing VP2, VP1 markedly stimulated dsRNA synthesis (Patton, 1996). These findings provided direct evidence that VP1 alone represents an inactive form of the viral RdRP, but when combined with VP2, undergoes conversion to a catalytically active form.

An important advancement in the analysis of the rotavirus capsid and its protein components was the discovery that VLPs can be formed in insect cells infected with rBVs encoding rotavirus structural proteins (Crawford *et al.*, 1994; Labbe *et al.*, 1991; Lawton *et al.*, 1997b; Vieira *et al.*, 2005). This technique has been particular useful for generating the large preparations of particles needed for cryo-electron microscopy. Replication assays performed with core-like particles made by coexpressing VP1, VP2, and VP3 (VP1–2–3 CLPs) have shown that they possess replicase activity that synthesizes dsRNAs from exogenous (+)RNAs (Chen *et al.*, 1994; Zeng *et al.*, 1996). VP1–2 CLPs were similarly found to possess replicase activity, thus excluding an essential role for VP3 in the synthesis of dsRNA *in vitro*. Not surprisingly, VP2 CLPs lacked replicase activity in the assay system.

Highly purified recombinant VP1 has been prepared using rBV expression systems. Like VP1 recovered from open-core preparations, BV-expressed VP1 does not show evidence of replicase activity when assayed in the absence of VP2 (Patton *et al.*, 1997; Tortorici *et al.*, 2003). However, when combined with purified BV-expressed VP2, VP1 directs dsRNA synthesis from (+)RNA, confirming the VP2-dependent nature of its polymerase activity. Stoichiometric analysis has shown that the molar ratio of recombinant VP1 to VP2 required for maximal levels of replicase activity is approximately 1:10 (Patton *et al.*, 1997). This ratio mimics that of VP1:VP2 present at each of the vertices of the rotavirus core, a result suggesting that the VP2-dependent activation of the RdRP is mediated not just by the presence of VP2, but by the assembly of VP2 into a pentamer unit of the core, vis-à-vis, five dimers. Deletion mutagenesis has shown that N-terminus of the VP2 contains a domain critical to the interaction of the protein with RNA and with VP1 (Labbe *et al.*, 1994; Lawton *et al.*, 1997b;

Zeng *et al.*, 1998). N-truncated forms of VP2 also fail to induce the replicase activity of VP1, demonstrating an important role for the N-terminus in polymerase function (Patton *et al.*, 1997). Although the precise role of VP2 in replicase activation is unknown, it is possible that the 10 copies (5 dimers) of VP2 interact to generate an ssRNA-binding platform on which the RdRP operates (Lawton *et al.*, 2000). The fact that no replicase activity is detected for VP1 by itself even when high levels of the protein are assayed *in vitro* suggests that the effect of VP2 is not just to stimulate some low-level basal activity associated with the RdRP. Instead, the absolute requirement for VP2 to achieve any level of dsRNA suggests that VP2 may bring about a conformational change in the RdRP that is essential for catalytic activity. Such changes may be reminiscent of the structural changes that T7 RNA polymerase undergoes as its transitions from an inactive to active state (Mukherjee *et al.*, 2002; Tahirov *et al.*, 2002; Yin and Steitz, 2002).

B. Initiation of Minus-Strand Synthesis

Replication assays performed with open cores or with recombinant VP1 and VP2 are typically carried out under low ionic conditions, as the presence of even small amounts of salt (e.g., 25-mM NaCl) can dramatically reduce levels of dsRNA synthesis (Chen and Patton, 2000). In the presence of 100-mM salt, the inhibition of dsRNA synthesis in these assays is nearly complete. The salt sensitivity can be overcome if certain components in replication assays are preincubated together prior to the addition of salt. Such preincubation allows the assembly of the initiation complex for (−) strand synthesis, a process that is salt sensitive. Once formed, the initiation complex can go on to support dsRNA synthesis even in high concentrations of salt (e.g., $\geq$1 M NaCl). Formation of a salt-resistant initiation complex has been shown to require GTP, Mg^{2+}, (+)RNA, VP1, and VP2 (Chen and Patton, 2000; Tortorici *et al.*, 2003). Given that rotavirus (+)RNAs terminate with CC, the need for the complementary GTP to form an initiation complex can be expected. Although Mg^{2+} is sufficient to promote assembly of the initiation complex, the addition of Mn^{2+} to the preincubation mixture increases the assembly of complexes even further. In the absence of Mg^{2+}, initiation complexes are not formed even when Mn^{2+} is present. Thus, both divalent cations are needed to achieve optimal formation of initiation complexes, but only Mg^{2+} is essential for the process (Tortorici *et al.*, 2003). The data indicates that the VP2-dependent nature of the replicase activity of VP1 includes the participation of VP2 in the formation of functional initiation complexes

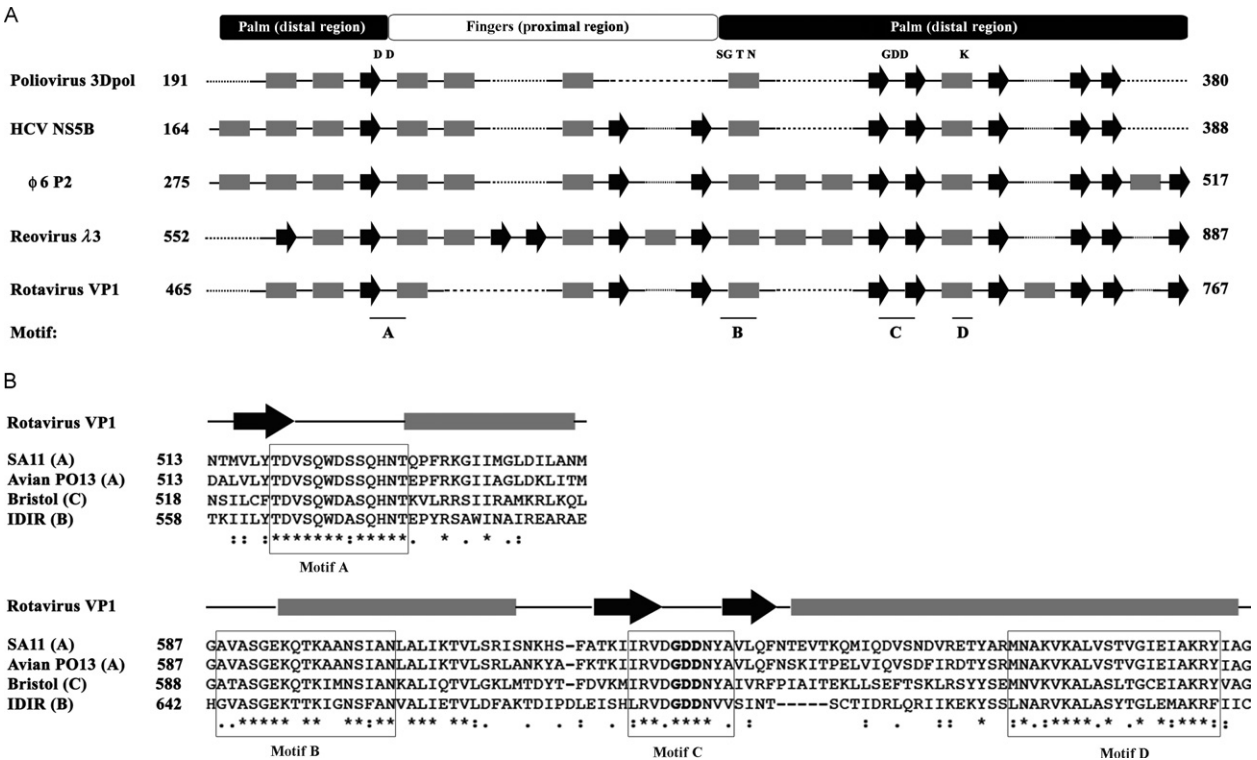

FIG 4. Conservation of secondary structures in the palm and fingers domain of VP1 and the presence of the canonical RdRP motifs. (A) Superposition of the predicted secondary structures of VP1 with the known secondary structures of the RdRPs of several plus and dsRNA viruses (1RDR for *Poliovirus* 3D pol, 1CSJ for HCV NS5B, 1HHS for ϕ 6 P2, and 1MUK for the reovirus λ3

for (−) strand synthesis. Whether the need for VP2 continues into the elongation phase of (−) strand synthesis is not resolved.

C. VP1

By comparison with other RdRPs, it is possible to resolve VP1 (1088 amino acids) into a central catalytic core bounded by N- and C-terminal domains unique to the polymerase. The predicted secondary structure of the central region (residues 440–800) matches closely with regions forming the catalytic core of the RdRPs of other segmented dsRNA viruses [e.g., *Orthoreovirus* and the ϕ6 phage (Fig. 4)] (Butcher *et al.*, 2001; Tao *et al.*, 2002). This structural similarity suggests that the catalytic core of the rotavirus RdRP has a right-handed conformation consisting of thumb-palm-finger domains. The predicted palm domain of the rotavirus RdRP includes the four canonical A, B, C, and D motifs that are characteristic of viral RNA polymerases in general. The rotavirus RdRP also contains an F motif within the finger region (not illustrated) but appears to lack the E motif present in the RdRPs of (+)RNA viruses. Although there is only limited sequence identity among the RdRPs of group A, B, and C rotaviruses, the sequences of the A, B, C, D, and F motifs are highly conserved, as expected given their importance to polymerase function.

Electrophoretic mobility shift assays (EMSA) performed with open cores and with recombinant proteins have shown that VP1 and VP3 can interact with probes to form stable RNA–protein complexes. Such assays have shown that both proteins have nonspecific affinity for ssRNAs, and lack affinity for dsRNAs (Patton, 1996; Patton and Chen, 1999; Tortorici *et al.*, 2003). This latter observation may explain in part why the open-core replication system is inefficient in using exogenous rotavirus dsRNAs as templates for (+)RNA synthesis. EMSA has

protein). The secondary structure was predicted for VP1 using *PSIPRED*. Alpha helices and beta strands are represented with tubular and arrow shape elements, respectively. The elements denote the presence of the structure and not necessarily its length. Dashed lines denote a gap. Numbers at ends of alignment represent location of amino acids within the indicated RdRP. Strictly conserved amino acids that form part of canonical RdRP motifs are shown across the top. (B) Alignment of VP1 sequences belonging to different rotavirus groups. The four canonical motifs present in a defined structural context are shown (boxed). The presence and boundaries of the motifs was initially predicted based on the defined structural context in which they are located and then confirmed using *MEME*, the motif searcher program (GCG/Wisconsin package).

shown that VP1 also has strong specific affinity for viral (+)RNAs, a property mediated by its interaction with recognition signals located at or near the 3′-end of the RNAs (Tortorici *et al.*, 2003). Although at least one such signal is present in the 3′CS, others signals have been mapped for some (+)RNAs to nonconserved regions upstream of the 3′CS. Because of the lack of sequence conservation and predicted secondary structures in these upstream regions, it is not clear that all 11 (+) RNAs have equivalent sets of RdRP-recognition signals. The absence of equivalent signals raises the possibility that the 11 (+)RNAs vary in their ability to recruit the RdRP, and therefore, may vary in their ability to support the formation of (−) strand initiation complexes. Indeed, variation of such upstream recognition signals may explain why the 11 (+)RNAs are replicated with significantly different efficiencies in cell-free replicase assays (unpublished results). However, given that the 11 genome segments are made in infected cells in equimolar levels (Patton, 1990), it is not clear how these differences in the replication efficiencies *in vitro* bear on replication events occurring in the infected cell.

Since the 3′CS is widely conserved among rotavirus (+)RNAs, its RdRP-recognition signal may be expected to promote to a similar extent the replication of all 11 (+)RNAs. The possibility cannot be excluded though that differences in the overall folding of the (+)RNAs may influence the accessibility of the 3′CS, thereby causing variation in the interaction of the RdRP with the recognition signal of the 3′CS. Although replicase assays have demonstrated that the 3′CC of the 3′CS (UGUGACC) is critical to (−) strand synthesis (Chen *et al.*, 1994, 2001), EMSA indicates that the 3′CC does not constitute part of a RdRP-recognition signal (Tortorici *et al.*, 2003). Instead, EMSA has shown that polymerase recognition is mediated by the UGUGA portion of the 3′CS. These results imply that rotavirus genome replication requires a multistep interaction of the polymerase with (+)RNAs, beginning with the VP2-independent binding of VP1 to the UGUGA portion of the 3′CS, followed by the VP2-dependent interaction of VP1 with the 3′CC portion to form an initiation complex. Successful elongation would require the polymerase to disengage from the UGUGA portion, an event that perhaps occurs as the polymerase interacts with the 3′CC.

D. Cis-*Acting Replication Signals*

Cell-free replicase assays performed with open cores or recombinant VP1 and VP2 have been used to locate *cis*-acting signals in rotavirus (+)RNAs that promote dsRNA synthesis (Chen *et al.*, 1994; Patton

et al., 1996, 1997). Results obtained with (+)RNAs containing 3′-terminal deletions have shown that the 3′CS is essential for dsRNA synthesis. Likewise, deletion of the 3′CC of (+)RNAs reduces replication, although to an extent not as a great as deleting the 3′CS (Tortorici *et al.*, 2003). The importance of the 3′CS as a promoter element is well illustrated by the fact that when this sequence is added to the 3′-end of nonviral RNAs, its presence alone conveys to the chimeric RNA some ability to be replicated. In comparison, the addition of just CC to the end of nonviral RNA has little stimulatory effect on dsRNA synthesis, pointing to the importance of the entirety of the 3′CS in promoting (−) strand synthesis (Patton *et al.*, 1996, 1997). Exhaustive mutagenesis of the 3′CS (UGUGACC) has shown that the terminal CC residues are those that are most important to the function of the 3′CS (Chen *et al.*, 2001; Wentz *et al.*, 1996). Though of comparative less importance, nearly any single change made to the remaining UGUGA portion of the 3′CS nonetheless reduces the replication efficiency of the (+)RNA. Considered collectively, replicase assays and EMSA indicate that the 3′CS represents an operationally independent promoter element involved both in the recruitment of the viral RdRP and in the VP2-dependent assembly of a (−) strand initiation complex that then drives dsRNA synthesis. As discussed above, these two activities require participation of different residues of the 3′CS, notably the UGUGA and CC portions, respectively. The discovery of mutant rotaviruses with one or more nucleotide insertions between these portions indicate that the processes of RdRP recruitment and initiation complex may proceed in manner that are spatially or temporally independent of one another (Kearney *et al.*, 2004).

Deletion mutagenesis has indicated that in addition to the 3′CS, other sequences are present in the rotavirus (+)RNAs that promote dsRNA synthesis *in vitro*. These enhancement signals have been mapped to the 5′-end of (+)RNAs and to regions of the RNAs immediately upstream of the 3′CS (Chen and Patton, 1998; Patton *et al.*, 1996; Tortorici *et al.*, 2006). These latter regions include sequences identified by EMSA that are important for specific binding of the RdRP to (+)RNAs (Tortorici *et al.*, 2003), providing a possible explanation why deletion of these regions is correlated with reduced dsRNA synthesis. In contrast to the 3′CS, the sequences that make up the enhancement signals differ among the 11 (+)RNAs, suggesting that these signals are gene specific. Computer modeling of the higher order structures of the (+)RNAs does not support the idea that the enhancements signals represent common secondary structures formed by the gene-specific sequences.

On the basis of extensive complementarity between the 5′- and 3′-terminal sequences of rotavirus (+)RNAs, it has been proposed that the ends of the (+)RNAs interact in *cis* to form 5′–3′ panhandle structures (Imai *et al.*, 1983b; Patton *et al.*, 1996; Tortorici *et al.*, 2006). Computer modeling indicates that the 3′CS extends from such panhandles as a single-stranded tail. Mutations made to the 3′CS that convert it from a single-stranded tail to a sequence partially or completely base paired to the 5′-end reduces the ability of the (+)RNA to serve as a template for (−) strand synthesis (Chen and Patton, 1998). Likewise, base pairing of complementary oligonucleotides to the 3′CS reduces the replication efficiency of the (+)RNAs (Barro *et al.*, 2001; Chen *et al.*, 2001). These observations suggest that the role of the 5′–3′ panhandle may be to stabilize (+)RNAs in a cyclized conformation that projects the 3′CS in a manner that is sterically accessible to the RdRP. In essence, the 5′–3′ panhandle with its single-stranded 3′-tail may be proposed to facilitate the assembly of the ternary complex that supports (−) strand synthesis.

Findings suggest that one or more of the highly conserved residues at the 5′-end of (+)RNAs interact in a sequence-specific but structure-independent manner with the initiation complex (Tortorici *et al.*, 2006). Thus, cyclization of the rotavirus (+)RNAs may be driven not only by 5′–3′ base pairing but also by bridging achieved through the simultaneous interaction of the 5′ and 3′ termini with one or more proteins of the initiation complex. Such bridging may be the principal mechanism by which cyclization is driven for those (+)RNAs that seem to lack the extensive 5′–3′ complementarity necessary to promote stable panhandle formation.

The location and characteristics of the gene-specific packaging signals in the 11 viral (+)RNAs that direct the assortment process remain a mystery. However, given the highly conserved nature of the 5′ and 3′ UTRs of homologous rotavirus (+)RNAs, it can be presumed that the gene-specific packaging signals are contained within these regions. Certainly, because viable rotaviruses have been isolated that package (+)RNAs appropriately despite the presence of extensive deletions in ORF sequences, the possibility that packaging signals are contained within coding regions seems untenable (Patton *et al.*, 2001; Taniguchi *et al.*, 1996a,b; Tian *et al.*, 1993). As addressed above, complementary sequences of the 5′ and 3′ UTRs of (+)RNAs are believed to be instrumental in inducing the formation of 5′–3′ panhandle structures. With the likelihood that the UTRs also include sequences directing packaging, it may be assumed that it is these sequences displayed in the context of the panhandle that promote gene-specific packaging.

Thus, panhandle formation may promote not only the assembly of (−) strand initiation complexes but also the assortment of the 11 (+)RNAs. The interaction of these assorted (+)RNA–RdRP complexes with the capping enzyme VP3 and the core lattice protein VP2 would generate a core-like RI, capable of supporting (−) strand synthesis and capturing the dsRNA product.

VII. Perspectives

Studies to date indicate that rotavirus VP1 represents the only RdRP of the *Reoviridae* family with activities *in vitro* that seem to reflect appropriately those activities needed to support virus replication *in vivo*. These include the ability of VP1 to specifically recognize (+)RNAs and to catalyze dsRNA synthesis in a manner connected to core assembly. With upcoming reports on the X-ray structure of VP1 and the DLP, our insight into these processes should become even clearer. However, a complete understanding awaits a description of how the interaction of VP2 with VP1 alters the polymerase such that it gains catalytic activity. Whether the (+)RNA interacts with the polymerase prior to or after RNA assortment, or perhaps represents a part of the assortment process, remains a mystery. Elucidation of assortment signals and the role of viral proteins in gene-specific packaging will be advanced by the development of a reverse genetics system.

Acknowledgments

This work was supported by the Intramural Research Program of NIAID, NIH.

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ADVANCES IN VIRUS RESEARCH, VOL 69

ORIGIN AND EVOLUTION OF 3′UTR OF FLAVIVIRUSES: LONG DIRECT REPEATS AS A BASIS FOR THE FORMATION OF SECONDARY STRUCTURES AND THEIR SIGNIFICANCE FOR VIRUS TRANSMISSION

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Abstract

The 3′ untranslated regions (3′UTRs) of flaviviruses are reviewed and analyzed in relation to short sequences conserved as direct repeats (DRs). Previously, alignments of the 3′UTRs have been constructed for three of the four recognized flavivirus groups, namely mosquito-borne, tick-borne, and nonclassified flaviviruses (MBFV, TBFV, and NCFV, respectively). This revealed (1) six long repeat sequences (LRSs) in the 3′UTR and open-reading frame (ORF) of the TBFV, (2) duplication of

0065-3527/07 $35.00
DOI: 10.1016/S0065-3527(06)69005-2

the 3′UTR of the NCFV by intramolecular recombination, and (3) the possibility of a common origin for all DRs within the MBFV. We have now extended this analysis and review it in the context of all previous published analyses. This has been achieved by constructing a robust alignment between all flaviviruses using the published DRs and secondary RNA structures as "anchors" to reveal additional homologies along the 3′UTR. This approach identified nucleotide regions within the MBFV, NKV (no-known vector viruses), and NCFV 3′UTRs that are homologous to different LRSs in the TBFV 3′UTR and ORF. The analysis revealed that some of the DRs and secondary RNA structures described individually within each flavivirus group share common evolutionary origins. The 3′UTR of flaviviruses, and possibly the ORF, therefore probably evolved through multiple duplication of an RNA domain, homologous to the LRS previously identified only in the TBFV. The short DRs in all virus groups appear to represent the evolutionary remnants of these domains rather than resulting from new duplications. The relevance of these flavivirus DRs to evolution, diversity, 3′UTR enhancer function, and virus transmission is reviewed.

I. Introduction

The flaviviruses (family *Flaviviridae* genus *Flavivirus*) have been the focus of extensive scientific research for more than 70 years because they produce severe disease in humans, with *Dengue virus* (DENV), *Japanese encephalitis virus* (JEV), *Yellow fever virus* (YFV), and *Tick-borne encephalitis virus* (TBEV) as the most significant human pathogens. The flaviviruses are subdivided into three ecological groups—mosquito-borne, tick-borne, and no-known vector flaviviruses (MBFV, TBFV, and NKV, respectively). Some have not yet been classified (Heinz *et al.*, 2000) and will be called the nonclassified flaviviruses (NCFV). The enveloped virions are about 50 nm in diameter with an internal C protein that encloses a single-stranded positive polarity RNA ~11 kb in length. The envelope of mature virions contains the viral E and M glycoproteins that provide receptor, fusion, and immunogenic functions. The genome RNA contains a single open-reading frame (ORF) that encodes a polyprotein of about 3400 amino acids which is cotranslationally processed into the individual structural (C, M, and E) and also nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The nonstructural proteins provide

the viruses with proteolytic and RNA-replicative functions (Lindenbach and Rice, 2001).

The ORF is flanked by 5′ and 3′ untranslated regions (UTRs) that function as recognition sites for virus translation, replication, and possibly assembly. They have attracted much scientific interest because genetic modifications within these regions can attenuate flaviviruses without altering their antigenic specificity making them potential candidates for the development of live attenuated vaccines (reviewed in Markoff, 2003). They are also efficient targets for antisense oligonucleotides used as antivirals (Deas *et al.*, 2005; Kinney *et al.*, 2005).

Structural and functional studies of the flavivirus 3′UTRs may be subdivided into experimental and theoretical approaches, each of which has been reviewed (Gritsun *et al.*, 2006; Markoff, 2003). Here on the basis of previous analysis, we have developed new concepts for the origin and evolution of the 3′UTR in relation to conserved RNA secondary structures that evolved from repeated sequences, and their roles in virus replication and transmission.

II. Short Direct Repeat Sequences

Originally the short direct repeats (DRs) were identified as 22–44 nucleotide conserved sequences (CSs) and repeated conserved sequences (RCSs) within the three divergent MBFV groups, that is, JEV, DENV, and YFV (Hahn *et al.*, 1987). CS1 and CS2 were identified in all three groups whereas RCS2 were identified only in the JEV and DENV groups. The CS3 and RCS3 were found only among JEV and triple tandem repeats YFV-R1, YFV-R2, and YFV-R3 among YFV (Fig. 1A). Subsequently, it was shown that three YFV repeats were present only in West African strains, whereas in South American and Central/East African YFV strains they were present as one and two copies, respectively. Different numbers of repeats were discovered in a YFV-related group of flaviviruses, that is, in *Banzi virus* (BANZV, three repeats), *Sepik virus* (SEPV, two repeats), and *Uganda S virus* (UGSV, one repeat) (Mutebi *et al.*, 2004; Wang *et al.*, 1996). Very few regions of homology were found in the sequences that flank the CS and RCS within the MBFV groups. Surprisingly, the CS2 and RCS2 were also described in the phylogenetically divergent NKV flavivirus group (Charlier *et al.*, 2002).

In the TBFV group, four R1, two R2, and two R3 were described as DRs (Gritsun *et al.*, 1997; Mandl *et al.*, 1991; Wallner *et al.*, 1995).

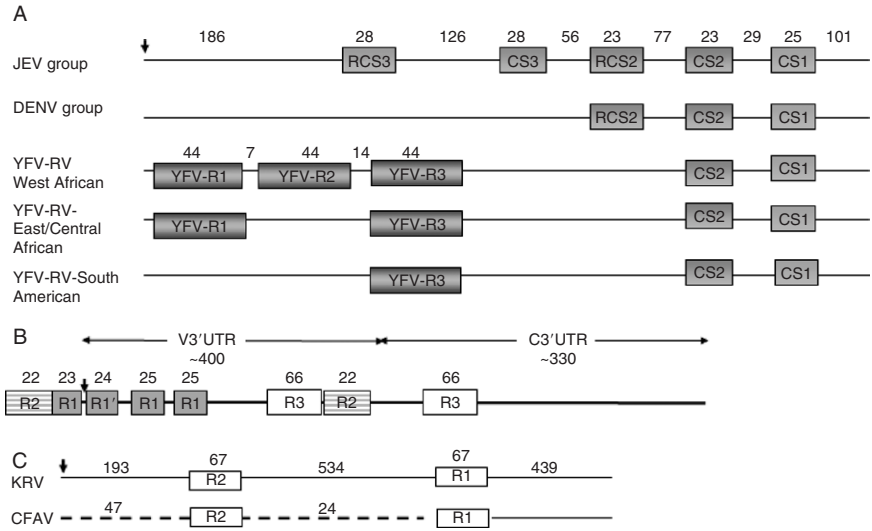

FIG 1. Schematic organization of DRs in the 3′UTR of (A) MBFV as adapted from Hahn *et al.* (1987) with modifications from Mutebi *et al.* (2004), (B) TBFV as adapted from Wallner *et al.* (1995), and (C) NCFV (KRV and CFAV) as adapted from Crabtree *et al.* (2003). Virus 3′UTRs are shown as solid lines and DRs are specified in boxes. Numbers depict nucleotide lengths. The variable (V3′UTR) and conserved (C3′UTR) regions for TBFV are specified. Positions of stop codon are shown by vertical arrow.

All TBFV DRs appeared to be distributed randomly along the 3′UTR and were separated by homologous but nonrepeated sequences (Fig. 1B).

In the NCFV group, that includes Kamiti River virus (KRV) and Cell fusing agent virus (CFAV), two 67-nucleotide DRs, separated in KRV by a 534-nucleotide region and in CFAV by a 24-nucleotide region, were also identified (Crabtree *et al.*, 2003) (Fig. 1C). However, until now (see below) there has been no attempt to interpret their biological significance.

III. Flavivirus Secondary RNA Structures

Computer simulation provided two overlapping models of stable RNA secondary structures in the flavivirus 3′UTR. The first model predicted linear folding of the 3′UTR (Charlier *et al.*, 2002; Gritsun *et al.*, 1997; Leyssen *et al.*, 2002; Proutski *et al.*, 1997a,b, 1999;

Rauscher *et al.*, 1997; Thurner *et al.*, 2004) in which both shared and distinct stem-loop (SL) conformations were identified between the four flavivirus groups (Fig. 2A–D). The most conserved conformation was the terminal long stable hairpin (3′LSH), about 100 nucleotides and an adjacent short stem-loop 2 (SL2) (Brinton *et al.*, 1986; Mohan and Padmanabhan, 1991). In the MBFV group, it is stabilized by pseudoknot interactions between loop 2 and the 3′LSH (Shi *et al.*, 1996; You *et al.*, 2001). The 3′LSH contains a conserved pentanucleotide CACAG that is exposed as a loop at the top of the 3′LSH. Experiments

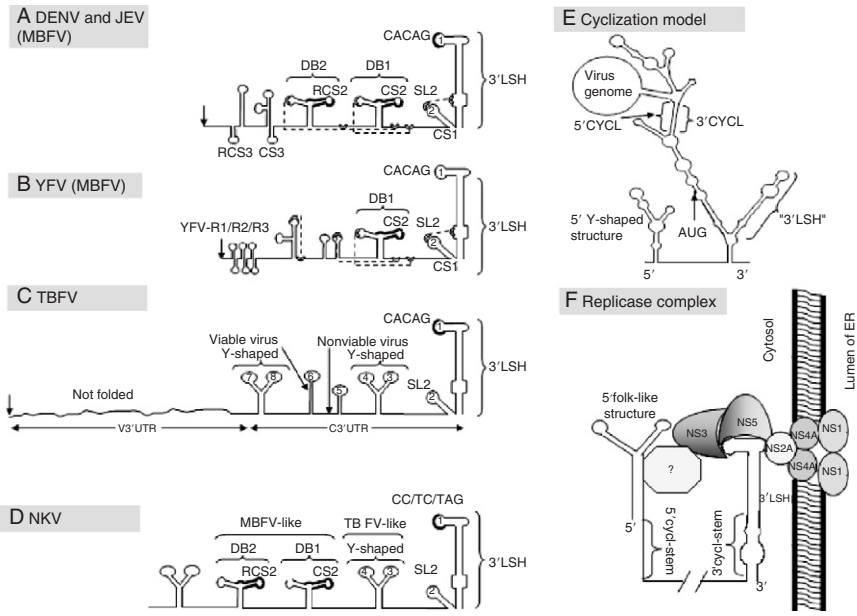

FIG 2. Linear (A–D) and cyclization (E and F) models of RNA structures in the 3′UTR for different flavivirus groups (specified in shadowed boxes). The positions and conformations of the RNA secondary structures are indicated. The CS2/RCS2, CS3/RCS3, and conserved pentanucleotide are highlighted by thick lines. Pseudoknot (thick semioval lines) interactions in MBFV group are shown by dashed lines. The boundaries between viable/nonviable TBFV mutants (C) are indicated. In cyclization model (E), the Y-shaped structure at the 5′UTR is folded independently from the cyclization stem and the part of the side SL that overlapped with the 3′LSH is specified as "3′LSH." The formation of a tentative replication complex (flavivirus proteins are specified as NSs) around the 5′UTR and 3′UTR (F) probably precedes the cyclization of the genome. Summarized from Charlier *et al.* (2002), Gritsun *et al.* (1997), Leyssen *et al.* (2002), Mutebi *et al.* (2004), Olsthoorn and Bol (2001), Proutski *et al.* (1997b, 1999), Rauscher *et al.* (1997), Thurner *et al.* (2004), and Westaway *et al.* (2003).

demonstrated that substitutions in this pentanucleotide either abolish or reduce virus infectivity (Gritsun *et al.*, 2001; Khromykh *et al.*, 2003; Tilgner *et al.*, 2005). The 3′LSH was shown to interact with viral and cellular proteins within the polymerase complex thus initiating replication of the viral genome (Blackwell and Brinton, 1995, 1997; Chen *et al.*, 1997; Cui *et al.*, 1998; De Nova Ocampo *et al.*, 2002; Ta and Vrati, 2000; Tan *et al.*, 1996; You and Padmanabhan, 1999).

The structures located upstream of the 3′LSH-SL2 are different between the MBFV and TBFV (Fig. 2A–D). The first upstream structure is similar for all the MBFV and described as dumbbell-like DB1, with the CS2 exposed presumably for molecular interactions (Fig. 2A and B). Viruses within the JEV and DENV groups formed pseudo-duplicated DB2, also with exposed RCS2s. For the YFV, the region equivalent to the DB2 presented a different conformation. The CS3/RCS3 of JEV and three YFV tandem repeats also formed distinct local secondary structures (Mutebi *et al.*, 2004; Proutski *et al.*, 1999). The exposed location of CS2/RCS2, CS3/RCS3, and YFV repeats is compatible with the concept that they might have a signaling function.

In the TBFV, the RNA structure upstream of the 3′LSH-SL2 (Fig. 2C) is Y-shaped (Gritsun *et al.*, 1997; Proutski *et al.*, 1997b; Rauscher *et al.*, 1997) and almost identical in conformation and sequence to the predicted loops 3 and 4 in the NKV group (Fig. 2D) (Charlier *et al.*, 2002). This structure in the TBEV group was interpreted as the DB-like equivalent of the MBFV (Olsthoorn and Bol, 2001). However, both Y-shaped and two DB-like structures were identified in the 3′UTR of the NKV group (Fig. 2D) suggesting that NKV shared features with both the TBFV and MBFV groups (Charlier *et al.*, 2002; Leyssen *et al.*, 2002).

The second (cyclization) model of stable secondary RNA structures (Fig. 2E) predicts interaction between inverted complementary (cyclization) sequences located in the 3′UTR and 5′UTR (also C gene for MBFV) that results in the formation of a dsRNA stem (Khromykh *et al.*, 2001; Thurner *et al.*, 2004). This model was substantiated experimentally both *in vitro* (Ackermann and Padmanabhan, 2001; You and Padmanabhan, 1999) and *in vivo* (Khromykh *et al.*, 2001; Lo *et al.*, 2003). Some of these inverted repeats, referred to as 3′CYCL and 5′CYCL (8–11 nucleotides long), are totally conserved within the MBFV and TBFV groups. In addition to their roles in cyclization, the 5′CYCL and 3′CYCL are believed to provide a signaling function, in the single- or double-stranded form. In the cyclization model, the top part of the 3′LSH folds as an independent structure (“3′LSH”; Fig. 2E) implying a level of compatibility between these two models. A model

of initiation of flavivirus RNA replication with participation of flavivirus proteins and cyclization between 5′UTR and 3′UTR has been proposed (Westaway *et al.*, 2002, 2003) and, with some modifications, is presented in Fig. 2F.

IV. What Is the Functional Significance of the DRs?

Initially the significance of DRs in flavivirus replication was studied by engineering a series of progressive deletions in the 3′UTR of the infectious clone of DENV (Men *et al.*, 1996). Deletion of CS1 completely abolished infectivity indicating that CS1 is essential for virus replication; this is compatible with the high conservation of CS1 in the widely divergent MBFV group. The deletion of only CS2 that is also conserved between all MBFV was not lethal, although DENV mutant had a reduced replication rate. Additionally, progressive upstream deletions were made from the CS2, toward the stop codon. All these viruses remained infectious although they exhibited a spectrum of growth restrictions in cell culture. The boundary between viable and lethal deletions lies immediately upstream of the CS1; deletion mutants that retain only CS1-SL2–3′LSH produced viable virus albeit with low infectivity and only in mosquito cells. For the infectivity in vertebrate cell, the presence of CS2 or RCS2 (corresponding to the presence of DB1 or DB2) was also essential. In general, the impact of deletions in 3′UTR downstream CS1 was more profound in simian than in mosquito cells, implying that this region could determine host susceptibility (Men *et al.*, 1996). Later, it was demonstrated that the level of perturbation of secondary RNA structures rather than the size of deletions might affect the yield of infectious virus (Proutski *et al.*, 1999).

Similar results were produced in experiments using an YFV infectious clone (Bredenbeek *et al.*, 2003). Deletion of the CS1 element abolished virus replication whereas mutants with deletions of CS2, RSC2, or YFV-R1, YFV-R2, and YFV-R3 still yielded viable viruses, although with reduced infectivity. This implies that while not essential for virus viability, CSs/RCSs and the three YF-specific tandem repeat sequences may have a function related to virus replication rate.

The use of replicons, rather than infectious clones, established which elements of the 3′UTR are responsible for virus replication as opposed to overall virus infectivity. For Kunjin virus (JEV group), deletion of RCS3/CS3 and RCS2 significantly inhibited RNA synthesis (Khromykh and Westaway, 1997), implying that these JEV-group-specific CSs/RCSs determine the efficiency of virus replication.

The individual deletion of CS2, RCS2, CS3, or RCS3 in a related *West Nile virus* (WNV) replicon did not abolish virus RNA synthesis but also reduced it (Lo *et al.*, 2003). Nevertheless, in contrast with the experiments on infectious clones, complete deletion of nucleotides between the RCS2 and the stop codon, with retention of the region CS2-CS1–3′LSH, abolished virus RNA synthesis in vertebrate cells (Lo *et al.*, 2003; Tilgner *et al.*, 2005), probably explained by the different sensitivities of the two methods. However, overall the results indicate that the role of the CSs/RCSs may be to accelerate viral RNA synthesis rates. Although it was also demonstrated that removal of DB1 and DB2 (i.e., CS2 and RCS2, respectively) in DENV 3′UTRs reduced efficiency of translation from the 5′UTR in a luciferase reporter system, this could be explained by nonspecific effects issuing from the reduced RNA stability in transfected cells (Chiu *et al.*, 2005). Moreover, these effects were not observed for WNV replicons (Lo *et al.*, 2003; Tilgner *et al.*, 2005).

Deletions in the 3′UTR of infectious clones representing the TBFV group produced results similar to those reported for the MBFV (Mandl *et al.*, 1998; Pletnev *et al.*, 2001). The boundary that determines the difference between viable and nonviable TBFV within the 3′UTR has been established, although not precisely; deletions upstream of loop 5 completely abolish virus infectivity whereas deletions within stem 6 still produce infectious virus but with reduced infectivity (Fig. 2C). Thus, in the TBFV group, which has a 730-nucleotide 3′UTR, only the terminal 189 nucleotides are essential for virus viability, similar to the 180-nucleotide region that is essential for virus "viability" in the MBFV group. This minimal region for the TBFV group includes a SL5-(Y-shaped structure)-SL2-3′LSH that is conformationally equivalent to the DB1-SL2-3′LSH of the MBFV group.

For the TBFV, two regions were defined in the 3′UTR (Fig. 1B), one of which is the "core" or conserved region (C3′UTR) that corresponds approximately to the terminal 330 nucleotides of the TBFV 3′UTR and shows a high level of homology between the different TBFV species (Gritsun *et al.*, 1997; Wallner *et al.*, 1995). The second region, located between the C3′UTR and the stop codon is more variable within the TBFV group and defined as the V3′UTR. Many TBFV isolates spontaneously lose significant fragments of the V3′UTR during laboratory passage, but the core remains intact (Gritsun *et al.*, 1997; Hayasaka *et al.*, 2001; Mandl *et al.*, 1998; Wallner *et al.*, 1995). The introduction of deletions into the core region produces viruses with reduced infectivity titers whereas viruses with truncated V3′UTRs retain similar levels of infectivity to wild-type viruses (Mandl *et al.*, 1998). It was

proposed that the variable region is essential for virus survival in ticks (Hayasaka *et al.*, 2001; Mandl *et al.*, 1998).

Subdivision of the flavivirus 3′UTR into the promoter and enhancer regions (Gritsun *et al.*, 2006; Proutski *et al.*, 1999) probably more adequately reflects the functional significance of individual structural elements. The promoter, essential for virus viability, interacts with the viral RNA polymerase complex and initiates RNA replication. In structural terms, for the MBFV, the promoter is the region that includes DB1-CS1-SL2-3′LSH, although with a truncated promoter, viz. CS1-SL2-3′LSH, virus infectivity can be demonstrated in mosquito cells. It correlates with the length of TBFV promoter that includes the SL5-(Y-shaped structure)-SL2-3′LSH. The flavivirus promoter functions through cyclization of the virus genome resulting in the initiation of viral RNA synthesis (Fig. 2E and F). As discussed above, there are two different models for the predicted RNA secondary structures, that is, linear and cyclization. They probably present two transient stages of complex flavivirus promoter for initiation of virus RNA replication (Fig. 2F and E) (Gritsun *et al.*, 2006).

The region between the stop codon and the promoter has an enhancer function (Proutski *et al.*, 1999). In other words, although this region may not be essential for viral RNA synthesis, it accelerates it. Therefore, the CSs/RCSs (DRs) of MBFV and the individual elements of the C3′UTR and V3′UTR of TBFV are important elements of replication, the role of which is not to provide virus replication per se, but to ensure rapid replication. The conserved RNA conformations predicted in the ORF of the flavivirus genome (Gritsun *et al.*, 1997; Thurner *et al.*, 2004) might also accelerate viral RNA synthesis (Gritsun *et al.*, 2006), although they might be dispensable in the laboratory-maintained viruses (Kofler *et al.*, 2002).

V. Evolution of the Flavivirus 3′UTR Occurred by Multiple Duplications

The concept of DR preservation is quite controversial in the context of the flavivirus 3′UTR organization. Although the DRs are conserved, this conservation is observed only within particular flavivirus groups and is not essential for virus viability. The function of the DRs appears to be restricted to increasing the rate of viral RNA synthesis. This presents us with a paradox; why should the RNA replication rate need to be maximized if the virus can survive without an increased rate of RNA synthesis? There is no obvious relationship between DRs and

secondary RNA structures. Their preservation strongly implies a signaling function but only parts of these repeats are predicted to be single-stranded regions available for molecular interactions and long enough to form their own local secondary structures. All these facts were quite puzzling and raised other questions. How did these repeats develop and why are they preserved?

We have attempted to answer some of these questions (Gritsun and Gould, 2006a; Gritsun *et al.*, 2006). We proposed that DRs may have arisen as long tandem repeats that were subsequently reduced to shorter sequences by extensive mutagenesis and then remained almost unchanged because they provide an essential role for virus survival. The key approach to understanding these problems was to construct robust alignments that revealed homology in regions outside the DRs.

The molecular mechanism by which repeat sequences arise in RNA viruses is known as copy choice or template switching. RNA-polymerases may dissociate from the template during elongation and, together with the nascent strand, reanneal to a new locus continuing to amplify the daughter strand from the new site of attachment (Copper *et al.*, 1974; Hajjou *et al.*, 1996; Lai, 1992; Pilipenko *et al.*, 1995). The reattachment might occur upstream of the site of dissociation on the same template (mechanism for generation of repeat sequences) or downstream (mechanism for deletion). If the reattachment occurs on different templates, this could lead to the insertion of heterogonous sequence. Misincorporation of a nucleotide during RNA elongation prompts the polymerase to pause and dissociate from the template, with subsequent reannealing at a locus where the sequence is at least partially complementary to the locus of dissociation. Large deletions arise when the secondary RNA structures bring together distant RNA loci corresponding to dissociation or annealing sites (Pilipenko *et al.*, 1995).

RNA-polymerase template switching (recombination) occurs in both ORFs and UTRs and, in conjunction with nucleotide substitutions, is the means by which quasispecies arise in virus populations. However, evolutionary constraints on the ORF and UTRs are very different. In the ORF, the selection pressure might be restricted only to nucleotide substitutions that gradually and slowly change genome sequence. In contrast, deletions, insertions, and duplications might significantly contribute to the variability of UTRs, since they may reduce but not abolish virus infectivity. Indeed, duplications have been recorded in the 3′UTR for a number RNA viruses (Bryan *et al.*, 1992; Faragher and Dalgarno, 1986; Peerenboom *et al.*, 1997; Santagati *et al.*, 1994; Shi *et al.*, 1997; Warren and Murphy, 2003).

On the basis of this idea, we proposed that in the ancestral lineages, repeats longer than the extant DRs might have been present in the 3′UTR of flaviviruses. Subsequently due to extensive mutagenesis, mediated by deletions, insertions, and substitutions, these long tandem repeats might have been reduced to shorter homologous regions that we now observe as a series of DRs, interrupted by nonhomologous regions. Indeed, the construction of the alignments based on these principles enabled us to trace the origin and evolution of the conserved repeated elements within each of the flavivirus groups and to reveal homologies in regions that had not previously been reported (Gritsun and Gould, 2006a; Gritsun *et al.*, 2006). In the following sections, we summarize the results of analysis of 3′UTR alignments that were originally constructed individually for each of the groups, that is, the TBFV (Gritsun and Gould, 2006a), MBFV (Gritsun and Gould, 2006b), and NCFV (Gritsun and Gould, 2006a) and then used to construct a pan-flavivirus alignment that includes these groups and the NKV group.

A. Alignment of the TBFV 3′UTRs

We recently demonstrated that two major duplications can explain the apparently random arrangements of several DRs in the 3′UTR of TBFV (Fig. 3A). First, we identified six imperfect long repeat sequences (LRSs) each about 200 nucleotides in length. These LRSs were shown to be not only in the 3′UTR but also in the ORF and they accommodate all the short DRs that were described previously (Gritsun *et al.*, 1997; Mandl *et al.*, 1991; Wallner *et al.*, 1995). In addition to the six LRSs, six other shorter tandem DRs were also shown to be present in the 3′UTR of the TBFV (Gritsun and Gould, 2006a).

We suggested that relatively high levels of conservation within the TBFV group, in comparison with other flavivirus groups, enabled identification of traces of early development of the 3′UTR. Probably the primordial sequence of the TBFV did not have a complex long 3′UTR in the form that we understand today. Early in the evolution, the function of the 3′UTR might have overlapped with the function of the ORF; alternatively, primordial 3′UTR sequence may have contained only a short nucleotide region that corresponds to the current 3′LSH. Apart from its coding function, this region also interacted with the viral and cellular proteins of the polymerase complex to initiate viral replication.

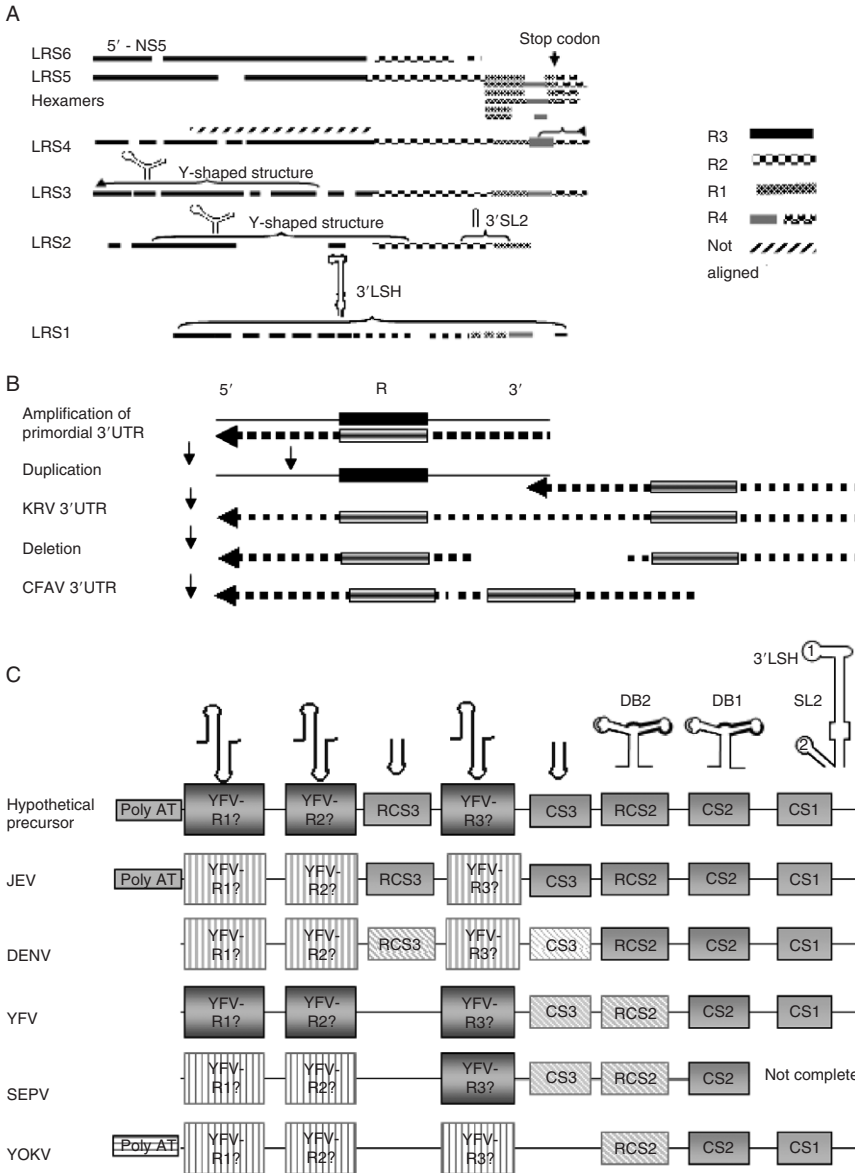

FIG 3. The origin of DRs in TBFV (A), NCFV (B), and MBFV (C) groups. (A) The presence of six LRSs in the 3′UTR of TBFV (Gritsun and Gould, 2006c). The positions of predicted RNA secondary structures 3′LSH, SL2, and two Y-shaped structures relative to LRS1–LRS6 are coordinated. (B) The origin of KRV repeats (R) by self-duplication of virus 3′UTR. The CFAV 3′UTR was developed from KRV by deletion of ~500 nucleotides between repeats

It appears that multiple duplication of the terminal region of the ORF was the major event that shaped the evolution of the TBFV genome. The selection of an early lineage, with an elongated 3′UTR was probably advantageous for the virus, because the dissociation of function of the 3′UTR from the function of the ORF provided more flexibility for the independent development of both, and increased the adaptation potential of the virus to a wider range of hosts.

The reason for preservation of the LRSs in the TBFV groups presumably reflects the slow evolution rate of the flaviviruses associated with tick-transmission in comparison with flaviviruses from other ecological groups. The tick vector that inhabits the Northern Hemisphere feeds once as a larva, once as a nymph, and once as an adult that then lays eggs. This life cycle may last up to 5 years during which for most of the time the tick is relatively immobile. In contrast, mosquitoes support flavivirus transmission in tropical and subtropical regions year-round and the virus is therefore subjected to a higher turnover, which reflects the higher evolution rate of MBFV (Gould *et al.*, 2003; Gritsun *et al.*, 1995; Zanotto *et al.*, 1996).

To identify relationships between LRSs and RNA conformations, we superimposed the predicted RNA secondary structures onto the alignments that we constructed between the TBFV (Gritsun and Gould, 2006a). This led to the suggestion that each LRS encodes an individual conformation, that is, the boundary of each LRS virtually coincides with the boundaries of the secondary structures and with boundaries between viable/nonviable viruses (Figs. 2 and 3A). It is possible that early RNA structures had a Y-shaped conformation, since two of them have been described for the TBFV and the NKV (Charlier *et al.*, 2002; Gritsun *et al.*, 1997) (Fig. 2) and later they evolved into a variety of other conformations in different virus groups.

B. Alignment of the NCFV 3′UTRs

The 3′UTRs of KRV and CFAV (NCFV group), also contained 67-nucleotide repeats, almost identical to each other. For KRV, they were separated by a 534-nucleotide region whereas for CFAV by only

(Gritsun and Gould, 2006a). (C) The common origin of MBFV DRs from a hypothetical monophyletic precursor (Gritsun *et al.*, 2006). The intact DRs (CSs/RCSs and YFV-R1/R2/R3) are depicted as grey-shaded boxes and the corresponding reduced DRs as striped boxes. The presence of poly-AT sequences in some MBFV is indicated. The RNA conformations corresponding to complete CSs/RCSs are coordinated relative to DRs.

24 nucleotides (Fig. 1C). The origin of these repeats was not clear neither was the reason for the almost double-length of the KRV 3′UTR in comparison with other flaviviruses (Crabtree *et al.*, 2003). However, the construction of a robust alignment has revealed that this double-length 3′UTR and the DRs originated from the virtually complete duplication of a primordial KRV 3′UTR (Gritsun and Gould, 2006a). It appeared that the 67-nucleotide-long DRs were the remnants of previous more extensive homology that was depleted by extensive mutagenesis in the flanking regions. We also proposed that the CFAV 3′UTR was derived from a KRV-like precursor sequence following a large deletion between two DRs (Fig. 3B). The third member of the NCFV Tamana bat virus (TBV) isolated from bats in Africa, demonstrated lower similarity with other flaviviruses but more close phylogenetic relationships with CFAV and KRV. It has a short 3′UTR and no duplications have been identified (de Lamballerie *et al.*, 2002).

C. Alignment of the MBFV 3′UTRs

A satisfactory alignment for the MBFV could not be derived using conventional alignment programs since this group is highly divergent. Therefore, we initially produced separate alignments for the JEV-, DENV-, and YFV-related viruses and subsequently aligned these with each other (Gritsun and Gould, 2006b). Using this method, we found traces of truncated CSs/RCSs in all the MBFV groups suggesting that all DRs were present in early MBFV lineages. As these viruses diversified, the CSs/RCSs were preserved in some MBFV and in others only remnants of homology remained (Fig. 3C).

D. Alignment of the NKV 3′UTRs

Currently the 3′UTRs of only four NKV have been sequenced (Charlier *et al.*, 2002; Leyssen *et al.*, 2002). Three, *Montana myotis leukoencephalitis virus* (MMLV), *Rio Bravo virus* (RBV), and *Modoc virus* (MODV), were isolated from rodents in the New World and *Apoi virus* (APOIV) from the Old World. Figure 4 presents an alignment based on the 3′UTR for these four viruses. The predicted RNA secondary structures (Charlier *et al.*, 2002; Leyssen *et al.*, 2002) have been superimposed on this alignment to reveal the correlation between linear and structural domains.

The lengths of the 3′UTRs were quite different for the NKV, longest for the APOIV (576 nucleotides) and shortest for the MODV (366 nucleotides). It is possible that the UTRs of the NKV are shorter due to passage of these viruses in laboratory culture systems. Alternatively,

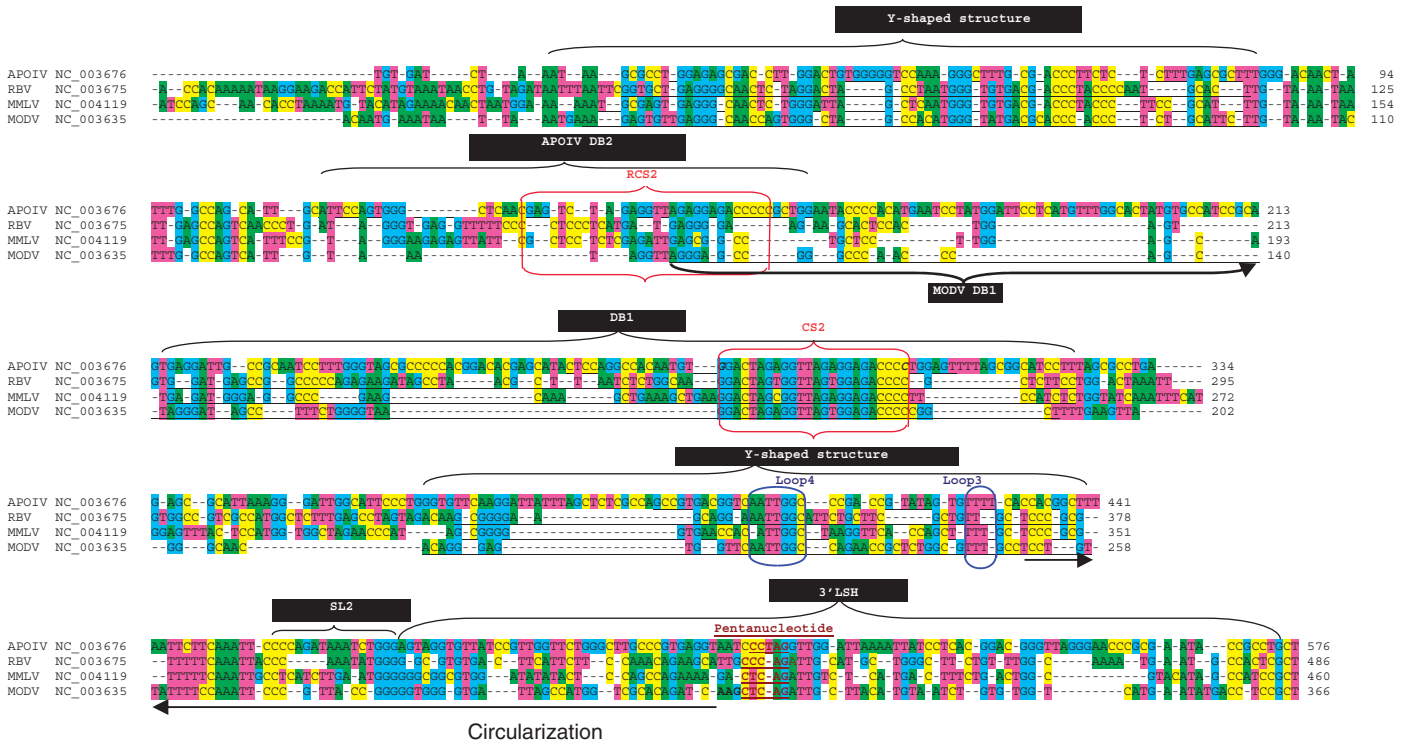

FIG 4. Alignment between 3′UTR of NKV viruses APOIV, RBV, MMLV, and MODV (accession numbers are specified). The boundary of RNA conformations (3′LSH, SL2, Y-shaped structures, loops 3 and 4) and CS2/RCS2 are shown. The conserved pentanucleotide is highlighted in brown letters. The cyclization sequences for APOIV are underlined (Charlier *et al.*, 2002; Leyssen *et al.*, 2002).

their short 3′UTRs might be the result of natural deletions within virus genomes that replicate only in mammalian cells. In either case, the "laboratory acquired" or "natural" deletions would be expected to occur in the variable region of the UTR, between the stop codon and the promoter region. However, our NKV alignments show that deletions are present throughout the whole 3′UTR rather than being concentrated beyond the stop codon. Thus, these deletions are probably not the result of laboratory passage and more likely to reflect the simplified requirement for NKV to multiply in a single host species, that is, rodents or bats.

Despite the wide divergence between the four NKV, regions of conserved nucleotide sequence could be identified following the manual introduction of large gaps. The CS2 element identified for all four NKV was similar to the CS2 of the MBFV; for APOIV, a recognizable RCS2 has been also identified (Charlier *et al.*, 2002). One and two DB-like structures, very similar to those in the MBFV were also predicted in the linear structure of the NKV 3′UTR (Charlier *et al.*, 2002). However, the construction of a robust alignment demonstrated sequence "remnants" RCS2 for the other three NKV (Fig. 4). Moreover, a TBFV-like Y-shaped structure with conserved loops 3 and 4 was also reported indicating that the 3′UTR of NKV preserved structural and linear features of two divergent flavivirus groups, TBFV and MBFV (Charlier *et al.*, 2002).

VI. DRs Might Serve Essential Functions for Viral Transmission

Interpreting experimental data in conjunction with the results of the robust alignment in our previous publications, we speculated that the DRs of MBFV (CSs/RCSs) acting as signals, directly or indirectly stimulate the production of factors that accelerate virus RNA synthesis. This function could be relevant to virus transmission between vertebrate and invertebrate hosts (Gritsun and Gould, 2006b; Gritsun *et al.*, 2006). For many RNA viruses, a number of cellular factors are associated with RNA synthesis. The most common are the proteins that exhibit RNA-trafficking activity or activity associated with modulating the cell life cycle (reviewed in Boguszewska-Chachulska and Haenni, 2005; Lai, 1998). Similar factors were identified for flaviviruses (Blackwell and Brinton, 1995, 1997; De Nova Ocampo *et al.*, 2002; Ta and Vrati, 2000). We suggested that the DRs might be sites of molecular interaction between viral RNA and cells, probably in the complex with viral proteins that direct RNA into appropriate cellular compartments thus ensuring their efficient replication. Alternatively (or additionally), DRs might also contribute to the efficiency of assembly

of the virus replication complex. The speed of this assembly and the transition from initiation of RNA synthesis to elongation determines the rate of virus replication and this could explain the conservation of DRs as elements of a virus replication enhancer. The other possibility is synergistic interaction of the DRs (through cellular factors) with the 5′UTR to initiate virus translation and/or to regulate the transition between translation and replication phases of the infection (Chiu *et al.*, 2005). Whatever their particular role, DRs clearly enhance virus replication, directly or indirectly, mediated by translation or protein interactions.

The reason for preservation of the DRs as double signals is less clear. It is possible that host/viral proteins that provide the virus with functions related to RNA synthesis are active as dimers and therefore the presence of a double signal would accelerate assembly of the replication complex. The effect of each individual element of the replication enhancer could be relatively moderate, but their cumulative action might significantly accelerate virus replication and this could be a vital requirement for efficient virus transmission and dissemination in the more fastidious natural environment. Each group within the MBFV diverged as the result of adaptation to particular vertebrate and invertebrate host species and therefore the enhancer, with its specific set of DRs has evolved according to the particular ecological and molecular requirements of the host.

Thus, the adaptation of the enhancer to maximize viral RNA synthesis and mediate vectored virus transmission was probably the driving force behind the evolution of the flavivirus 3′UTR. In relation to MBFV, this would explain why some DRs (CSs/RCSs) are shared between the MBFV groups whereas others are different. This could also explain the apparent discrepancy between conservation of the DRs and their apparent redundancy in laboratory-maintained viruses, for which transmission has not been tested. In the case of arboviruses, their success is wholly dependent on their ability to replicate rapidly in vertebrate and invertebrate hosts. Mosquitoes feed for very short periods of time, consequently, the frequency of successful viral transmission is increased if the virus is present in the vertebrate host at a high titer which directly depends on the virus replication rate. Indeed, laboratory experiments demonstrated that deletions in the enhancer part of the 3′UTR reduce the replication rate of experimentally mutated viruses to a greater extent in simian than in mosquito cells (Men *et al.*, 1996). This implies that the evolution of enhancer elements is of greater significance for replication in vertebrate cells where the virus must reach a high titer in a short period of time, before the immune system can control the infection. In contrast, when mosquitoes become

infected, they have a period of weeks to amplify the virus before they feed again (Gritsun and Gould, 2006b; Gritsun *et al.*, 2006).

VII. Alignment of All Flavivirus 3′UTRs

Although the analysis of individual alignments, for each flavivirus ecological group enabled us to clarify many issues related to the origin, evolution and functional role of flavivirus DRs, the construction of a comprehensive flavivirus alignment offered the opportunity to test some of the hypotheses regarding the monophyletic origin of flavivirus 3′UTRs. In this section, we have presented a 3′UTR alignment between all flaviviruses using the previously constructed alignments for each of the four individual flavivirus groups, that is, MBFV (YFV, JEV, and DENV), TBFV [TBEV, *Powassan virus* (POWV), *Langat virus* (LGTV), *Louping ill virus* (LIV), *Omsk haemorrhagic fever virus* (OHFV), and *Kyasanur Forest disease virus* (KFDV)], NKV (MODV, RBV, APOIV, and MMLV), and NCFV (KRV, CFAV, and TBV). This can be viewed online as Fig. 1A (Gritsun and Gould, 2006d).

To align all the available flavivirus 3′UTRs, we commenced at the 3′-end working toward the 5′-end, since 3′-terminal regions contained more homology than those in proximity to the stop codon. The alignment was edited manually, aligning the previously derived subgroup alignments rather than individual sequences, and superimposing onto this alignment the known RNA secondary structures. We used the sequences conserved between distantly related groups as anchors, followed by alignment of other, less conserved sequences, in both directions, that is, toward the 3′ and 5′ ends. Inevitably, in some regions of high variability, the alignment was unreliable but we adjusted these regions taking into consideration that they flanked regions of higher conservation. The LRSs that were revealed for the TBFV (Gritsun and Gould, 2006b) were used as anchors to align the MBFV (Gritsun and Gould, 2006b) and NKV (Fig. 4). To simplify the explanation of this alignment, each of the TBFV LRSs (except LRS1 and LRS2) is presented separately in Fig. 5A (available as a supplement on http://www.sciencedirect.com/science/MiamiMultiMediaURL/B6WXR-4M6452B-1/B6WXR-4M6452B-1-9/7165/cca12aff5aed833152d6cd67fbcc1714/applic1.pdf). This form of presentation identifies the possible correlation between the CSs/RCSs of the MBFV and the LRSs of the TBFV. Since we had already demonstrated the duplication of the entire KRV 3′UTR (Gritsun and Gould, 2006a), the KRV/CFAV 3′UTRs were also included in the alignment as two aligned halves (Fig. 3). A schematic presentation of the alignment with superimposed

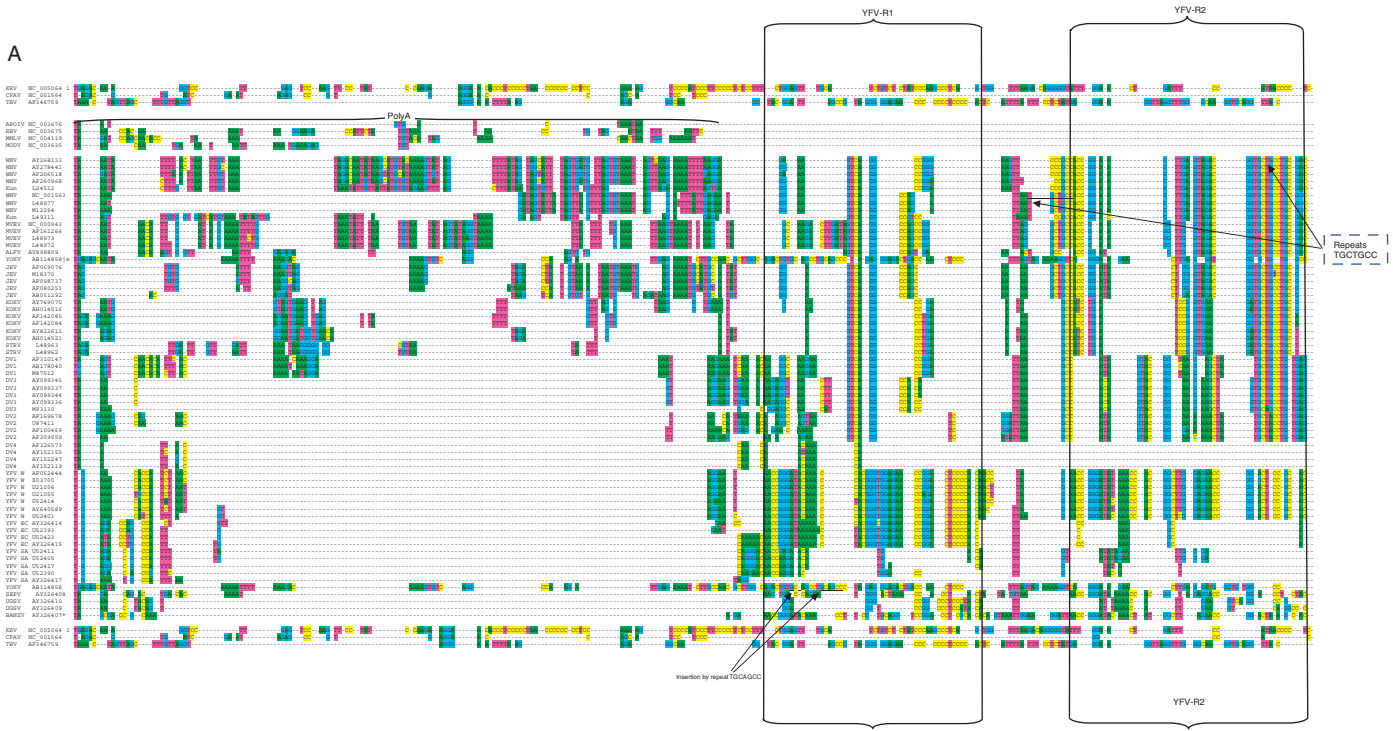

FIG 5. (*continued*)

RCS3
YFV-R3(a)
YFV-R3(b)
USR
ZA AF527415
Ya L40361
Cahil-1 AB049391
Sof-HO AB049401
OMPV-B AY193805
Neu M77799
Hypr U39292
LIV Y07863
LGTV NC_003690
Alkhur NC_004355
POWV NC_003687
KSV NC_005064.2
CPAV NC_001564
TBV AF346759
APOIV NC_003676
SSV NC_003675
MMLV NC_004119
MODV NC_003635
WNV AY268133
WNV AY278441
WNV AF206518
WNV AF260968
Kun L24512
WNV NC_001563
WNV L48977
WNV M12294
Kun L49111
MVEV NC_000943
MVEV AF161266
MVEV L48973
MVEV L48972
ALFV AY898809
JEV AF069076
JEV M18370
JEV AF098737
JEV AF080251
JEV AB051292
KOKV AY765070
KOKV AB014516
KOKV AF142085
KOKV AF420384
KOKV AY822611
KOKV AB014521
STRV L48963
STRV L48962
DV1 AF310147
DV1 AB178040
DV1 M87512
DV3 AY099345
DV3 AY099337
DV3 AY099344
DV3 AY099336
DV1 M91130
DV2 AF149678
DV2 U87411
DV2 AF100469
DV2 AF309959
DV4 AF126573
DV4 AY152155
DV4 AY152247
DV4 AY152119
YFV N AF052444
YFV N X03700
YFV N U21056
YFV N U21055
YFV N U52414
YFV N AY640589
YFV N U52401
YFV EC AY326414
YFV EC U52393
YFV EC U52423
YFV EC AY326415
YFV SA U52411
YFV SA U52405
YFV SA U52417
YFV SA U52390
YFV SA AY326417
YOKV AB114858
SEPV AY326408
USGV AY326410
USGV AY326409
BANZV AY326407
KSV NC_005064.2
CPAV NC_001564
TBV AF346759
ZA AF527415
Ya L40361
Cahil-1 AB049391
Sof-HO AB049401
OMPV-B AY193805
Neu M77799
Hypr U39292
LIV Y07863
LGTV NC_003690
Alkhur NC_004355
POWV NC_003687
10243
10242
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10241
10231
10225

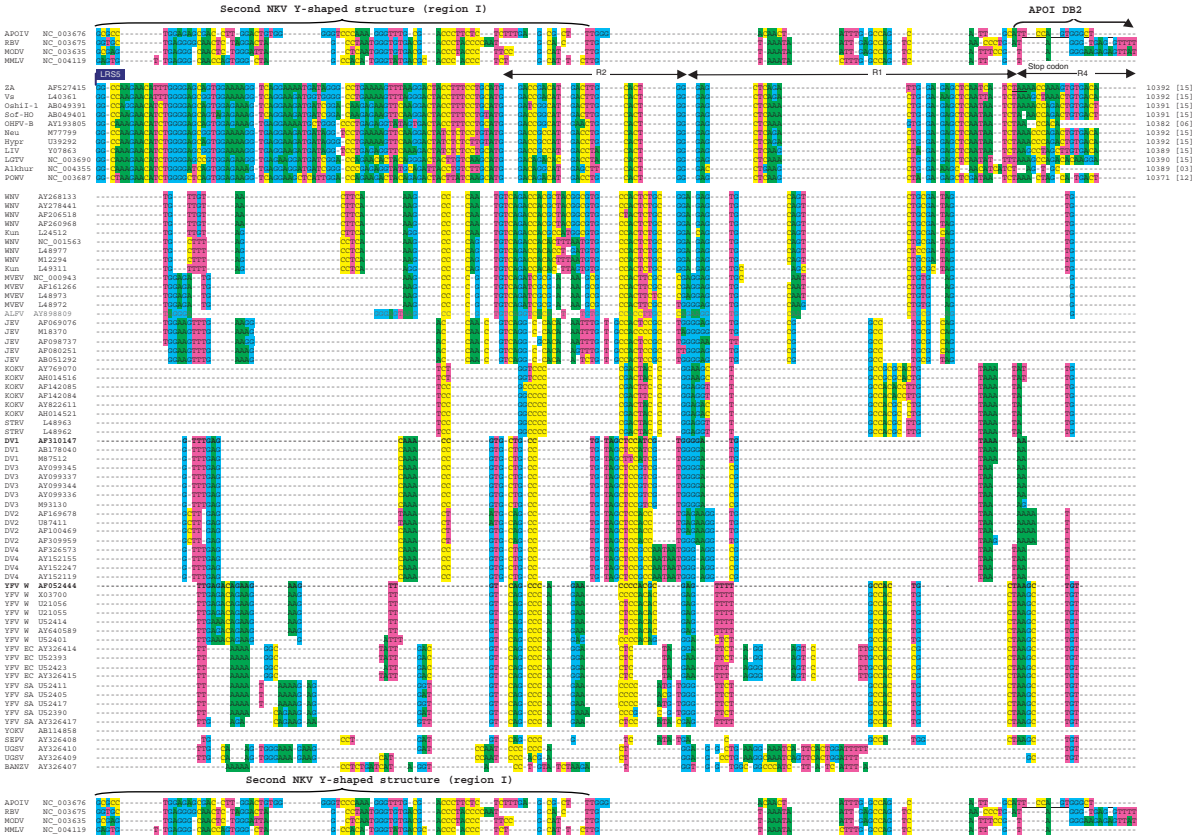

FIG 5. (*continued*)

R1
R2
IR99-1m4 AB049398
Vs L40361
OshiI-1 AB049391
Sof-HO AB049401
OHFV-B AY193805
Neu M77799
Hypr U39292
LIV Y07863
LGTV NC_003690
Alkhur NC_004355
POWV NC_003687

FIG 5. (*continued*)

KRV/CFAV-R1
KRVCFAV-R2
RCS2
APOIV DB2
NKV DB1
LRS4
R3
R2
Second TBFV Y-shaped structure
DB2
DB1

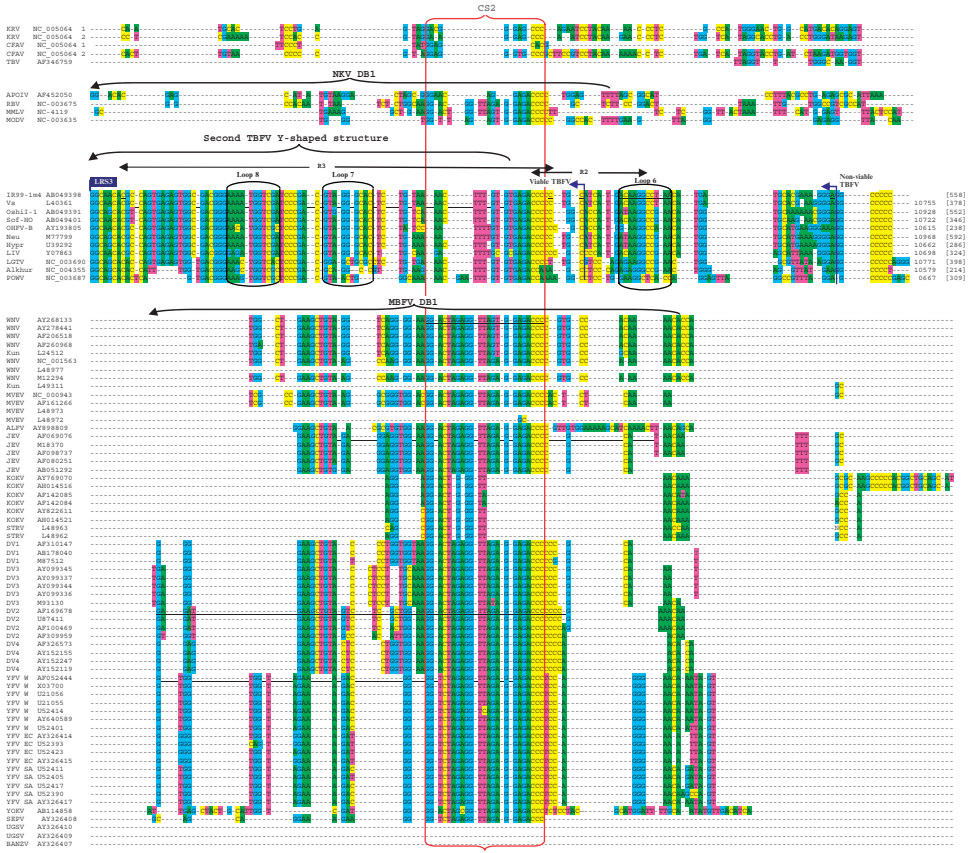

FIG 5. (*continued*)

CS1
First NKV Y-shaped structure
Loop 4
Loop 3
First TBFV Y-shaped structure
Loop 5
Loop 6
Loop 2
SL2
CYCL
Pentanucleotid e
Viable/nonviable DENV

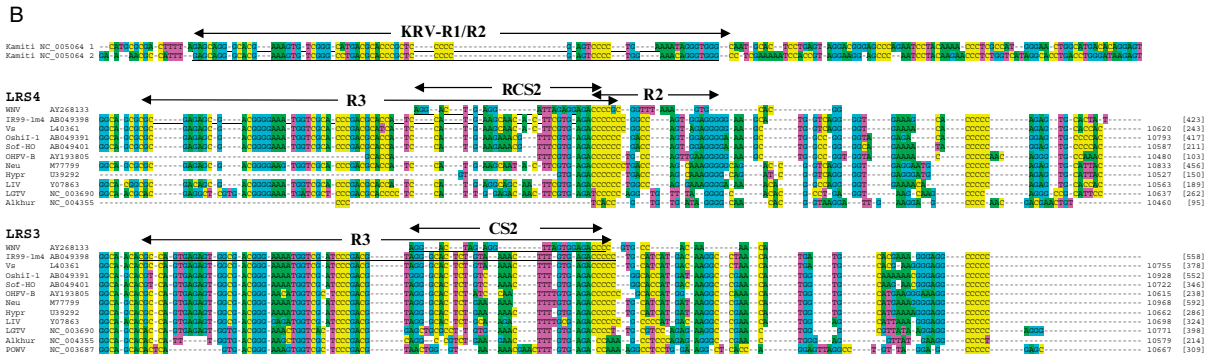

FIG 5. Available as supplementary material (http://www.sciencedirect.com/science/MiamiMultiMediaURL/B6WXR-4M6452B-1/B6WXR-4M6452B-1-9/7165/cca12aff5aed833152d6cd67fbcc1714/applic1.pdf) to view with magnification 130–150%. (A) 3′UTR alignment of all flaviviruses. The alignments constructed separately for MBFV, NKV, and NCFV were then aligned along the LRSs of the TBFV. For clear illustration, the same alignment fragments for TBFV, NKV, and NCFV on each page were placed twice, that is, above and below the alignment for MBFV. TBFV genomes and their UTRs are enumerated. The DRs and boundaries of RNA secondary structures for each flavivirus group are specified as described in the text. The boundary between viable/nonviable engineered mutants for TBEV (Mandl *et al.*, 1998; Pletnev, 2001) and DENV-4 (Men *et al.*, 1996) are indicated. (B) Alignment corresponding to the region LRS3/LRS4. The DRs of MBFV, NCFV, and TBFV are indicated. See text for explanations.

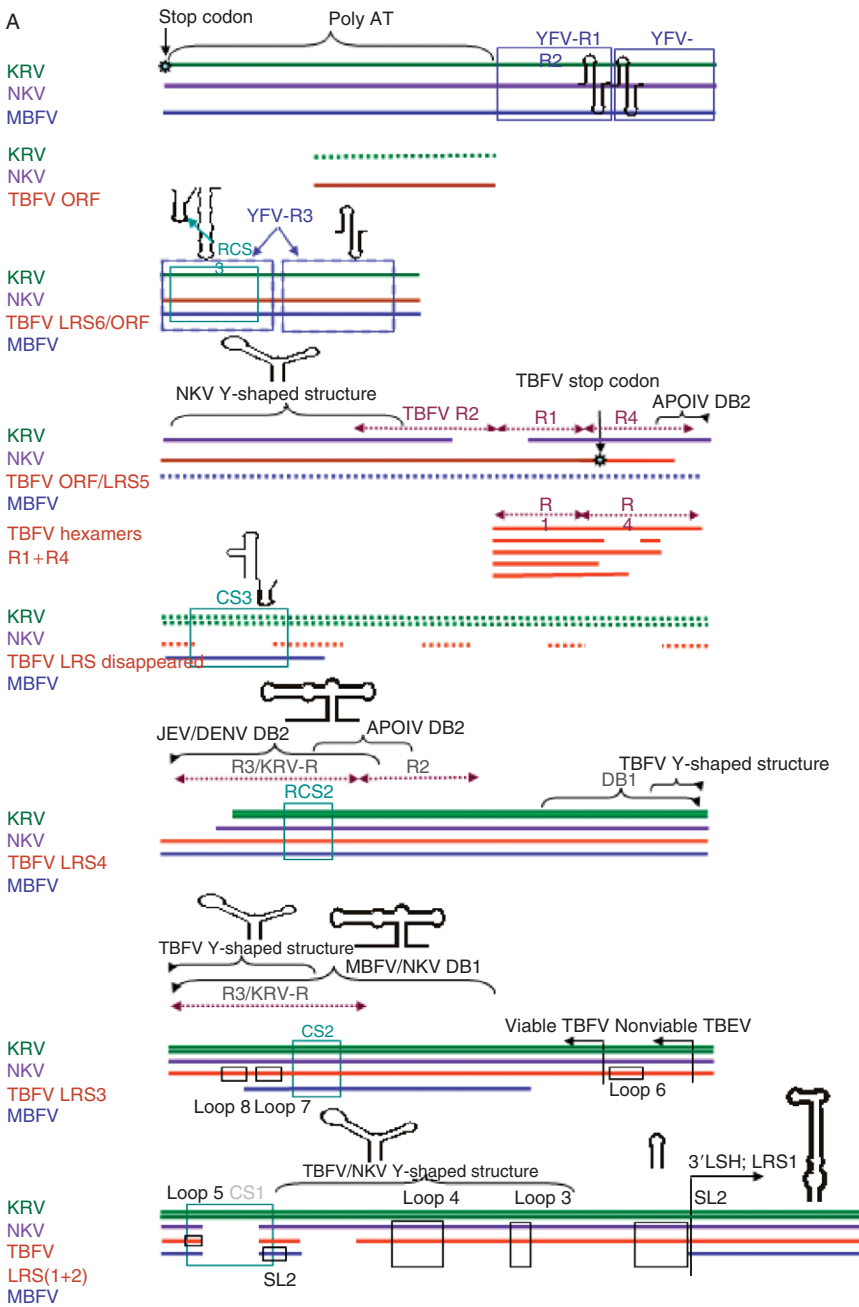

FIG 6. (*continued*)

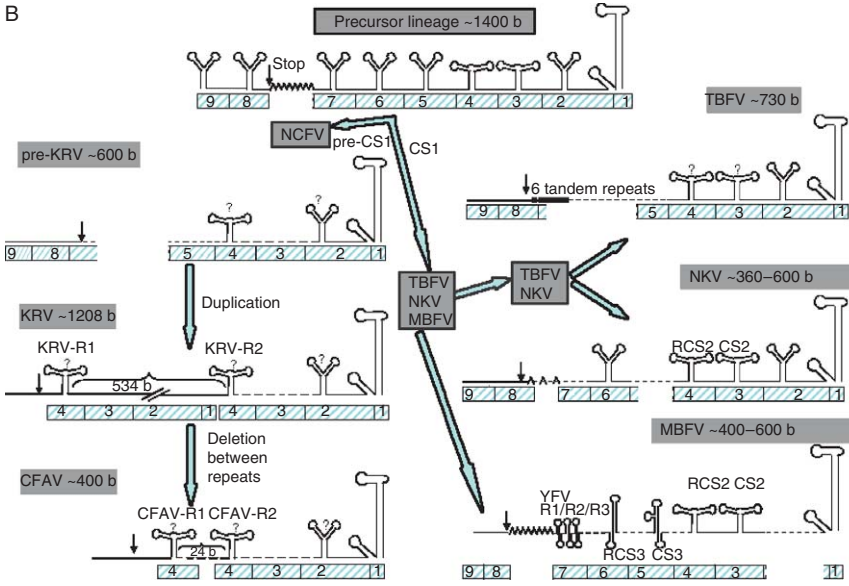

FIG 6. Evolution of flavivirus 3′UTR. (A) Schematic presentation of alignment deduced from Fig. 5A. Each flavivirus group is indicated in different colors. The boundary of DRs and RNA conformations are shown as in Fig. 5A. (B) The tentative development of RNA secondary structures in the 3′UTR from the pan-flavivirus precursor lineage. It probably originally contained nine LRSs, with developed 3′LSH-SL2, Y-shaped structures, two DB-like conformations, and a poly-AT stretch (zigzag line). The hypothetical structure corresponding to the LRS is depicted as Y-shaped. The obsolete regions are depicted by dashed lines. The tentative (not predicted) RNA conformations are specified by "?." See text for more explanations.

secondary RNA structures is presented in Fig. 6A. We also attempted to correlate the development of the particular linear and predicted structural elements of the 3′UTR of each of the four ecological groups of flaviviruses with the phylogenetic analysis; this enabled us to reconstruct the formation of the current 3′UTRs in chronological terms (Fig. 6B).

The results are presented in the 5′→3′ direction of the genome, but they are described in the 3′→5′ direction, since as explained earlier, the alignments were derived starting at the 3′-end.

A. The MBFV Lost a Y-Shaped Structure

The TBFV LRS1 and LRS2 were arranged in tandem from left to right on one page and the MBFV, NKV, and NCFV groups were then aligned against these two regions (Fig. 5A, page 228). For the TBFV

and MBFV, a conserved pentanucleotide CACAG and the corresponding pentanucleotide in the NKV were used as anchors to align the flanking sequences of the other groups in the 3′ and 5′ directions. The conserved sequences of loops 3 and 4 of the Y-shaped structure, shared between the TBFV (Gritsun *et al.*, 1997; Proutski *et al.*, 1997b; Rauscher *et al.*, 1997) and the NKV (Charlier *et al.*, 2002), were used as a second anchor. The boundaries of secondary structures in this region, that is, 3′LSH, SL2, and Y-shaped structure are also specified.

A robust alignment spanning the LRS1/LRS2 was achieved by introducing a number of small gaps (Fig. 5A, page 228). However, to align the MBFV group in this region it was necessary to introduce a large gap that corresponded with the Y-shaped structures described for the TBFV and NKV groups, indicating that the MBFV probably lost this structure during their evolution. In contrast, the NCFV were readily aligned with the TBFV and NKV groups in this region. Moreover, the alignment in the region of the Y-shaped structure and the downstream region shows even more homology between the NKV, TBFV, and NCFV than the region of the 3′LSH, the most conserved conformation between all flaviviruses. We assume that the NCFV also forms a Y-shaped structure. This conservation, between three quite divergent ecological flavivirus groups, indicates its functional importance and supports the experimental data based on engineered deletions in this region, which completely abolish virus infectivity (Mandl *et al.*, 1998; Pletnev, 2001).

The alignment (Fig. 5A, page 228) also reflected the evolution of the "mosquito-borne" element, CS1 (Hahn *et al.*, 1987). The longer CS1 region of the NCFV was aligned with the shorter CS1 region of other flaviviruses by introducing a gap of about 20 nucleotides. The data suggest that the CS1 region of NCFV represents the "primordial" CS1 (pre-CS1). Its development into the "modern" CS1 in MBFV arose through deletion of the middle region of sequence. Each of the three other groups, that is, MBFV, TBFV, and NKV preserved remnants of pre-CS1 that aligned individually with different stretches of the pre-CS1. However, one 20-nucleotide deletion could be attributed to all three groups, implying, in evolutionary terms, simultaneous separation of the TBFV/MBFV/NKV precursor from the NCFV lineage; otherwise, and less likely, independent but identical deletions occurred in the MBFV, TBFV, and NKV groups. The alignment in the CS1 region also shows that TBV is genetically closer to CFAV and KRV than to the NKV/TBFV groups along the entire LRS1/LRS2 region, consistent with conclusions based on phylogenetic analysis (de Lamballerie *et al.*, 2002).

Importantly, the boundaries of the 3′LSH for all flaviviruses virtually coincide with the boundaries between the LRS1 and LRS2 (Fig. 5A, page 228), described initially in the TBFV group (Gritsun *et al.*, 2006). The pan-flavivirus alignment also shows that the boundary that determines the difference between viable and nonviable TBFV (Mandl *et al.*, 1998) and DENV (Men *et al.*, 1996) also maps in proximity to the boundary between LRS2 and LRS3. The nucleotides GGAGA that formed the 5′-proximal part of 3′LSH for the MBFV are similar to the GGAGG of TBFV (Fig. 5A, page 228), which appears quite distant from the 3′LSH of the TBFV/NKV groups corresponding to the deleted Y-shaped structure. However, the GGAGA region also aligned with the GAGGGGGGG adjacent to the 3′LSH of the TBFV group. This demonstrates how the deletion of the Y-shaped structure might have happened. These two polypurine regions might represent the partition/anchoring sites for "switching" of RNA polymerase across the RNA template (Pilipenko *et al.*, 1995). This also illustrates that each LRS encodes an individual RNA conformation, that is, the entire LRS1 encodes a 3′LSH whereas the LRS2 encodes the SL2 and Y-shaped structure.

It is interesting to note that the AATTGGC heptanucleotide in loop 4 that is highly conserved between the TBFV, NKV, and some NCFV, is also present in the C protein of the TBFV group as an inverted repeat sequence, exposed by a conserved SL6 (Gritsun *et al.*, 1997, 2006; Thurner *et al.*, 2004). These could represent kissing loops that might contribute to the cyclization of the genome and/or might carry out signaling functions. Experimental results again demonstrated that SL6 is not essential for virus viability (Kofler *et al.*, 2002) but its conservation implies that it might contribute to the cumulative effect of other conserved signals that provide enhancer functions for virus RNA replication and virus transmission.

B. The CS2 and RCS2 of the MBFV and NKV and the Two Repeats of KRV and CFAV Might Represent Regions Equivalent to the TBFV LRS3 and LRS4

The alignment within the LRS3–LRS4 (Fig. 5A, page 227) region was "patchy" because it was achieved by introducing large gaps between regions that showed robust homology only in the individual virus groups. The CS2/RCS2 elements described for the MBFV (Hahn *et al.*, 1987) and for NKV (Charlier *et al.*, 2002) were used as anchors around which other sequences were aligned upstream and downstream. The highest homology was observed in the CS2 and RCS2

regions, even for the non-MBFV groups, that is, the TBFV and NKV, inferring that this region was probably the last to change.

Remarkably, all the short DRs, that is, CS2 and RCS2 of the MBFV and NKV (Charlier *et al.*, 2002; Hahn *et al.*, 1987), the R3 (DR1/DR2) and R2 repeats of the TBFV group (Gritsun *et al.*, 1997; Wallner *et al.*, 1995), and the two KRV/CFAV repeats (Crabtree *et al.*, 2003) show homology with the TBFV LRS3 and LRS4 sequences (Fig. 5A, pages 226–227). The KRV-R1/R2 virtually coincides with the TBFV region R3 + R2, within the LRS4. These data confirmed our previous predictions that all flavivirus DRs originated from a duplicated LRS that is most preserved in the TBFV group. For clarity of illustration this specific region of the alignment is shown separately with only a limited number of representative sequences (Fig. 5B).

As can be seen (Fig. 5B), the two KRV 67-nucleotide repeats R1/R2, which as described above appear to have been generated by duplication of virtually the entire 3′UTR (Gritsun and Gould, 2006a), have a slightly higher homology with the LRS4 than with the LRS3. Both of these repeats have more homology to each other then to LRS4. This provides additional evidence that the KRV DRs resulted from duplication of the original 3′UTR rather than evolving separately from the LRS3 and LRS4, respectively.

These observations are remarkable since they imply that a similar molecular event, that is, preservation of similar duplicated sequence, occurred three times in the same flavivirus. The first preservation presumably occurred in an early flavivirus lineage, with the formation of multiple LRSs, eventually resulting in the more rigid conservation of LRS3/LRS4 than other LRSs. Subsequently, the LRS3 was almost completely deleted in the NCFV group (Fig. 5A, page 227), but the LRS4 was reduced by deletion to produce the KRV-R1 (Fig. 5A, page 226). However, this NCFV descendant sequence, with one KRV-R1, was subsequently duplicated and evolved further, leaving the KRV-R1/R2 intact. Thus, a duplicated region was once again selected. In CFAV, the large deletion of 510 nucleotides shortened the 3′UTR but R1/R2 was again preserved. Finally, a YFV strain was isolated with a large internal duplication of 216 nucleotides that again includes the region equivalent to LRS3/LRS4 (Bryant *et al.*, 2005). These facts imply that this region provided the viruses with a selective advantage to survive in the environment in contradiction to the experimental evidence which indicated that deletion of the DRs still produced viable virus. Thus, the alignment confirmed that the duplication of sequences in the 3′UTR is a random process, but preservation of duplicated sequences is an essential requirement for viruses to be selected for successful dissemination.

The most likely significance of this is to preserve an RNA structure, with exposed double signaling function, that provides a selective advantage. The DB1/DB2 structures, described for both NKV and MBFV, are each encoded by regions homologous to the LRS3 and LRS4. Indeed, for all three groups of MBFV (DENV, JEV, and the YFV), almost the entire DB1 is encoded by the LRS3 (Fig. 5A, pages 226–227). Similarly, the DB2 (shared only between DENV/JEV groups, i.e., excluding YFV) is encoded mainly by the LRS4 region (Fig. 5A, pages 225–226). Moreover, the sequence of DB2 in the JEV group is quite similar to that of the TBFV, resembling a "sequence bridge" between the MBFV and TBFV groups, indicating a common origin for this region. No equivalent RNA-folding prediction has been made for the LRS3/LRS4 region of MBFV and TBFV, although different folding algorithms applied to LRS3 in the TBFV group predicted the second Y-shaped structure, with loops 7 and 8, that overlap on the alignment with the position of DB1 (Fig. 5A, page 227).

Thus the concept, originally developed for the TBFV group (Gritsun and Gould, 2006c) could be extended to the whole *Flavivirus* genus; each LRS corresponds to each of the functionally essential RNA secondary structures and the extant RNA secondary structures probably originated from a single conformation. These "ancient" RNA structures could be Y-shaped (for TBFV) or DB-like (for MBFV/NKV), although the differences between these conformations are not highly significant, and mutual transition between them would require only minimal numbers of nucleotide substitutions. The presentation of the Y-shaped structure represented as DB1 for the TBFV (Olsthoorn and Bol, 2001) indicates that such a possibility is feasible. The second coincidence is that the DB1 of the MBFV/NKV groups and the second Y-shaped structure of TBFV overlap in their location, and therefore could also be derived from a common structure.

Thus, LRS3 and LRS4 in the TBFV group may represent an early doubled Y-shaped/DB-like conformation from which the other extant flavivirus RNA conformations were derived. An improved refolding of this region for the TBFV and NCFV groups might provide evidence to support this conjecture.

C. The Previously Nonaligned TBFV Region Aligns with the NCFV Alignment

The TBFV alignment upstream of the LRS4 contains a region of sequence that was previously defined as "nonaligned" (Fig. 3A) due to its poor homology to any LRSs (Gritsun and Gould, 2006c). Surprisingly,

KRV and CFAV could be aligned in this region (Fig. 5A, page 225), although attempts to find similar homology with other virus groups were unsuccessful. Perhaps this "nonaligned" region was formed by extensive mutagenesis of the sequence that was initially located between the LRS4 and LRS5. This presumably "obsolete LRS" might have been virtually deleted early during the evolution of monophyletic flavivirus precursor.

D. The Region Containing Six R1 + R4 Repeats Is Unique to the TBFV Group

The "obsolete LRS" is followed, in the 5′ direction, by six sequential R1 + R4 regions, implying that the entire region was a "hot spot" for intramolecular recombination or "polymerase slippage" (Fig. 5A, page 224). It is likely that the arrangement of secondary RNA structures in this region causes the RNA polymerase to pause and then to dissociate and reassociate on the same template. Indeed, the six R1 + R4 regions within the TBFV group are divergent almost certainly due to deletions and insertions, resulting from RNA-polymerase translocations rather than only from nucleotide misincorporations. One can imagine that this region of the 3′UTR was functionally nonessential for virus survival, and therefore could readily be modified by rearrangements of the RNA secondary structures. The six short repeats appear unique to the TBFV group and therefore possibly arose after this group diverged from the other flaviviruses. Both spontaneous and engineered deletions in this region demonstrated its relative unimportance for virus replication efficiency in mammalian cells. However, it was suggested that this region might be essential for maintaining the life cycle of the TBFV in ticks (Hayasaka *et al.*, 2001; Wallner *et al.*, 1995).

E. The RCS3 but Not the CS3 Aligns with the TBFV LRSs

The region corresponding to MBFV CS3-RCS3 (Fig. 5A, page 222–223) aligns poorly between all flavivirus groups. Initially, homology could not be identified between the CS3 and the other 3′UTR regions of other flaviviruses. However, the progressive development of the alignment from CS3 toward the RCS3, within the ORF of TBFV, identified the presence of a number of regions of partial alignment between the different flavivirus groups (Fig. 5A, page 223). A region of higher homology was found between the RCS3 and LRS6 and also downstream there was higher homology with the JEV-related groups than with other MBFV groups. Additionally, the TBFV group was also homologous with regions

in the NKV group, although there was no direct homology between the NKV and MBFV groups (Fig. 5A, page 223). These homologous regions were used as anchors to identify pan-flavivirus homology in the regions between LRS4 and LRS6.

The alignment of the TBFV ORF (LRS5/LRS6 are parts of the ORF) with the 3′UTR of three other flavivirus groups implies that an early 3′UTR lineage was probably longer than the 3′UTR of extant flaviviruses. One can hypothesize that there were originally nine LRSs in the early flavivirus lineage, with six complete and one "obsolete" being preserved only in the TBFV group (Fig. 6B). Subsequently, two LRSs within the 3′UTR were entirely lost from the TBFV group, whereas others were preserved in the ORF, due to the presence of a conserved protein domain, and in the distal part of the 3′UTR, due to functions related to viral RNA synthesis.

Following the linear order of the alignment, CS3 was placed against the "nonaligned" (obsolete LRS) sequence of the TBFV group. Since RCS3 was homologous to the LRS6, we placed the truncated versions (contain only limited amount of viruses) of RCS3, LRS5, and LRS6 into this region. These sequences are not in their "natural" location (marked by the figure brackets in Fig. 5, page 225), but they illustrate how the evolution might have occurred in this region.

The main conclusion to be drawn from this analysis is that both the CS3 and RCS3 could be a part of the TBFV LRSs but the homologue for CS3 has been deleted in the TBFV group during evolution of the viruses. An additional observation is that CS3/RCS3 and CS2/RCS2 probably represent two different parts of the original LRSs since it is difficult to imagine two identical sequences originating independently of each other on two occasions.

F. The Poly-AT Region of the Flaviviruses Was Probably Present in the Earliest Flavivirus Lineages

Further alignment from the RCS3 toward the stop codon between different flavivirus groups was difficult since this region was divergent even between viruses within the same group. To resolve this problem, we used the stop codons of the MBFV, NKV, and KRV/CFAV groups as anchors and aligned them with the sequences that we had already aligned from the 3′-end. To simplify the presentation, the alignments of the NKV and NCFV groups have been placed both above and below the MBFV alignment in Fig. 5A, page 221.

Previously, we revealed that the region of high but "truncated" homology in the MBFV group contained an AT-enriched region most

noticeably in the JEV-related group (Gritsun and Gould, 2006b). In molecular terms, this region may have developed through multiple stuttering of viral RNA polymerase sequence around the stop codon (TAA), creating a variety of short poly-A and poly-T regions that were subsequently duplicated. WNV and MVEV have the longest AT-enriched region but they show extensive variability even though they are genetically closely related. This region is less apparent in the DENV- and YFV-related groups suggesting that AT-enrichment arose only in the JEV subgroup. However, the remnants of the AT-enriched region as a truncated fragment similar to the JEV were identified (Fig. 5A, page 221) in *Yokose virus* (YOKV), a member of the YFV-related viruses (Tajima *et al.*, 2005) and also in the distantly related NKV group.

It has not proven possible to identify a long AT-region in the 3′UTR or ORF of the NCFV or TBFV groups. Instead, KRV and TBV have variants of a poly-TCCCC tract with residual AT-homology indicating that the NCFV group might initially have had an AT-region that was substituted with a poly-TCCCCC tract, which in CFAV was deleted. The presence of a short AT-region in the TBV group, without a poly-TCCCCC tract supports this conclusion.

Thus, AT-enrichment probably occurred early in the evolution of the flaviviruses. For reasons that are not yet clear, this region was preserved better in the JEV-related virus group, possibly providing an advantage for virus circulation in the natural environment. In structural terms, the AT-enrichment of this region might promote the formation of relaxed, that is, unstable secondary structures functioning as spacers to ensure independent folding of the 3′UTR. The TCC-region of KRV and TBV could also serve the same purpose.

G. Are the YFV Triple Repeat Sequences Present in the NCFV Group?

The AT-enriched sequence in the JEV-related virus group is followed by the first YFV repeat YFV-R1. Previously we demonstrated that the original precursor sequence of the MBFV might contain all the DRs including the YFV repeats (Gritsun *et al.*, 2006). In this work, we attempted to discover whether the YFV repeats were present in early flavivirus lineages.

Some homology with YFV-R1 was found in TBV (Fig. 5A, page 221). Interestingly, direct comparison between KRV and YFV-R1 did not reveal significant similarity, but alignment of KRV and YFV-R1 together with TBV sequence, revealed some homology. If a YFV-R1 was at some time present in KRV, then it was presumably modified by deletions

and substitutions. The short A(TT)A region between YFV-R2 and YFV-R1 was also similar between the YFV and NCFV groups. In the region of the YFV-R2, NCFV- and YFV-related viruses also showed some remnants of homology.

The region following the YFV-R2 toward the 3′-end contains a large insertion that separates YFV-R2 and YFV-R3 within the YFV-related viruses (Gritsun *et al.*, 2006). Surprisingly, this region could be aligned with the NCFV and also with the region of the TBFV located upstream of the LRS6 into the ORF, toward the 5′-end (Fig. 5A, page 222). In this region, the alignment between the flavivirus groups is not as robust as in other regions. The small duplications, CTGT and TAGG (underlined in Fig. 5A, page 222) in different virus groups probably form this insertion.

The assigned position of YFV-R3 is tenuous and for the JEV- and DENV-related groups there are two possibilities; one overlaps with RCS3 (Fig. 5A, page 223) and the other is downstream of this position. It is quite possible that more copies of YFV repeats were present in earlier lineages and this could explain the difficulty of identifying the YFV-R3 position. It is noteworthy that further alignment toward the 5′-end of the 3′UTR between YFV-R2/R1 and the TBFV ORF becomes increasingly difficult. The difficulty of demonstrating remnants of homologous sequence might be explained by the different mode of mutagenesis: the 3′UTR, as we established, tends to preserve deletions and insertions whereas homologous regions in the ORF are mainly subjected to point mutations and therefore previous remnants of homology may have totally disappeared.

H. Evolution of the Flavivirus 3′UTR

The NCFV KRV and CFAV are widely divergent from the recognized viruses in the genus *Flavivirus* and therefore phylogenetic assumptions concerning these viruses may not be reliable. The pan-flavivirus 3′UTR alignment demonstrates significant regions of homology between the NCFV and the recognized flaviviruses implying evolutionary relationships between these ecologically distinct viruses. Therefore, we can create a picture of the likely molecular evolutionary events at the 3′UTR as flaviviruses evolved to become the viruses that we recognize today. Figure 6A presents a schematic version of the pan-flavivirus 3′UTR alignment. By combining this with the known phylogenetic relationships of the flaviviruses, we can trace the chronology of molecular events that changed the linear and structural organization of the early 3′UTR lineages eventually resulting in the formation of extant

3′UTRs for the four different flavivirus groups (Fig. 6B). The spatial localization of the linear conserved elements CS/RCS within the MBFV/NKV, KRV/CFAV-R (Fig. 6A), and their relation to the LRS of TBFV as presented on the alignment (Fig. 5A) is also shown. These figures reveal the similar positions of secondary RNA structures conserved within each of the major flavivirus groups, that is, 3′LSH, Y-shaped structure, and DB1/DB2, in relation to linear sequences. The RNA conformations and location of the YFV repeats, the CS3/RCS3 of JEV, and the second Y-shaped structures for the TBFV and NKV groups are also presented in Fig. 6.

As discussed, early flavivirus lineages probably did not have a complex 3′UTR or a very short 3′UTR similar to the 3′LSH. The function of the 3′UTR probably completely or partially overlapped with the function of the ORF. The duplication of the terminal region of the ORF of 200 nucleotides (LRS) is the first landmark that could be traced in the molecular evolution of the 3′UTR for all flaviviruses, not only for TBFV as assumed previously (Fig. 6B). We do not know exactly how many times this duplication occurred. The homology between the TBFV group and other flavivirus groups beyond the TBFV stop codon indicates that at least nine LRSs could have arisen in the early lineages, with six being identified in the TBFV group, one being virtually deleted during evolution and two being completely lost. In addition, extensive mutagenesis within the LRSs also occurred in the early lineages. The early flavivirus lineage also had an elongated poly-AT region immediately after the stop codon (Fig. 6B).

In respect to RNA secondary structures, the early lineages might have had a fully developed 3′LSH-SL2 region and its preservation between the four flavivirus groups could be associated with the requirement for an essential function such as cyclization of the genome and interaction with viral RNA polymerase to initiate replication.

The other conserved RNA secondary structure predicted for TBFV and NKV is Y-shaped and maps upstream of the 3′LSH-SL2. The sequences that encode this structure also show a high homology between the TBFV, NKV, and NCFV groups and together with the 3′LSH-SL2 make up the flavivirus promoter, experimentally proven to be vital for TBFV group viability (Mandl *et al.*, 1998). It is also possible that the two DB-like structures that are preserved between the MBFV and NKV groups, with the exposed signaling CS2/RCS2 sequences were also present in the early flavivirus lineage, upstream of the Y-shaped structure. However, additional predictions on RNA secondary structures for the TBFV and NCFV groups will be required to validate this.

The divergence of early lineages into the four ecological virus groups is largely associated with the change of vertebrate host and invertebrate vector which in molecular terms means that the promoter and enhancer must be adapted to interact with new cell proteins. Whether all arboviruses originated as mosquito-vectored viruses is debatable. However, this might be the case for flaviviruses since KRV and CFAV (NCFV) isolated only from mosquitoes represent early lineages in the genus *Flavivirus* (Crabtree *et al.*, 2003; de Lamballerie *et al.*, 2002). Like the NCFV, the early lineages might have pre-CS1 (Fig. 6B). The transition that added the ability to infect vertebrate hosts was accompanied by deletion within CS1 that separated the NKV/MBFV/TBFV lineage (Fig. 6B) from the NCFV lineage (Fig. 6B). Whether this molecular event followed this divergence or was one of the reasons for it is not clear.

The deletion of the Y-shaped structure in the LRS2 of the MBFV group was a remarkable evolutionary event that was probably directly related to the diversification of the MBFV clade (Fig. 6B) from the common ancestral lineage of the TBFV/MBFV/NKV (Fig. 6B) and its function has probably been transferred into the upstream DB1. In the MBFV group, the promoter that is essential for virus infectivity in both mammalian and mosquito cells contains a combination of the 3′LSH-SL2-CS1 and DB1. However, the truncated promoter 3′LSH-SL2-CS1 still supports virus replication in mosquito cells, probably reflecting the adaptation of early lineages to mosquitoes.

It is not clear if the precursor of the TBFV/MBFV/NKV totally lost its connection with mosquitoes and became a purely "vertebrate" virus, like the NKV. If this was the case, the evolution of the MBFV group required a reverse escape to reacquire mosquito transmission. Alternatively, the TBFV/MBFV/NKV precursor changed directly to mosquito-borne (arbo)virus. In this case, the MBFV group could be considered as the only one which subsequently developed adequate fitness for these two particular hosts and the TBFV/NKV precursor lineage will have had to lose, at least to some extent, the capacity to be transmitted by mosquitoes. In the LRS2–LRS5 regions, the NKV showed more overall sequence homology to the TBFV and NCFV than to the MBFV, indicating the likelihood of a common TBFV/NKV precursor lineage after the separation of the MBFV (Fig. 6B). Further diversification of the NKV and TBFV groups was again associated with the adaptation of some early flaviviruses to the tick vector. As we discussed above, the acquisition of the transstadially quiescent tick host, with its limited seasonal feeding activity and its capacity to transmit TBFV nonviremically (Jones *et al.*, 1997; Labuda *et al.*, 1996) reduced the rate of virus

evolution; this is why we can see more extensive homology between the DRs (LRSs) in the TBFV group than in the other flavivirus groups. The next molecular event in the TBFV group was a large deletion downstream of the stop codon that resulted in the loss of the poly-AT region and two hypothetical LRSs, following the stop codon. The third event was the generation of the region containing six short tandem repeats R1 + R4s, followed by mutagenesis in each of the repeats, for the formation of an enhancer that is probably significant for the efficient replication of the TBFV in ticks.

Two LRSs lost in the TBFV group were possibly lost independently in the NKV and NCFV groups, since they were located in "the nonessential zone" of the 3′UTR and also replication of both NKV and NCFV was limited to one host, with fewer requirements for a complex enhancer. However, further evolution of the enhancer of MBFV occurred exactly in this region where different parts of the LRS might be transformed into conserved repeat elements, that is, CS3/RCS3 of the JEV group and YFV-repeats. Evidence for this can be found in Fig. 5A, page 222: WNV, a member of the JEV group, shows homology with TBFV in this region including its unique repeated element RCS3 that is aligned with the LRS6 region. Other evidence is presented in Fig. 5A, page 221—the insertion between YFV-repeats specific only for the YFV-related group was also homologous (although through the small gaps) to the TBFV region upstream of the LRS6.

After separation of the TBFV group, the NKV continued to circulate in vertebrates and the extant NKV are probably the direct descendants of this branch. The association of the NKV with only one host in comparison with the two-host TBFV ensured a higher tempo of mutagenesis and regression of elongated 3′UTR in some NKV species (Fig. 4).

Meanwhile the precursor lineage of all flaviviruses persisted in mosquitoes (Fig. 6B) and its direct descendant is KRV that evolved through the reduction of a poly-AT stretch and extensive mutagenesis and truncation within the LRS5–6-7. This was followed by duplication of the 3′UTR (Gritsun and Gould, 2006a) that probably resulted in the duplication of the DB-like (or Y-shaped) structure. Subsequently, the homology of the duplicated 3′UTR was reduced to two shorter regions that currently are recognized as 67-nucleotide KRV repeats and still show high homology to the LRS4 (Fig. 5A/5B). Finally, TBV represents a lineage that appears to have been derived from the NCFV but has lost the capacity to infect mosquitoes becoming a purely vertebrate virus. Perhaps significant reduction of the 3′UTR is a common occurrence in viruses confined to a single host, since the shortest 3′UTRs

were revealed in the non-arthropod-borne bat/rodent- or mosquito-associated flaviviruses.

Although the schedule in Fig. 6B is compatible with the phylogenetic analysis performed on the ORF, there is no evidence that the KRV/CFAV lineage is directly descended from an earlier mosquito flavivirus lineage. It is possible that TBV is the direct descendant of a precursor lineage that initially circulated in vertebrates implying that NKV and TBV might both be independent direct descendants of early lineages. Similar scenarios were also discussed previously (de Lamballerie *et al.*, 2002).

VIII. Conclusions

In summary, this chapter has examined the series of events that may define the origin and evolution of short DRs revealed within the 3′UTR of the four different ecological groups of flaviviruses. We suggest that the 3′UTR of flaviviruses was initially formed due to multiple duplications of a 200-nucleotide region of the ORF, six mutated copies of which (LRSs) were preserved in the genome of TBFV, as the least evolved group due to association with the tick vector. More extensive evolution in the 3′UTR of the MBFV, NKV, and NCFV groups resulted in the reduction of LRSs to smaller regions that are now observed as short DRs. We suggested that LRSs might encode RNA conformations in their earliest form and from which a variety of other conformations subsequently evolved.

The terminal region of the 3′UTR, consisting of ~190 nucleotides was defined as a flavivirus promoter, an essential RNA domain that after interaction with the 5′UTR and viral/cellular proteins of the polymerase complex, initiates virus RNA replication; it forms secondary RNA structures conserved between all flaviviruses. The enhancer region of the 3′UTR located between the stop codon and promoter contains a pattern of DRs and secondary RNA structures specific for each flavivirus group. We suggest that the enhancer and particularly the DRs are the means by which flaviviruses change their vector and host preferences. DRs probably represent specific RNA-recognition signals interacting with cellular proteins, possibly in complex with viral proteins that might traffic flavivirus RNA to the appropriate cellular compartments and/or accelerate the assembly of the virus replicase complex. It is still not clear why DRs, acting as double signals, are required for rapid virus replication; they probably interact with dimeric protein molecules, but more research into this area is

required. Although the enhancer function is not essential for virus viability under experimental conditions, it might play a significant role in nature where high rates of virus replication could be critical for virus transmission between vertebrates and invertebrates.

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TROPICAL WHITEFLY IPM PROJECT

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Abstract

The Tropical Whitefly IPM Project (TWFP) is an initiative of the Systemwide IPM Programme of the Consultative Group on International Agricultural Research (CGIAR), financed by the Department for International Development (DFID) of the United Kingdom, the Danish International Development Agency (DANIDA), the United States Department of Agriculture (USDA) and Agency for International Development (USAID), the Australian Centre for International Agricultural Research (ACIAR), and the New Zealand Agency for International Development (NZAID), to manage whitefly pests and whitefly-transmitted viruses in the Tropics. Participating CGIAR and other international centers include the Centre for International Tropical Agriculture (CIAT); the International Institute of Tropical Agriculture (IITA); The International Potato Centre (CIP); the Asian Vegetable Research and Development Centre (AVRDC); and the International Centre of Insect Physiology and Ecology (ICIPE), in close collaboration with the National Resources Institute (NRI-UK); national agricultural research institutions; agricultural

0065-3527/07 $35.00
DOI: 10.1016/S0065-3527(06)69006-4

universities; and advanced agricultural research laboratories in Africa, Asia, Europe, the Pacific Region, and the Americas. The TWFP was launched in 1996 as five separate but closely linked subprojects targeting: (1) *Bemisia tabaci* as a vector of viruses affecting cassava and sweet potato in sub-Saharan Africa (IITA, NRI, CIP, CIAT); (2) *B. tabaci* as a vector of viruses in mixed cropping systems of Mexico, Central America, and the Caribbean (CIAT); (3) *B. tabaci* as a vector of viruses in mixed cropping systems of eastern and southern Africa (ICIPE, AVRDC); (4) *B. tabaci* as a vector of viruses in mixed cropping systems of Southeast Asia (AVRDC); (5) *Trialeurodes vaporariorum* as a pest in mixed cropping systems of the Andean highlands (CIAT); and (6) whiteflies as pests of cassava in South America (CIAT). Diagnostic surveys conducted in Phase I (1997–2000) clearly showed that the two main whitefly pests in the Tropics are *B. tabaci* and, in the highlands, *T. vaporariorum*. Other whitefly species investigated by the TWFP included *B. afer* and *Aleurotrachelus socialis*. *B. tabaci* is the main vector of plant viruses inducing African cassava mosaic disease, sweet potato virus disease, bean golden mosaic disease, and many other diseases of horticultural crops, mainly tomato, hot and sweet peppers, squash, melon, and several other cucurbits. Genetic resistance was identified as the most important component of an IPM programme, followed by phytosanitary, cultural and legal measures. The two most important factors contributing to whitefly/virus epidemics were shown to be pesticide abuse and the use of virus-infected planting materials. Biological control is only effective in cropping systems with minimal or rational use of insecticides, and should be considered only as a complementary IPM strategy. Farmer education and technical assistance are considered the most critical steps toward the implementation of sustainable and economically viable IPM strategies in tropical countries affected by whitefly pests and whitefly-transmitted viruses.

I. Introduction

There are over 1140 different species of whiteflies in the world (Mound and Halsey, 1978), of which, 724 species have been described from the Tropics (Strong *et al.*, 1984). However, not all of these whitefly species are agricultural pests of socioeconomic importance, and only a handful are recognized vectors of plant viruses, namely: *B. tabaci* (Gennadius), *B. afer sens lat.*, *T. vaporariorum* (Westwood), *T. abutilonea* (Hald.), and *T. ricini* (Misra). Yet, *B. tabaci* alone can transmit more than 200 different plant viruses responsible for the loss of millions of hectares

of valuable food and industrial crops in tropical, subtropical, and temperate regions of Europe, North America, Asia, Africa, Oceania, and Latin America (Bird and Maramorosch, 1978; Brown, 1994; Jones, 2003; Legg and Fauquet, 2004; Morales and Anderson, 2001; Morales and Jones, 2004; Muniyappa, 1980; Polston and Anderson, 1997).

Over 90% of the plant viruses transmitted by *B. tabaci* belong to the genus *Begomovirus*, family *Geminiviridae*. These are unique single-stranded DNA viruses encapsidated in two quasi-isometric twin particles (Fauquet *et al.*, 2005). *B. tabaci* can also transmit filamentous, single-stranded RNA plant viruses belonging to three different genera: *Crinivirus, Carlavirus*, and *Ipomovirus* (Fauquet *et al.*, 2005; Jones, 2003). The criniviruses transmitted by *B. tabaci* are: *Cucurbit yellow stunting disorder virus, Lettuce chlorosis virus, Lettuce infectious yellows virus, Sweet potato chlorotic stunt virus* (SPCSV), and *Tomato chlorosis virus* (ToCV). The only known carlavirus transmitted by *B. tabaci* is *Cowpea mild mottle virus*, although a carla-like virus transmitted by *B. tabaci* has been detected in Brazil affecting melon (Nagata *et al.*, 2005). The ipomoviruses associated with *B. tabaci* are: *Cassava brown streak virus, Cucumber vein yellowing virus, Squash yellow leaf curl virus, Sweet potato mild mottle virus* (SPMMV), and *Sweet potato yellow dwarf virus* (Jones, 2003).

B. tabaci also causes direct damage to crops such as soybean, common bean, mungbean, tomato, pepper, chilies, okra, eggplant, sweet potato, cassava, cotton, tobacco, melon, watermelon, and squash by extracting large amounts of sap or inducing noticeable physiological disorders (i.e., irregular ripening, silver leaf, and severe chlorosis) in tomato, cucurbits, and various plant species, respectively (Costa and Brown, 1991; Legg *et al.*, 2004; Ramirez *et al.*, 1998). Moreover, the large populations of *B. tabaci* observed on suitable hosts induce the growth of superficial fungi (sooty molds) on the honeydew excreted by immature and adult whiteflies. These fungi do not grow at the expense of the plant, but greatly reduce its photosynthetic capacity and, ultimately, may cause plant death and total crop failure.

Whereas *B. tabaci* affects food and industrial crops in tropical and subtropical lowland and mid-altitude regions (0–1000 m) of the world, the whitefly *T. vaporariorum* attacks several important crops, such as potato, tomato, peppers, snap beans, common dry beans, strawberry, and cucurbits, in temperate and tropical highland (1000–3000 m) regions (Cardona *et al.*, 2005). *T. vaporariorum* is probably a native of the Americas (Vet *et al.*, 1980), but it is currently found in all continents. Both whitefly species can survive and cause damage in temperate regions inside greenhouses during winter (Fargues *et al.*, 2005;

Montalti, 2005). *T. vaporariorum* was first observed to transmit a plant virus in California, in the early 1960s (Duffus, 1965). The virus, *Beet pseudoyellows virus*, was later found in different European countries and Asia (Wisler *et al*., 1998). In the mid-1990s, *T. vaporariorum* was again associated with two similar viral diseases of tomato in the United States, tomato infectious chlorosis and tomato chlorosis (Wisler *et al*., 1998). The causal viruses (TICV and ToCV, respectively) were later on detected in Europe (Dalmon *et al*., 2005; Dovas *et al*., 2002; Font *et al*., 2003; Vaira *et al*., 2002). Interestingly, ToCV is also transmitted by *B. tabaci* and *T. abutilonea* (Wisler *et al*., 1998), which constitutes a relatively rare case of vector nonspecificity within the genera of whitefly-transmitted viruses (Jones, 2003).Two criniviruses associated with the strawberry pallidosis disease have been transmitted by *T. vaporariorum* (Tzanetakis *et al.*, 2004). These apparently new virus problems were thought to be restricted to temperate regions of the world, but following the classification of the causal agents as species of the genus *Crinivirus* (Martelli *et al*., 2002), it became apparent that criniviruses had existed in South America since the 1930s, when "potato yellow vein" was first observed in Colombia and Ecuador (Diaz, 1966; Diaz *et al*., 1990; Silberschmidt, 1954). Potato yellow vein is now known to be induced by a crinivirus (Salazar *et al*., 2000), and it has been observed to affect tomato in Colombia (Morales *et al*., 2005). There are other criniviruses in developing countries of Asia, Africa, and South America, such as SPCSV, but they are transmitted by the whitefly *B. tabaci* (Jones, 2003). Sweet potato is a very important staple in developing countries, particularly in China and eastern Africa, which produce over 80% of the sweet potato cultivated in the world (FAO, 2004).

B. afer is considered a pest of several crops in Italy, Greece, Turkey, the Middle East, and southern Asia (Martin, 1987). *B. afer* is also a common pest of cotton, peanut, cowpea, and cassava in Africa, where it has been described as *B. hancocki* (Corbett, 1936). The role of *B. afer* as a vector of cassava viruses, namely African cassava mosaic begomoviruses and brown streak potyvirus, has been investigated in Africa without conclusive results (Jones, 2003), although there is preliminary evidence suggesting that *B. afer* is a vector of *Cassava brown streak virus* in Africa (Maruthi *et al.*, 2004). In Central America, whitefly specimens belonging to the *B. afer* group had been observed in the early 1990s. However, the first unambiguous identification of *B. afer* in Latin America was made from Peru, South America (Anderson *et al*., 2001), using specimens collected on sweet potato and pepino (*Solanum muricatum* Ait.). Studies on the possible role of *B. afer* as a vector of sweet potato viruses in Peru have shown that this whitefly species can

transmit the crinivirus that causes sweet potato chlorotic stunt, albeit at a lower efficiency than the main vector (*B. tabaci*) of this virus (Gamarra *et al*., 2005).

T. abutilonea had been associated with the transmission of sweet potato viruses in the United States in the late 1950s (Girardeau, 1958; Hildebrand, 1960), although unequivocal evidence was only provided in 1999, when this whitefly species was shown to be the vector of the sweet potato chlorotic stunt crinivirus (Sim *et al*., 2000). This virus was first associated with *B. tabaci*, and found to be part of a complex (with the aphid-borne sweet potato feathery mottle potyvirus) that caused sweet potato virus disease (SPVD) in Nigeria, Africa, in the late 1980s (Cohen *et al*., 1992; Schaefers and Terry, 1976). The transmission of Abutilon yellows crinivirus by *T. abutilonea* was reported by Duffus (1987) in the United States. A third virus transmitted by *T. abutilonea*, Diodia vein chlorosis virus, was reported to infect *Diodia virginiana* in Arkansas, United States (Larsen *et al*., 1991).

T. ricini, the castor bean whitefly, has been implicated in the transmission of *Tomato yellow leaf curl virus* (TYLCV) in Egypt (Idriss *et al*., 1997). This would be a unique case of a different whitefly vector for a species of the *Begomovirus* genus generally transmitted by *B. tabaci*.

The above-mentioned whitefly species combine the ability to cause direct and indirect feeding damage with their capacity to transmit plant viruses that often cause total crop failure. These whitefly species were not always considered as pests, and some of the viruses they transmit were initially described as occasional pathogens of little economic importance. In Latin America, it has been observed that whiteflies such as *B. tabaci* and *T. vaporariorum* began to gain pest status in the late 1960s, due to the introduction and intensive use of insecticides on crops such as cotton in the lowlands and probably potato in the highlands (Morales and Anderson, 2001).

In Africa, the main crop production problem associated with the whitefly *B. tabaci* has been African cassava mosaic disease (ACMD), recorded in this continent since 1894 (Warburg, 1894; Zimmerman, 1906). In this case, it would be difficult to associate insecticides to the rather rapid dissemination of ACMD throughout Africa by the 1930s (Storey and Nichols, 1938), because DDT was not used extensively in Africa until the 1950s for the eradication of malaria and general pest control. The early dissemination of ACMD in Africa was probably associated to the vegetative propagation of cassava via stem cuttings taken from systemically infected cassava plants, particularly from cassava plants showing mild or unapparent ACMD symptoms. Studies showing that the level of initial inoculum in surrounding cassava fields

was a more important determinant of ACMD incidence, than whitefly abundance (Legg *et al*., 1997), seem to support this assumption.

In India, one of the first countries affected by *B. tabaci* (Husain, 1931), whitefly-borne viruses have attacked several crops of economic importance since the 1940s. The most important viral diseases associated so far with *B. tabaci* in India are: okra yellow vein mosaic (1940), lima bean yellow mosaic (1948), tomato leaf curl (1948), dolichos yellow mosaic (1950), pumpkin yellow vein mosaic (1955), mungbean yellow mosaic (1960), and horsegram yellow mosaic, described in 1968 (Muniyappa, 1980).

The real extent of the economic impact of whiteflies as pests and vectors of plant viruses became more apparent following the emergence and dissemination of a new biotype of *B. tabaci* in 1986. The first sign of this new biotype was the unusually high whitefly populations observed on imported poinsettias (*Euphorbia pulcherrima*) in Florida, and the occurrence of silver-leaf symptoms in squash and irregular ripening in tomato (Maynard and Cantliffe, 1989). By 1992, the "poinsettia" whitefly had moved west into the states of Texas, Arizona, and the Imperial Valley of California, causing losses estimated at US$500 million (Oliveira *et al.*, 2001). The new B biotype attacks more plant species than the original *B. tabaci* biotype A, including alfalfa, bell pepper, broccoli, cabbage, cauliflower, and tomato (Johnson *et al*., 1982). The neighboring valley of Mexicali, in northwestern Mexico, suffered crop losses in excess of US$30 million, when its melon, watermelon, sesame, and cotton crops were badly damaged by the B biotype of *B. tabaci*. South of this valley, in the state of Sonora, cotton and soybean were severely affected by the aggressive new biotype, eventually causing a reduction in their planting areas of over 50% (Silva, 1997). Some of the viruses transmitted by *B. tabaci* in southwestern United States, such as *Squash leaf curl virus* (SLCV) and Texas pepper virus (currently renamed *Pepper golden mosaic virus*), rapidly spread down through Mexico and Central America (Morales and Anderson, 2001). The arrival of biotype B of *B. tabaci* also coincided with the increasing diversification and expansion of horticultural crops in this region, particularly tomato, sweet pepper, chili, squash, and melon, thus facilitating the reproduction and survival of the polyphagous new biotype (Morales and Anderson, 2001; Polston and Anderson, 1997). TYLCV, the first *B. tabaci*-transmitted virus to trespass the geographic barrier between the Old and New World, was first identified in Israel in the early 1960s (Cohen and Harpaz, 1964). This virus has caused crop losses in Asia, Africa, Europe, and the Americas (Polston and Anderson, 1997), worth billions of dollars.

The B biotype rapidly invaded the Caribbean region and eventually South America, devastating traditional and nontraditional crops in the main agricultural areas where *B. tabaci* can thrive. For instance, in Brazil, yield losses in crops such as common bean, tomato, cotton melon, watermelon, okra, and cabbage were estimated at US$5 billion in the period 1995–2000, following the arrival of the new biotype of *B. tabaci* (Oliveira *et al*., 2001). The B biotype of *B. tabaci* is now a global pest responsible for direct and indirect damage caused to a large number of crops of major socioeconomic importance in Asia, Africa, Europe, the Pacific region, and the Americas.

T. vaporariorum is undoubtedly the second most important whitefly pest, particularly in the Andean region of South America, and temperate and subtropical regions of the world. Its importance as a virus vector has also increased in recent years (Morales *et al*., 2005). The economic impact of other whitefly species, as direct pests of crops such as cassava and citrus in the Tropics, is also considerable. Even crops, such as rice and sorghum, which had escaped the attack of whiteflies, have recently been attacked in Central America and South America by the neotropical whitefly species *Aleurocybotus occiduus*, resulting in total crop losses (Tropical Whitefly IPM Project, unpublished results).

These crop production problems took place at a time when many countries, and particularly developing nations, were suffering an economic crisis caused by the ongoing wars in the Middle East and resulting increase in oil prices. The impact of these crises has greatly affected national and international agricultural research and extension, and, ultimately, resource-poor farmers around the world. Amid the mounting yield losses caused by new whitefly pests and over 100 new viruses transmitted by these vectors, and in the absence of technical assistance for farmers in developing countries, agrochemicals became the only alternative available against these pests. As a result, insecticide use, production costs, environmental pollution, and human health problems created by the constant exposure to pesticides and ingestion of contaminated food products have increased significantly in the past three decades throughout the Tropics. One of the main effects of pesticide abuse has been the development of resistance to all of the traditional insecticides used to control whitefly pests. Altogether, whitefly pests and the viruses they transmit have resulted in widespread crop losses and increased poverty in developing countries (Morales and Anderson, 2001). Ultimately, however, the fact that triggered a worldwide campaign to combat whitefly pests and plant viruses transmitted by these insects was the emergence of the B biotype of *B. tabaci* in the Mediterranean region, and the southern

agricultural areas of the United States. This chapter describes the experiences of one of the most ambitious global projects launched by European, North American, Australasian, African, Asian, and Latin American agricultural research and development institutions to manage whiteflies as pests and vectors of plant viruses.

II. The Tropical Whitefly IPM Project

The Consultative Group for International Agricultural Research (CGIAR) coordinates the activities of 15 international agricultural research centers (IARCs) around the world. Each center investigates specific commodities, although some IARCs share research responsibilities on common crops such as cassava. To further promote collaboration among IARCs, a Systemwide IPM Programme was created in 1995, to pursue intercenter research initiatives (SP-IPM, 2001). In 1996, a Task Force meeting was held at the International Centre for Tropical Agriculture (CIAT) in Palmira, Colombia, to discuss the pan-tropical threat of whiteflies as pests and vectors of plant viruses affecting CG-mandated crops and other important commodities around the world. A total of 24 participants representing IARCs, national agricultural research institutions (NARIs), and advanced research institutions, discussed the nature of the problem, the goal of the proposal, possible collaborative activities, and potential sources of financing. The participating IARCs possessing expertise on the management of whitefly pests and whitefly-borne viruses were: the International Institute of Tropical Agriculture (IITA) in Nigeria, the International Centre of Insect Physiology and Ecology (ICIPE) in Kenya, the Asian Vegetable Research and Development Centre (AVRDC) in Taiwan, the International Potato Centre (CIP) in Peru, and CIAT as the coordinating IARC. It was decided that the goal of the TWFP should be "to improve the living conditions of resource-poor farmers and urban communities in developing countries affected by whitefly pests and whitefly-transmitted viruses."

The Task Force defined three major crop production problems: (1) whiteflies as pests and vectors of cassava viruses, (2) whiteflies as vectors of plant viruses in mixed cropping systems in the lowlands and mid-altitude regions of the world, and (3) whiteflies as pests in mixed cropping systems in tropical highlands. These three categories were further divided into six subprojects, based on geographic and comparative technical advantages:

1. *B. tabaci* as a vector of viruses affecting cassava and sweet potato in sub-Saharan Africa, led by Dr. James Legg of IITA with support from NRI (Drs. Richard Gibson, John Colvin, and Barbara Adolph), CIP (Drs. Luis Salazar, Segundo Fuentes, and Regina Kapinga), and CIAT (Dr. Anthony Bellotti).
2. *B. tabaci* as a vector of viruses in mixed cropping systems of eastern and southern Africa, led by ICIPE (Drs. Bernhard Löhr, Mohamed Ali Bob, and Lisbeth Riis), with the collaboration of AVRDC (Drs. Remi-Nono-Womdim and Sylvia Green).
3. *B. tabaci* as a vector of viruses in mixed cropping systems of Mexico, Central America, and the Caribbean, led by CIAT (Dr. Francisco J. Morales).
4. *T. vaporariorum* as a pest in mixed cropping systems of the Andean highlands, led by CIAT (Dr. Cesar Cardona).
5. *B. tabaci* as a vector of viruses in mixed cropping systems of southern Asia, led by AVRDC (Drs. Peter Hanson and Sylvia Green) with the collaboration of NRI (Dr. John Colvin).
6. Whiteflies as pests of cassava in South America, led by CIAT (Dr. Anthony Bellotti), with the collaboration of NRI (Dr. John Colvin).

The TWFP was designed to conduct research activities in three phases of 3-year periods each, and six outputs to be developed over the length of the project, two outputs per phase, as follows:

Phase I

Output 1: Formation of an international network for management of whitefly pests and whitefly-transmitted viruses in the Tropics.
Output 2: Characterization of whitefly pests, whitefly-borne viruses, and cropping systems affected by these pests.

Phase II

Output 3: Improvement of the knowledge on whitefly population dynamics and epidemics of whitefly-transmitted viruses in the Tropics.
Output 4: Selection and validation of IPM strategies used around the world for whitefly and whitefly-borne virus control.

Phase III

Output 5: Strengthening the research capacity of NARIs and other institutions responsible for the control of whitefly-related problems in the Tropics.
Output 6: Assessment of project impact.

The TWFP was officially launched in 1997 with the financial support of the Danish Agency for International Development (DANIDA), the United States Department of Agriculture (USDA), the United States Agency for International Development (USAID), the Australian Centre for International Agricultural Research (ACIAR), and the Ministry of Foreign Affairs and Trade of New Zealand (MFAT). In 2001, the Department for International Development (DFID) of the United Kingdom joined the TWFP as the main donor until the expected completion of Phase III in April 2008.

A. B. tabaci *as a Vector of Viruses Affecting Cassava and Sweet Potato in Sub-Saharan Africa*

Sub-Saharan Africa produces almost half of the cassava (*Manihot esculenta*) grown worldwide, and East Africa is one of the main producers of sweet potato (*Ipomoea batatas*) in the world. Both crops are grown by subsistence farmers in marginal soils, and represent the food security of millions of people in this continent (Jennings, 1970; Legg, 2005). Cassava came to Africa in colonial times from its center of origin, South America. Unfortunately, this crop encountered a severe disease in Africa, caused by a whitefly-transmitted virus, *African cassava mosaic virus* (ACMV) (Storey and Nichols, 1938). The broad distribution of cassava in Africa by colonial authorities in the 1920s and 1930s greatly contributed to the dissemination of the causal viruses in this vegetatively propagated crop. In the late 1990s, it was estimated that the different ACMD viruses transmitted by *B. tabaci* caused annual yield losses in excess of 20 million tons, worth over US$2 billion (Thresh *et al.*, 1997). Nevertheless, ACMD had become an endemic but manageable disease of cassava over a century after it was first observed in Africa (Legg and Fauquet, 2004; Warburg, 1894).

The occurrence of a severe attack of ACMD in Uganda, in the late 1980s, was rather unexpected and set the stage for a major catastrophe in that country. By 1990, the ACMD epidemic had already reached important cassava production regions in the northern Luwero district and other cassava-growing districts in northern and eastern Uganda (Otim-Nape *et al.*, 1997). The epidemic and unexpected drought conditions caused severe food shortages and famine in Uganda and neighboring regions from the mid-1990s, coinciding with the initiation of the sub-Saharan Africa subproject of the TWFP. In 1997, it was demonstrated that a new, more virulent form of ACMD was the cause of the increasing levels of plant damage observed in Uganda and that the new variant, currently classified as *East African cassava mosaic virus*

(EACMV)-UG, was the result of interspecific recombination between EACMV and ACMV (Zhou *et al*., 1997).

The ACMD epidemic subsequently moved onto neighboring Kenya, Tanzania, Sudan, and the Democratic Republic of Congo (Legg, 1999), and Rwanda (Legg *et al*., 2001). The severity of this epidemic led to the virtual abandonment of cassava cultivation in many areas and, thus, had a major impact on food security in the region, with financial losses estimated at US$60 million per year (Otim-Nape *et al*., 1997). Although the situation stabilized in Uganda after the late 1990s, the epidemic continued to spread through northwestern Tanzania. In 1999–2000, the USAID Office of Foreign Disaster Assistance (OFDA) financed an "Emergency Programme to Combat the Cassava Mosaic Disease Pandemic in Eastern Africa."

In East Africa, the ACMD epidemic was rapidly addressed through the introduction of ACMD-resistant varieties produced by IITA-Ibadan, Nigeria. These varieties were evaluated under Ugandan conditions, and those exhibiting resistance were selected for mass multiplication and distribution. By the early 2000s, these varieties were beginning to have an impact at farm level, and it is currently estimated that more than one quarter of all cassava cultivated in Uganda (>100,000 ha) is under ACMD-resistant varieties. Resistant germplasm has also been introduced into the neighboring countries of Kenya and Tanzania. Complementary IPM strategies included introduction of sources of resistance to whitefly pests of cassava (recently identified in South America), and crop management practices, mainly phytosanitation.

The molecular variability of viruses inciting ACMD in "postepidemic" Uganda was investigated (Sseruwagi *et al*., 2005b), revealing the occurrence of two previously reported viruses: ACMV and EACMV-UG2. EACMV-UG2 was the predominant (73%) virus and occurred in all the surveyed regions, whereas ACMV was present mostly in cassava plants showing mild symptoms. Mixed infections of ACMV and EACMV-UG2 were detected in only 18% of the field samples, and did not necessarily result in enhanced symptom expression. The widespread occurrence and distribution of the EACMV-UG2 strain associated with the epidemic of severe ACMD in Uganda confirmed the "postepidemic" nature of the current situation in this country.

1. Whitefly-Borne Viruses Affecting Cassava in East Africa

In **Kenya**, cassava improvement for resistance to ACMD and cassava brown streak started in the mid-1950s (Doughty, 1958). The improved varieties, however, were only partially accepted by Kenyan farmers, who did not adopt most of the improved materials produced, with the

exception of cultivar "Kaleso" in the Coast Province (Bock, 1994). In the late 1990s, the TWFP detected the severe form of the ACMD in western Kenya (Legg, 1999), and, thus, efforts to introduce ACMD-resistant germplasm started at that time. The severe ACMD and the pandemic-associated virus, EACMV-UG, continued to spread further to the south, and the pandemic rapidly reached the Western Province and Nyanza Province. Although overall ACMD incidence for western Kenya remained unchanged from the previous year at about 52%, there was a significant increase in the percentage of fields growing ACMD-resistant varieties derived from the TWF-IPM project, up to 20% (Kamau *et al*., 2005).

ACMD was observed in **Tanzania** as early as 1894 (Warburg, 1894). However, this disease did not become an important production constraint until the 1920s. The genetic improvement of cassava against ACMD was initiated in Tanzania around the 1930s (Jennings, 1994), and it may have been successful in keeping Tanzania relatively free of ACMD in relation to its neighbors. Unfortunately, the epidemic of ACMD that started in Uganda in the late 1990s now threatens northwestern Tanzania and other cassava-producing regions in the eastern part of the country (Ndunguru *et al*., 2005). Work conducted by J. Ndunguru (LZARDI, Tanzania) and C. Fauquet (ILTAB, United States) in conjunction with the TWFP involved the collection of ACM-diseased samples from the major cassava growing areas in Tanzania in order to identify the causal viruses using PCR and RFLP analyses. The information generated was used to map the virus distribution in the country. RFLP analyses of the ACM viruses revealed more molecular variability in EACMV than in ACMV. In addition to the previously characterized EACMV-[Tanzania], 11 EACMV-like virus types, designated here as EACMV-[TZ1–11], were identified following restriction analysis of PCR products using *Eco*RV and *Mlu*I endonucleases. These viruses were associated with distinct symptoms on different cassava cultivars, and had a nonoverlapping and overlapping geographical distribution, with most of these isolates occurring in the coastal regions of the country. Of these, EACMV-[TZ1] was the most widespread followed by EACMV-[TZ2]. EACMV-[TZ4], EACMV-[TZ5], and EACMV-[TZ8] were the most virulent types, and were associated with severe mosaic symptoms in field-grown cassava. Two previously undescribed ACM begomoviruses were detected in the Mara Region of the Lake Victoria zone. These viruses were tentatively designated *East African cassava mosaic Tanzania*-Mara and an ACMV-like isolate, ACMV-[Tanzania]. The EACMV-UG2 associated with the pandemic of severe ACMD has expanded its range into Tanzania, covering most of the Lake Victoria region. Coinfection frequently involved ACMV and

EACMV-UG2, mainly at the front of the ACMD pandemic. ACMV was not found in any of the coastal regions or in the south of the country (Ndunguru and Fauquet, 2004).

In **Malawi**, ACMD is more prevalent in the lowlands (central and northern lakeshores), where the warmer conditions favor the whitefly vector *B. tabaci*. ACMD incidences have reached up to 90% (Nyirenda *et al.*, 1993). In **Madagascar**, ACMD was first observed in 1932 (François, 1937), and the disease spread rapidly throughout the island in that decade. A breeding project was then started, and the first ACMD-resistant varieties were released in the mid-1940s. This breeding effort and the enhanced awareness of the transmission of the causal virus via infected cuttings seemed to have reduced the incidence of ACMD in Madagascar (Sahondramalala *et al.*, 2005).

On the basis of the results of the surveys described above, it became apparent that there was a "hotspot" in the Lake Victoria Basin of southern Uganda and northwestern Tanzania. This region was therefore targeted for more detailed research during Phase II of the TWFP. A key aspect of this work was complementation with the Crop Protection Programme (CPP) supported by the DFID, the main donor financing Phase II of the TWFP, in order to implement sustainable integrated ACMD management practices in sub-Saharan Africa.

Three distinct ACMD-affected zones were detected in **Burundi**. The northeast zone was characterized by high incidences of ACMD, predominance of EACMV-UG, and high whitefly populations. EACMV-UG was present in the second zone to the south and west, albeit at a lower frequency. To the south and far west in the third zone, ACMD incidence and severity were both low, and only ACMV was present. This pattern suggests that the pandemic is currently spreading through Burundi toward the south and west, threatening the rest of the country.

Surveys conducted in southern and eastern **Cameroon** and throughout **Gabon** revealed the sole occurrence of ACMV, associated with mild ACMD symptoms in the area. EACMV-UG was detected in eastern Gabon, a region that represents the westernmost "front" of the ACMD pandemic. Since much of central Gabon and southern and eastern Cameroon are covered by dense primary rainforest, it is likely that further spread westward will be limited. The more likely route for pandemic spread into West Africa may be through the savannas of Central African Republic and Central Cameroon.

The last survey conducted in **Rwanda** in 2002 indicated that the pandemic was confined to the northeastern region. Informal reports received in July 2002 suggested that parts of southeastern Rwanda were also affected. Similarly, informal reports from southern

Sudan suggest the occurrence of severe ACMD conditions in this country.

The development and adoption of ACMD-resistant varieties are important milestones toward the successful implementation of the IPM strategies promoted by the TWFP. Trials conducted in Tanzania showed that all improved cassava varieties had average ACM disease severity values of 2.0/5.0, whereas local varieties had average disease severities that ranged from 2.6 to 3.6. "Rushura" had the highest disease severity of 3.6, followed by "Kaitampunu Nyeupe" (2.8) and "Mukarukwatage" (2.6). "Rushura" had the highest disease incidence of 4.5/5.0. This contrasted strongly with improved ACMD-resistant varieties, three of which were not diseased at all. However, the trial suggests that even under high ACMD pressure, the less susceptible local varieties (e.g., "Kaitampunu Nyeupe" and "Mukarukwatage") might be sustained through selection of healthy stems at the end of the season. Fortunately, farmers seem to be pursuing this approach, which in part explains the increasing frequency of cultivation of these two varieties in the postepidemic situation in northwestern Tanzania. This practice may contribute to the endemic nature of ACMD in certain regions of sub-Saharan Africa, and points out the importance of incorporating the necessary quality traits sought by farmers in improved varieties possessing resistance to ACMD.

ACMD surveys were conducted in Kenya, Tanzania, ROC, Burundi, eastern Cameroon, and Gabon in 2003. In Kenya, severe ACMD and the associated virus, EACMV-UG, continued to spread further south, covering the Western Province and Nyanza Province. However, there was a noticeable amelioration of the situation in the Western Province near the Ugandan border. Although ACMD incidence for western Kenya remained unchanged from the previous year at about 52%, there was a significant increase in the percentage of fields growing ACMD-resistant varieties derived from the management program; up to 20% from 15% the previous year (Legg, 2004).

2. Whitefly-Borne Viruses Affecting Sweet Potato in East Africa

Sweet potato (*I. batatas*), an important food crop grown extensively in eastern Africa, is affected by different viruses (Mukasa *et al*., 2005), such as *Sweet potato feathery mottle virus* (SPFMV), an aphid-borne potyvirus, and SPCSV, a crinivirus transmitted by *B. tabaci*. These two viruses act synergistically to cause the so-called SPVD, the main biotic constraint to sweet potato production in the Great Lakes region of Africa (Rwegasira *et al*., 2005). Other viruses found affecting sweet potato in East Africa are: SPMMV, an ipomovirus transmitted by *B. tabaci*, and *Sweet potato chlorotic fleck virus* (SPCFV), a carlavirus

not yet recognized by the ICTV (Fauquet *et al*., 2005). SPVD is one of the main biotic constraints of sweet potato production in **Uganda** (Bashaasha *et al*., 1995). Surveys were conducted in 1997 and 1998 to evaluate the SPVD situation in Uganda, revealed the presence of SPVD in 60 of the 80 sweet potato fields sampled. Virus incidence was higher in southern areas along Lake Victoria, particularly in the Mpigi District on the northern shore of the Lake. The lowest incidence was registered in Masindi and Lira, further to the north. At least, four different sweet potato viruses occur in most Ugandan sweet potato fields, namely: SPFMV, SPCSV, SPMMV, and SPCFV (Mukasa *et al*., 2003).

In **Kenya**, research on SPVD began in the 1950s, and its incidence remains at ∼22% (Ateka *et al*., 2004). This disease was shown to reduce tuber yield by over 90% in three different sweet potato varieties (Njeru *et al*., 2004). At least four different sweet potato viruses have been detected in Kenya, including those present in Uganda. The most common virus is SPFMV (detected in 86% of the fields), followed by SPCSV, detected in 50% of the sweet potato fields surveyed, the causal virus complex of SPVD. SPMMV and SPCFV were present in a relatively low percentage (3–11%) of the plant samples assayed (Ateka *et al*., 2004).

Viral diseases of sweet potato are also a major concern in **Tanzania**, but little research on SPVD had been conducted in this country until the initiation of the TWFP. A survey conducted by the Mikocheni Agricultural Research Institute of Dar-es-Salaam, with Swedish Government support, led to the detection of the main group of viruses found in East Africa: SPFMV, SPCSV, SPMMV, and SPCFV, particularly the aphid-borne SPFMV, and showed that the most affected region in Tanzania is the zone around Lake Victoria (Tairo *et al*., 2004).

The identification and deployment of SPVD-resistant sweet potato varieties are viable mechanisms to reduce the impact of SPVD in East Africa. For instance, whereas SPVD devastated sweet potato plantings in central Uganda (Mpigi District) in the 1960s, the disease is now a minor problem there, due to the introduction of the SPVD-resistant variety "New Kawogo" (Aritua *et al*., 1998). In southern Uganda, the CPP-funded, NRI-executed SPVD project (led by Dr. Richard Gibson) has been successful in establishing SPVD-resistant varieties in southern Uganda. As in the case of cassava, the enhancement of biological control and the development of guidelines for the effective use of phytosanitation were additional IPM activities pursued in Tanzania.

Interestingly, and regarding the performance of local sweet potato varieties, very high SPVD incidences were recorded in the evaluation trial of the local sweet potato varieties "Kombegi" and "Zerida," in contrast to SPVD incidences registered for improved varieties SP

93/34 (40%) and "Polista" (5%). However, the local varieties produced higher yields than the improved varieties, providing further evidence for the apparent occurrence of "SPVD-tolerance" mechanisms in local sweet potato varieties. Hence, breeding programs should continue to evaluate Ugandan sweet potato varieties (recently introduced to Tanzania), which yield adequately and provide tolerance to SPVD (Legg, 2005). The promotion of sweet potato varieties possessing resistance to SPVD remains a priority in Phase III of the TWFP, together with cultural practices and other IPM strategies developed in collaboration with national program scientists and farmers in a participatory way.

The CIP with headquarters in Lima, Peru, has the global responsibility for sweet potato improvement. CIP joined Phase III of the TWFP, following limited collaborative activities in Phase I, to support the sweet potato activities conducted in East Africa by IITA and NRI. CIP's role is to: participate in the characterization of viruses affecting sweet potato in East Africa; identify SPVD-resistant germplasm, particularly orange-fleshed (vitamin A-rich) sweet potatoes currently being promoted by CIP in developing countries; and train farmers in the production and selection of virus-free planting material. The main partner of CIP in Nigeria is the National Root Crops Research Institute.

3. Whiteflies as Pests and Vectors of Plant Viruses in Sub-Saharan Africa

In **Uganda**, surveys conducted by the TWFP had identified two whitefly species on cassava: *B. tabaci* (95.8%) and *B. afer* (4.2%). However, only one species, *B. tabaci*, was found on sweet potato. Average whitefly counts were higher for both cassava and sweet potato in southern and southwestern Uganda, coinciding with higher cassava and sweet potato disease incidences in these areas.

Following the unexpected epidemic of ACMD in Uganda in 2002, the genetic variability and geographical distribution of *B. tabaci* haplotypes in "postepidemic" Uganda were established (Sseruwagi *et al.*, 2005a). The study used the mitochondrial cytochrome oxidase I (mtCOI) molecular marker to investigate the genetic variability of *B. tabaci* populations associated with the ACMD epidemic in Uganda. The occurrence of two previously described haplotypes "local" and "invader" (Legg *et al.*, 2002) in the "post-epidemic" Uganda was confirmed. Comparison of the nucleotide identity of mtCOI DNA revealed that the two populations were ~8% divergent and shared very close sequence similarity (98–99.9%) with their closest relatives. The "local" haplotype predominated (83%) and was more widely distributed than the "invader" (17%). Unlike the late 1990s at the height of the

spread of the epidemic of severe ACMD in Uganda, when a clear association was demonstrated between the "invader" *B. tabaci* haplotypes and the ACM viruses, in the "postepidemic" situation there was no clear association between the two. The existence of alternative hosts for the cassava *B. tabaci* biotype was investigated by the TWFP. On the basis of the preliminary results, the *B. tabaci* populations clustered in four main clades: (1) B-like types, (2) non-B-like types, (3) Ivory Coast-okra-like types, and (4) cassava-like types. All the cassava types clustered with the "local" haplotype described by Legg and coinvestigators (2002).

B. tabaci and *B. afer* coexist in **Kenya** on both cassava and sweet potato in a 6:1 ratio, approximately, for both crops. Whitefly populations on cassava were higher in Coast Province, and on sweet potato in Nyanza Province. Mean ACMD incidence was 51.3% in Western Province. SPVD incidence ranged between 3% and 53% in farmers' fields, with the highest incidences occurring in Western Province and Nyanza Province (Kamau *et al.*, 2005).

In **Tanzania**, the TWFP has detected the presence of three different whitefly species: *B. tabaci, B. afer*, and *T. ricini* (Misra.). The first two species are present on cassava in similar proportions, and in a 4:1 ratio on sweet potato. *T. ricini* was found only once in a cassava field. Adult whiteflies were more abundant on cassava in the northern coast, and on sweet potato in the Lake zone.

In **Madagascar**, both *B. tabaci* and *B. afer* were found on cassava, but, unlike other zones of East Africa surveyed, *B. afer* was the predominant whitefly species. Whitefly populations were higher in the central region (Antananarivo) on cassava; and whitefly populations on sweet potato were relatively low during the surveys conducted by the TWFP in 1998 (Sahondramalala *et al.*, 2005).

In West Africa, the TWFP conducted surveys in Ghana, Benin, Nigeria, and Cameroon, in 1997. In **Ghana**, cassava is the most important crop, followed by maize and yam (*Dioscorea* spp.). *B. tabaci* is by far the most common whitefly species, whereas *B. afer* and *T. vaporariorum* were found in less than 4% of the 151 specimens collected (Cudjoe *et al.*, 2005). In **Benin**, *B. tabaci* coexists with a much lower population of *B. afer* on cassava, and in **Nigeria**, where up to 85% of the plants show ACMD symptoms (Echendu *et al.*, 2005), *B. tabaci* was the most common whitefly species in all sites surveyed, followed by a single sample of *T. vaporariorum* on cassava. *B. afer* was not found. In **Cameroon**, two whitefly species *B. tabaci* and *B. afer* were recorded, with the former species accounting for over 90% of the specimens collected (Ntonifor *et al.*, 2005).

4. Socioeconomic Aspects Related to Whitefly-Related Problems in Sub-Saharan Africa

Only 22% of the cassava and sweet potato farmers interviewed in 12 districts of **Uganda** recognized whiteflies as a problem. However, 100% of the farmers contacted were aware of ACMD as a serious cassava production problem. On the other hand, only 48% of the sweet potato farmers interviewed were aware of SPVD. Cassava farmers assessed their losses due to ACMD at 75%, and many had abandoned the crop. Sweet potato farmers believed that SPVD was reducing their yields in ~25%. The main virus disease control method for both cassava and sweet potato was the elimination of symptomatic plants (roguing). Only about a fifth of the cassava farmers interviewed were planting virus resistant varieties, and, of these farmers, only 24% thought that the resistant varieties yielded better than their local varieties (Sseruwagi *et al*., 2005b). In Uganda, the TWFP seeks to build on previous work funded by DFID under its Crop Protection Programme (R8243, R8243, R8457) on the control of SPVD. The main outputs include the use of cultural control measures, training of farmer groups, distribution of information, and deployment of SPVD-resistant varieties in disease-affected areas. One of the main problems experienced by sweet potato farmers has been the conservation of planting material by farmers during the dry season, which affects the adoption of SPVD-resistant varieties. Some SPVD-resistant local sweet potato varieties have been detected in the Lake area of Uganda.

In Kenya, about 50% of all farmers interviewed recognized whiteflies, and 61% considered whiteflies as pests. Most cassava farmers (88%) recognized ACMD, whereas only 58% of the sweet potato farmers recognized SPVD. Cassava farmers in the Western Province believed that they were losing up to 75% of their crops to ACMD. On the contrary, sweet potato farmers do not perceive a large (<25%) yield loss due to SPVD. About a third of the cassava and sweet potato farmers interviewed use roguing of diseased plants, but do not plant ACMD-resistant varieties. Very few cassava farmers in East Africa use insecticides (Kamau *et al.*, 2005). Less than 30% of the farmers interviewed during a survey conducted by the TWFP in **Tanzania** recognized the whitefly as an important pest, although over 58% of the farmers recognized ACMD as an important disease. Only 38% of the sweet potato farmers recognized SPVD as a problem. Cassava farmers thought that ACMD costs them between 25% and 50% of their yields, whereas the sweet potato farmers believe that the viral diseases of sweet potato reduce yields only up to 25% (Tairo *et al*., 2004).

In **West Africa**, the TWFP conducted surveys in Ghana, Benin, Nigeria, and Cameroon, in 1997. In **Ghana**, cassava is the most important crop, followed by maize and yam (*Dioscorea* spp.). Over 54% of the farmers interviewed mentioned ACMD as a serious crop production problem, but very few of them (2%) associated this disease with the presence of *B. tabaci*. Highest whitefly incidence occurred in the eastern coastal savannah, but whitefly populations seem to play a secondary role to the introduction and maintenance of ACMD via infected cuttings (Cudjoe *et al.*, 2005). Approximately 89% of the farmers interviewed mentioned the lack of technical assistance with respect to the management of ACMD, and, consequently, only 11% of the farmers contacted, planted improved cassava varieties developed by IITA. In **Benin**, cassava is also the main staple grown in land-holdings under 0.3 ha, followed by maize and cowpea. ACMD is the main biotic constraint, registering 49% incidence in the dry season and 57% in the wet season (probably from plants infected during the dry season). However, most farmers (60%) did not recognize *B. tabaci* as a pest or vector of cassava viruses (Gbaguidi *et al.*, 2005).

In **Cameroon**, 70 fields were surveyed in three different cassava-growing regions. Two whitefly species *B. tabaci* and *B. afer* were recorded, with the former species accounting for over 90% of the specimens collected (Ntonifor *et al.*, 2005). ACMD ranged from 44% in the rainforest and wet savannah to 72% in the transition forest, although the highest whitefly population densities were found in the rainforest. Most cassava farmers in Cameroon were women, working farms with less than 0.6 ha. Fifty-one percent of the farmers recognized ACMD as a constraint, but very few knew the role of the whitefly vector. Farmers practiced roguing; some used improved varieties; and others took care in the selection of their planting material in order to combat ACMD (Ntonifor *et al.*, 2005).

Cassava is also the most important crop grown in **Nigeria**, where up to 85% of the plants show ACMD symptoms (Echendu *et al.*, 2005). Two different ACMD viruses were detected in Nigeria in the late 1990s: ACMV and EACMV (Ogbe *et al.*, 1998). A survey of 80 farms conducted by the TWFP in 1997 showed that *B. tabaci* was the most common whitefly species in all sites surveyed, followed by a single sample of *T. vaporariorum* on cassava. *B. afer* was not found. Only 17.5% knew that *B. tabaci* caused problems in cassava. Both local and improved cassava varieties were found in farmers' fields, although the former predominated. Average ACMD incidence ranged from 45% to 83% across ecozones, with cuttings providing most of the initial inoculum. About 53% of the cassava farmers practiced roguing of symptomatic plants (Echendu *et al.*, 2005).

Farmers' groups were organized in Umuekete Aguleri (Anambra State), Obeagu Eleke, Ndufu Echara (Ebonyi State), and Otere Ikom (Cross River State), in southeast Nigeria. Group discussions revealed that all farmers in Umuekete Aguleri (Anambra State) and Obeagu Eleke (Ebonyi State) cultivate cassava and sweet potato, whereas in Otere Ikom (Cross River State) only 70% of the farmers cultivate sweet potato. Cassava was ranked first both as a food and a cash crop in Umuekete Aguleri and Otere Ikom, whereas in Obeagu Eleke, cassava was ranked first as a food crop but second to rice as a cash crop. Sweet potato was ranked third as a food crop and second as a cash crop in Umuekete Aguleri, fourth as a food crop and third as a cash crop in Obeagu Eleke, and fourth as a food crop and sixth as a cash crop in Otere Ikom. Farmers in the three communities had no knowledge about whiteflies, ACMD or SPVD. Apart from Otere Ikom where it appeared that farmers had been having some contacts with the Cross River State Agricultural Development Project (ADP) extension agents, farmers in Umuekete Aguleri (Anambra State) and Obeagu Eleke (Ebonyi State) were not receiving technical assistance from ADP extension agents of their respective states. The most important source of information was farmer to farmer.

B. B. tabaci *as a Vector of Viruses in Mixed Cropping Systems of Eastern Africa*

Traditional agriculture is giving way to the establishment of diversified agricultural systems in developing countries, as a response to falling prices for basic agricultural commodities, and the need to maximize the profitability of small-scale agriculture in the Tropics. Vegetables are an economically viable alternative for small-scale farmers in developing countries. Unfortunately, whiteflies have found additional reproductive and feeding hosts among these horticultural crops, particularly the fastest growing crops: tomato (*Lycopersicon esculentum*), peppers (*Capsicum* spp.), and cucurbits. The TWFP investigated the situation of nontraditional crops in selected countries of eastern Africa, namely: Sudan, Kenya, Tanzania, and Malawi.

Sudan has traditionally been a hotspot of *B. tabaci* due to its warm and dry lowlands, and irrigated agriculture, which compensates for their low relative humidity (10%). *B. tabaci* has been recognized in Sudan as an important pest and vector of plant viruses, such as *Cotton leaf curl virus*, since the 1920s (Kirkpatrick, 1931). ACMD is also present in southern Sudan (Dafalla and Sidig, 1997). Whitefly-borne

viruses of tomato in Sudan were first reported in the mid-1960s (Yassin and Nour, 1965); and there was enough evidence to suspect the presence of other whitefly-transmitted viruses affecting okra, pepper, melon, and watermelon (Dafalla and Ahmed, 2005). The surveys conducted by the TWFP demonstrated the presence of the whitefly-borne begomoviruses TYLCV, *Watermelon chlorotic stunt virus*, *Okra leaf curl virus*, and *Pepper leaf curl virus*. The main region affected by whitefly-related problems is undoubtedly the Gezira Irrigation District (Gezira, Hasaheisa, Managil, Umelgura, Dindir, and Butana). The incidence of TYLCV in this region is often 100%, and *B. tabaci* populations concentrate mostly in the central and southern areas that receive less rainfall. The eastern, western, and northern states have lower disease incidence and whitefly populations (Dafalla and Ahmed, 2005).

Over 95% of the vegetable production in Sudan takes place in landholdings under 5 ha, and only about 40% of the farmers interviewed by the TWFP owned the land. Tomato was the most profitable crop, but they also cultivate cucumber, melon, okra, common bean, pepper, eggplant, sweet potato, radish, carrot, onion, and some leafy vegetables. Over 75% of the farmers recognize whiteflies and 89% were aware of TYLCV. However, only 35% of the tomato farmers knew that the whitefly transmitted TYLCV. Perceived tomato yield losses due to these pests reached 62%, and 26% of the tomato farmers in Gezira reported up to 100% losses. Whitefly populations build up during the winter (October–January) and damage crops during the hot season (March–August). Tomato production costs include insecticides to control whiteflies, amounting to 30% of total costs. Only 10% of the farmers receive any technical assistance. There are higher (>500 m) areas in Sudan with enough rainfall to produce vegetables without irrigation, such as the Gedaref region and some areas of the Kordufan Province.

In **Kenya**, a total of 94 farms were surveyed by the TWFP in Phase I, mostly in the southwestern and eastern parts of the country. In these regions, tomato is planted throughout the year. The Eastern Province is a semi-arid, low-to-mid-altitude region. Western Province and Nyanza Province are subhumid and humid mid-altitude zones. Tomato production in Kenya is an important activity for many farmers, both for local and foreign markets. Whiteflies are important pests of several crops in the Solanaceae, Leguminosae, Malvaceae, Cucurbitaceae, and Euphorbiaceae, particularly in the semi-arid Eastern Province. Whitefly-transmitted viruses reported in Kenya include TYLCV, *Cowpea mild mottle virus* (carlavirus), SPMMV (ipomovirus), and *Watermelon chlorotic stunt virus* (begomovirus). Whitefly surveys in these vegetable-producing

regions of Kenya revealed the presence of *B. tabaci* (57%), *T. vaporariorum* (34%); *B. afer* (7%) on cassava only; and other species including *T. ricini*. Curiously, *B. tabaci* individuals collected from tomato plants, preferentially colonized common bean, followed by *Lablab purpureus, Vigna unguiculata*, and *Abelmoschus esculentus* (okra). Vegetable production in Kenya is dominated by smallholders, who account for over 70% of total vegetable production in this nation. Tomato is the most profitable crop, but all cultivars were susceptible to TYLCV in the late 1990s, with yield losses of up to 75%. About 88% of the farmers interviewed recognized whiteflies as a pest, and 64% could identify TYLCV. However, only 1% of the farmers knew that whiteflies transmit the virus. Most tomato producers (85%) choose their own pesticides and application times (Ali-Bob *et al*., 2005).

C. Whiteflies as Vectors of Viruses in Mixed Cropping Systems in the Lowlands and Mid-Altitude Valleys of Mexico, Central America, and the Caribbean

Tropical and subtropical America are undoubtedly the regions most affected by whitefly pests and whitefly-transmitted viruses in the world, particularly in terms of the number of countries and crops affected by different whitefly species and whitefly-borne viruses. Perhaps only one country in the Old World could make a similar claim: India, but in Latin America, there are over 20 countries that suffer the ravages of these pests. In this region, begomoviruses have been studied since the mid-1930s, particularly in Brazil, where a group of agricultural scientists captivated by the investigations conducted on the "infectious variegation" of *Abutilon* spp. by Baur (1904) in Germany, noticed the similarities of this disease with the common variegations of malvaceous plant species in Brazil (Costa, 1955; Silberschmidt, 1943; Silberschmidt and Tommasi, 1955).

In 1946, Orlando and Silberschmidt (1946) published on the transmission of the causal agent of the "infectious chlorosis of the *Malvaceae*" in Brazil, by the whitefly species *B. tabaci*. Puerto Rico, in the Caribbean region, was another "hotspot" of whitefly-transmitted viruses in Latin America. In the 1950s, Bird and Sanchez (1971) conducted a series of investigations on the etiology of several diseases of wild and cultivated plants infected by viruses transmitted by *B. tabaci*. They referred to these viruses as "rugaceous," based on the pioneering work of Holmes (1949). At present, the main crops affected by begomoviruses in Latin America are: common bean (*Phaseolus vulgaris*), tomato (*Lycopersicon esculentum*), sweet and hot peppers (*Capsicum* spp.),

and some native cucurbits. There are currently over 3 million ha of common bean affected by different begomoviruses capable of inflicting total crop losses (Morales and Anderson, 2001). The area planted to tomato (ca. 290,000 ha) and peppers (ca. 195,000 ha) in Latin America is relatively small, but the economic losses caused by several begomoviruses in these crops are equivalent to those reported for common bean. This fact is due to the high value of vegetables and the cultivation of begomovirus-resistant common bean varieties in Latin America. In the case of cucurbits, melon plantations have been severely damaged by whiteflies and whitefly-borne viruses in Latin America (Morales and Anderson, 2001).

1. The Whitefly Vector: B. tabaci

The economic importance of begomoviruses in Latin America is directly associated to the population dynamics of the whitefly species *B. tabaci*. The geographic distribution of this whitefly species depends largely on climatic conditions that favor its reproduction: warm temperatures, moderate relative humidity, and relatively low-to-moderate rainfall. These conditions are commonly found in tropical America, at altitudes that range from sea level to over 1200 m of altitude. The ecology of *B. tabaci* in Latin America has been studied by Morales and Jones (2004). Besides favorable weather conditions, the existence of a suitable reproductive host is another critical factor responsible for the rapid growth and dissemination of *B. tabaci* populations. For instance, the rapid increase in the area planted with soybean in Brazil, from 1 million to 15 million ha in the last three decades, has been the main factor responsible for the increasing whitefly and begomovirus problems in Brazil and neighboring countries in South America, particularly Argentina and Bolivia (Morales and Anderson, 2001).

One of the most important factors responsible for the increasing incidence of whitefly damage and begomoviruses in Latin America was the introduction of the B biotype of *B. tabaci* in the early 1990s (Morales and Anderson, 2001). The new biotype is more aggressive in terms of its fecundity and adaptation to different hosts and environments (de Barro, 1995). Biotype B also induces physiological disorders of significant economic importance, previously unknown in the agricultural regions invaded by this new pest. These disorders include the "silver leaf" of squash; "irregular ripening" of tomato (Costa and Brown, 1991; Ramirez *et al.*, 1998); and severe chlorosis of some fruits produced by some plant species (e.g., common bean and some cucurbits) affected by the new biotype (Morales, F. J., personal observation).

The highly polyphagous nature of the B biotype of *B. tabaci* has facilitated the adaptation of this new pest to a changing agricultural environment characterized by the transition of traditional agriculture (e.g., maize and common bean) to mixed cropping systems (e.g., maize, common bean, tomato, pepper, and peanut in one farm or region) in Latin America. The high-reproductive capacity of the new biotype also makes it a serious pest in the absence of begomoviruses. Populations of over 2000 whitefly individuals per trifoliolated leaf have been observed to cause severe cases of "sooty mold" and, ultimately, plant death. As a vector, the new biotype may not be as efficient as the original biotype in the transmission of begomoviruses (Duffus *et al.*, 1992), but the large populations of the B biotype largely make up for the lower efficiency of transmission observed for the B biotype. Moreover, as the B biotype begins to encounter and transmit begomoviruses, its transmission efficiency increases over time (Morales, F., and Cuellar, M. H., unpublished data). Furthermore, the B biotype has displaced the A biotype of *B. tabaci* from important agricultural regions of Latin America, where it has been introduced, and it has also displaced the second most important whitefly pest species, *T. vaporariorum*, from mid-altitude (950–1000 m) agricultural regions where the original *B. tabaci* biotype could not thrive (Rodriguez *et al.*, 2005). Occasionally, the B biotype of *B. tabaci* has been observed to transmit begomoviruses at altitudes over 1500 m above sea level, in the central highlands of Colombia, but this observation seems to be related to the movement of tomato seedlings from lowland nurseries to production fields in the highlands (Morales, F. J., unpublished information).

2. Collaborative IPM Activities on Whiteflies and Associated Viruses in Latin America

Organizing an international network on whitefly-transmitted viruses in a region as extensive as Central America, Mexico, and the Caribbean is no easy task. First, whitefly-borne viruses have affected more than a dozen countries and commercial crops in this region. Second, most of these countries lack the necessary research infrastructure or resources to study plant viruses. And last but not least, most of the research conducted in the region has focused on the whitefly *B. tabaci* as a pest and not as a vector of plant viruses. Fortunately, a "Regional Action Plan for Whitefly Management" had been implemented in 1992 on the initiative of various international institutions such as the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) in Costa Rica, the Pan American Agricultural School at Zamorano in Honduras, and the Instituto Interamericano de Cooperación para la Agricultura

(IICA). The network promoted annual meetings of regional scientists from countries affected by these pests, under the coordination of CATIE. Another important network that operates in the region is the Regional Bean Cooperative Programme (PROFRIJOL), financed by the Swiss Government to promote the production of common bean, *P. vulgaris* in this region. The main production problem of common bean in the lowlands and the mid-altitude valleys of Central America, Mexico, and the Caribbean has been *Bean golden yellow mosaic virus* (BGYMV). Both PROFRIJOL and CIAT have been breeding for resistance to this disease since 1978. The existence of these networks greatly facilitated the task of organizing an international network to focus specifically on viruses transmitted by the whitefly *B. tabaci* in legume and horticultural crops in the region.

Mexico is the largest country in Latin America, north of the Equator, with an area of 1,958,201 km^2. The country can be divided into different regions, the largest being the Central Plateau, which extends from the US border to the Isthmus of Tehuantepec in the south, rising to more than 2650 m south of Mexico City. Although the Central Plateau contains many agricultural valleys, the altitude limits the survival and activity of the whitefly *B. tabaci*. Instead, other whiteflies, such as *T. vaporariorum*, predominate, and are often confused with *B. tabaci*. Although *T. vaporariorum* is a major pest on several crops, including common bean (*P. vulgaris* L.), tomato (*Lycopersicon esculentum* Mill.), potato (*S. tuberosum* L.), and other horticultural plant species grown above 900-m altitude, this whitefly species is not a vector of begomoviruses.

The Yucatán Peninsula, bordering the Gulf of Mexico, consists of flat, limestone terrain, seldom exceeding 160 m above sea level. The northernmost coastal area of the Yucatán Peninsula became an important horticultural area in the late 1980s, and a decade later was already under attack by *B. tabaci* and different begomoviruses transmitted by this whitefly (Diaz-Plaza *et al.*, 1996). However, the main horticultural areas affected by *B. tabaci* in Mexico are the coastal lowlands that lie east and west of the Central Plateau. The Gulf Coastal Plain extends some 1400 km from the Texas border to the Yucatán Peninsula. The narrower Pacific coastal lowlands begin near the Mexicali Valley in the north and end, also some 1400 km to the south, near Tepic, Nayarit. Parts of these arid regions have been irrigated since the 1930s and support intensive cropping systems of traditional and nontraditional (export) crops. Finally, Baja California is an isolated strip of arid land, about 1280-km long and 160-km wide.

The first report of *B. tabaci* as a vector of plant viruses in Mexico, in the early 1950s, was associated to the production of cotton (*Gossypium*

hirsutum L.) in the Valley of Mexicali, Baja California (Cárdenas *et al*., 1996). The disease observed was probably "cotton leaf crumple," caused by a begomovirus transmitted by *B. tabaci*, previously observed in the United States near the Colorado River Valley (Brown, 1994). Soon after, *B. tabaci* was also associated with a disease of tomato, called "chino del tomate" in the Valley of Culiacán, Sinaloa, in northwestern Mexico (Gallegos, 1978). In 1962, *B. tabaci* was observed attacking cotton in the southern state of Chiapas (Cárdenas *et al*., 1996).

In 1978, *B. tabaci* caused considerable yield losses to horticultural crops in the states of Hidalgo, Tamaulipas, San Luis Potosí, and Veracruz, where the Gulf Coastal Plain tapers to form a narrow strip that reaches all the way to the northernmost point of the Yucatán Peninsula. On the opposite coast, a disease believed to be "bean golden mosaic" was observed for the first time in the northwestern state of Sinaloa (Lopez, 1974). When this disease was later (1990) detected in the state of Sonora, it was shown to be caused not by *Bean golden mosaic virus* or BGYMV, but by a distinct *Bean calico mosaic virus* (BCaMV) species (Brown *et al.*, 1990). BCaMV induces more striking yellowing and bleaching symptoms, which eventually cause foliar necrosis. The virus is not closely related to any known bean begomovirus but, rather, to SLCV, a virus previously described attacking cucurbits in California (Flock and Mayhew, 1981). However, BGYMV emerged on the Gulf Coast of Veracruz in 1977, and in 1979–1980, it affected common bean plantings in Las Huastecas and Chiapas (Lopez-Salinas and Becerra, 1994). Finally, in 1980, *B. tabaci* reached the Yucatán Peninsula, particularly the state of Yucatán, where it caused severe yield losses to horticultural crops as a pest and virus vector (Diaz-Plaza *et al*., 1996).

The rapid dissemination of whitefly-transmitted begomoviruses in Mexico has been associated with the boom in horticultural and other nontraditional export crops that took place in the 1980s. The cultivation of melon, soybean, tomato, and peppers, and excessive pesticide use associated with these high-value crops created favorable conditions for the reproduction of *B. tabaci*. The most important whitefly-borne viruses affecting horticultural crops in Mexico are: *Tomato chino virus*, first described in Sinaloa in 1970 (Gallegos, 1978); Serrano golden mosaic virus, in northeastern Mexico (Sánchez *et al*., 1996) and Texas pepper virus (Stenger *et al*., 1990), both of which are currently classified as *Pepper golden mosaic virus* (PepGMV) (Fauquet *et al*., 2005); and *Pepper yellow vein Huasteco virus* (PYVHV) in pepper and tomato in Las Huastecas and Sinaloa (Hou *et al*., 1996). In Yucatán, chili pepper, tomato, squash, melon, and watermelon have been attacked since the

1980s by *B. tabaci* and begomoviruses such as PYVHV and PepGMV (Diaz-Plaza *et al.*, 1996).

Tomato mottle virus (ToMoV), a begomovirus originally described from Florida, was detected in the Yucatán Peninsula, also affecting tomato (Garrido-Ramirez and Gilbertson, 1998). PYVHV had already been detected infecting Jalapeño varieties of chili pepper in the neighboring state of Quintana Roo (Diaz-Plaza *et al.*, 1996). Meanwhile, an Old World begomovirus, TYLCV, made its appearance in the Yucatán Peninsula (Ascencio-Ibañez *et al.*, 1999). This virus has caused yield losses in the Caribbean estimated at millions of US dollars, but so far it has not adapted well to the agroecosystems of the Yucatán Peninsula in Mexico.

The participation of Mexican researchers in Phase I of the Tropical Whitefly Project provided an opportunity to gain an updated and more systematic view of the status of whitefly-borne viruses in the country. The Centro de Investigaciones y Estudios Avanzados (CINVESTAV), located at Irapuato, Guanajuato, was selected as the lead institution in Mexico, under the coordination of Dr. Rafael Rivera Bustamante. In the course of this project, samples were taken from crop plants showing begomovirus-like symptoms at a range of sites in major horticultural production areas of Mexico and assayed using monoclonal antibodies (MABs) previously developed for various isolates of BGYMV (Cancino *et al.*, 1995). In addition, a survey was conducted of viruses affecting tomato and chili in Mexico. The results obtained demonstrate that two begomoviruses, PYVHV and PepGMV, are the main viral pathogens attacking horticultural crops in the states of Quintana Roo, Campeche, Jalisco, Colima, Nayarit, Guanajuato, Veracruz, Morelos, Hidalgo, San Luis Potosí, and Querétaro.

As noted above, golden mosaic symptoms in common bean in northern Mexico are caused by BCaMV, which is related to SLCV, a cucurbit virus originally recorded from the United States. In Phase I, three bean-producing areas were surveyed in the northwestern region of Mexico: Etchojoa in Sonora, and Los Mochis and Culiacán in Sinaloa states. A partial molecular characterization of the viral isolates collected showed that BCaMV is still present in common bean plantings in northwestern Mexico, specifically in Los Mochis. This isolate is still closely related to an isolate of BCaMV collected in the same locality in 1986. The sample from Culiacán represents a begomovirus closely related to BCaMV but already evolving into a new species (as indicated by a sequence homology of less than 90%). An interesting finding is that the common bean begomovirus from Sonora is still identifiable as SLCV, constituting the first direct evidence of the evolution of this virus (Morales *et al.*, 2005). Selected whitefly samples were also collected on

different crops in northwestern Mexico, revealing the presence of mixed populations of biotypes A and B of *B. tabaci*.

The southeastern region of **Guatemala** (El Oriente), formed by low- and mid-altitude (200–1000 m) valleys, has suffered continuous attacks from the whitefly *B. tabaci* and different begomoviruses transmitted by this whitefly species since the mid-1970s. This region has drastically changed its traditional cropping systems to join the boom in export crops, thus creating complex and fragile agroecosystems. Already in the 1860s, cotton produced in the Pacific lowlands of the country comprised 20% of Guatemala's exports. A second boom of cotton production took place in the 1950s and 1960s, and at this time the first viruses transmitted by whiteflies were observed in Guatemala and neighboring countries (Gill, 1994). Tobacco production in southeastern Guatemala has also been blamed for the increasing populations of *B. tabaci* observed since the 1970s in this region. The first outbreaks of BGYMV in common bean were observed at that time (Morales, 1994).

In 1974, the Horticultural Research Division of the Instituto de Ciencia y Tecnología Agrícolas (ICTA) initiated extension work in the Zacapa Department to produce export crops such as melon. A similar industry to produce tomato was also initiated in the late 1970s in southeastern Guatemala (Gaytán, 1979). These crops became suitable reproductive hosts for *B. tabaci* than either common bean or wild plant species. The populations of the vector soon soared and new begomoviruses emerged to attack the new crops. The arrival of the B biotype of *B. tabaci* further increased yield losses (Dardón, 1992).

The first collaborative project designed to meet the growing problem of whitefly-borne viruses was the PROFRIJOL, initiated by CIAT in 1978 with support from the United Nations Development Programme (UNDP) and, as of 1980, the Swiss Development Cooperation (SDC). This program is credited with the development of several BGYMV-resistant (DOR) lines that have subsequently been widely adopted as commercial varieties in Mexico, Central America, the Caribbean, and South America. In Guatemala, the project began work under the umbrella of ICTA in the southeastern Monjas Valley, about 960 m above sea level. Until the 1970s, this valley had been planted to traditional crops, mainly common bean and maize, but in the 1980s, horticultural crops such as tomato and broccoli made their appearance in the valley. Other nontraditional crops, such as pepper, cucumber and, more recently, watermelon, have also been planted in this valley over the latter part of the 1990s.

In the mid-1970s, BGYMV was the first whitefly-transmitted virus to cause severe yield losses in the Monjas Valley (Rodriguez, 1994).

The local common bean cultivars, "Pecho Amarillo" and "Rabia de Gato," soon succumbed to the disease. Fortunately, the PROFRIJOL project released the first BGYMV-resistant breeding lines: ICTA-Quetzal, ICTA-Jutiapán, and ICTA-Tamazulapa (Rodriguez, 1994). The Guatemalan (GA) BGYMV isolate was shown to be different from the Brazilian isolates of *Bean golden mosaic virus* (BGMV) (Faria *et al.*, 1994). Characterization of whiteflies collected by the TWFP from sites across southeastern Guatemala confirmed the suspicion that the B biotype had displaced the A biotype almost completely. The presence of PepGMV in pepper was confirmed in samples taken from the locality of Laguna de Retana.

A special study was conducted in the municipality of Monjas, Jalapa Department, where Amerindians still make up over 70% of the total population. The Monjas Valley is located at an altitude of 960 m and has an average annual precipitation of 900 mm, most of which falls between May and October. The mean temperature is 23.7°C and the mean relative humidity is 80%. Maize and common bean were the most traditional crops in the valley. Tobacco has been planted for over 20 years as a commercial crop but almost disappeared in the late 1970s because of market problems, coming back in the late 1990s. Broccoli and pepper have been very popular crops in the valley in the past decade. Cucumber has been planted commercially for the past 10 years, and watermelon for the last 6 years, although it has been planted for local consumption for decades in the valley. The results of a survey clearly showed that pesticide abuse occurs in most of the crops affected by whiteflies. In the case of tomato, only 5 of the 11 pesticides used are specific to the control of whitefly; the remaining 6 pesticides being applied as "repellents" of the whitefly.

Whereas growers recognize *B. tabaci* as a pest, they do not fully understand its role as a vector of viruses. However, they readily associate the bright yellowing shown by infected common bean plants with a disease (bean golden yellow mosaic). In the case of tomato, growers call symptoms expressed by begomovirus-infected plants "*acolochamiento*" (curling). According to most growers, whiteflies are a regular pest, year after year, particularly during the summer time because of the higher temperatures and relative humidity. Growers also perceive the complex cropping systems in the valley as a negative factor because they claim that these crops represent more food sources for the whitefly. Finally, growers blame ratoon crops for the survival of whiteflies between summer and winter seasons. Growers believe in the existence of whitefly-resistant or more generally pest- and disease-resistant crops, particularly tomato, tobacco, and common bean. However, the "resistance" of tomato and tobacco seems to be associated with the implementation of more successful chemical

control practices in the valley. In reality, the case of common bean is a good example of genetically controlled virus resistance obtained through a well-designed, long-term breeding program developed in Central America by PROFRIJOL.

El Salvador occupies a volcanic region and, thus, most of its territory is formed by mid-to-low-altitude valleys (up to 1000 m), where *B. tabaci* can thrive. El Salvador is one of the smallest countries in Latin America, but one of the most densely populated. Given the shortage of land, the higher slopes are dedicated to crops such as coffee. Farmers make up over 50% of the population, primarily producing subsistence crops such as maize and common bean. Recently, the production of melon, pepper, and tomato has greatly increased because of their relatively high market value; even so, some of these products continue to be imported. Common bean is widely grown in El Salvador, occupying about 75,000 ha (1995). However, total common bean production does not satisfy internal demand and, in 1994–1995, El Salvador imported 14,600 tons of common bean (Parada and Perez, 1996).

Among the biotic problems that limit common bean production in the country, the main pest and disease problems are *B. tabaci* and bean golden yellow mosaic. Zaumeyer and Smith (1964) were the first to report BGYMV in El Salvador. This virus can now be found wherever common bean is grown in the country. The introduction of new crops further complicated the dynamics of whitefly populations in El Salvador. The Zapotitán Valley (La Libertad) is a good example of a traditional agricultural area where crops such as maize and common bean were largely displaced by high-value horticultural crops (e.g., tomato, pepper, and cucurbits). However, these vegetable crops have been eliminated gradually from the valley because of the damage caused by whitefly-transmitted viruses.

A case study was conducted in El Salvador, in the San Vicente Department, in an area about 55,340 ha, including 20,346 ha planted to basic grains: maize, sorghum, common bean, sesame, and soybean. Among the horticultural crops grown here, *ayote* (*Cucurbita moschata*), *pipián*, sweet pepper (*Capsicum annuum*), hot pepper (*Capsicum* spp.), tomato, cucumber, melon, and watermelon are the most important. Only 25% of the farmers interviewed had secondary (high school) education and 41% had received technical assistance in the selection of pesticides to control the whitefly problem. Pesticide salesmen are highly influential in the selection and use of insecticides for all crops. Most farmers (54%) apply insecticides with a frequency between 10 and 15 times per crop cycle. A significant proportion (16%) of the farmers interviewed apply pesticides more than 20 times in a single crop cycle.

One hundred and forty-seven different commercial pesticides were mentioned during this survey. Sixty-two percent of the farmers interviewed mentioned preventive pesticide applications as a risk-averting practice.

Of the farmers interviewed, about 97% recognizes the whitefly problem but only 36% associates this pest with viral diseases. Farmers believe that whitefly-related problems are more severe during the dry months of the year but they cannot predict the incidence of whiteflies and viral diseases based on climatic factors. Over 60% of the respondent farmers considered that yield losses in susceptible crops range from 25% to 50% because of the whitefly/begomovirus problems.

The current situation of the whitefly/begomovirus problems in El Salvador merits an effort to implement integrated pest management strategies in the main horticultural areas of the country, mainly in the departments of San Vicente, San Salvador, La Libertad, Sonsonate, and Santa Ana. The use of virus-resistant common bean cultivars has attenuated the problem in this food crop, but genetic resistance is not available in any of the other crops affected by begomoviruses in El Salvador. One of the main goals of the TWFP in El Salvador is to teach farmers to make rational use of pesticides.

The isolated situation of the interior valleys of **Honduras** and the absence of an extensive cotton industry in the Pacific coast, probably protected this country from the early outbreaks of *B. tabaci* and begomoviruses recorded in neighboring countries in the 1970s. It was not until the mid-1980s that the intensification and diversification of cropping systems in the highland central valleys coincided with the emergence of begomovirus problems. BGYMV emerged around 1985. In 1989, most common bean-producing regions in Honduras were affected by BGYMV. The most affected areas were those in the central and southern agricultural regions of the country, where annual rainfall does not surpass the 1500-mm range (Rodriguez *et al.*, 1994).

In the last two decades, Honduras also experienced a boom in the production of nontraditional crops such as tomato, pepper, and melon. The tomato industry in the Comayagua Valley (580 m) was developed as early as 1978, with a view to producing high-quality produce for export (Standard Fruit Company, 1978). This industry has come practically to an end now because of the persistent attacks of whitefly-borne viruses. A similar phenomenon has been recorded for the valleys of Jamastrán, El Paraiso, and La Lima (El Paraiso Department), although the most affected crop in these areas is pepper. The melon industry of southern Honduras also has been brought to the point of extinction by whitefly-related problems (Caballero, 1995). These production problems have forced many growers to abandon the cultivation of susceptible food crops

leaving as alternative crops such as tobacco. However, tobacco is currently under attack by whiteflies and begomoviruses in neighboring countries, particularly in Guatemala.

Research on whitefly-transmitted begomoviruses in Honduras has been scant. During this project, common bean plants exhibiting golden mosaic-like symptoms were tested with the monoclonal BGYMV antibodies. In these tests, the Honduran BGYMV isolate reacted with the broad spectrum monoclonal antibody (MAB-BS) but not with the GA, as observed in this project for other BGYMV isolates from Guatemala and El Salvador. Begomoviruses were detected in common bean and tomato but not in two cucurbits tested, namely cucumber and cundeamor (*Momordica charantia*). The latter seemed to have a low incidence of a phytoplasma disease, and the cucumber sample was infected by a potyvirus, most likely transmitted by aphids. The BGYMV isolates from Honduras did not react with the MAB-BS prepared in 1993 to the GA isolate. The original GA isolate reacted to this monoclonal when used as a control in this test. This result suggests that the present Honduran BGYMV isolates also have altered their capsid protein composition, probably in response to the arrival of the more aggressive B biotype of *B. tabaci*. However, eight whitefly samples from common bean, four samples from cucumber, three samples from tomato, and one sample from chili biotyped by random amplified polymorphic DNA (RAPD) analysis only detected the presence of the A biotype of *B. tabaci.*

A case study was conducted in the Comayagua Valley with the collaboration of the Escuela Agrícola Panamericana (EAP), Zamorano. In the Comayagua Valley, more than 70% of the 100 producers surveyed had been cultivating the land longer than 5 years. The number of literate farmers in the population surveyed was 75%, of which 66% had received only primary education. Only half or less (in some areas) of the farmers surveyed had received technical assistance to control whitefly problems (Jara, 1998). Regarding whitefly incidence, 87% of the farmers believed that whiteflies and whitefly-borne viruses increased in the warmer months of the year. About 80% of the farmers noted that whitefly populations increase in periods of low rainfall. The worst whitefly/virus epidemics in the Comayagua Valley occurred in 1989, when drought and high temperatures struck the region. These results demonstrate that most farmers are aware of the key climatic factors that determine whitefly epidemics.

Over 40% of the farmers interviewed apply insecticides on a calendar basis for whitefly control. This practice can be interpreted as a risk reduction measure, particularly in the case of tomato growers, who

invest between US$2100 and US$3500 per *manzana* (0.764 ha). In tomato plantings, 63% of the growers apply insecticides against whiteflies as soon as the tomato plants are transplanted, 31% apply 1 week after transplanting, and only 6% wait until they see whiteflies in their fields. In the case of common bean, 12% of the farmers apply pesticides at planting time, 42% start controlling whiteflies 1 week after germination of the plants, and 46% apply insecticides later on during the vegetative phase of the crop. These results demonstrate farmers' perception regarding the investment required to plant tomato (usually 10 times higher than in the case of common bean).

In economic terms, 53% of the production costs for tomato and 47% for common bean are related to the chemical control of whitefly/begomoviruses. Total production costs for tomato are up to 20 times more than for common bean or cucumber. It is evident from this study that the implementation of an integrated whitefly management package in the Comayagua Valley would significantly contribute to lessening the environmental impact of insecticides and to a significant reduction in production costs of high-value crops such as tomato and peppers.

A good indicator of the presence of whitefly-transmitted viruses in a region is the occurrence of BGYMV. This virus is distributed in the departments of El Paraiso, Francisco Morazán, Choluteca, Valle, Olancho, Comayagua, and Copán, marking a vast region where whitefly/begomovirus problems can emerge on susceptible crops. The results obtained showed that the Honduran BGYMV isolate is a strain of the BGYMV species found in Middle America.

Nicaragua is the largest country in Central America and one of its richest nations in terms of agricultural resources. Cotton became a highly important crop in the Pacific lowlands, between Lake Managua and the Gulf of Fonseca (Chinandega to León) and along the eastern side of Lake Managua (Tipitapa) in the early 1950s. By 1977, Nicaragua was the largest (217,000 ha) producer of cotton in Central America (Gill, 1994).

As in the rest of Central America, there are a number of fertile mid-altitude valleys in Nicaragua such as Boaco and Sébaco, where a more intensive agriculture eventually developed, consisting of nontraditional crops such as tomato, pepper, and other horticultural crops. Common bean remains a major staple and consequently is grown throughout the country. However, the main regions producing common bean are the Pacific region (30% of total production), the Central Highlands (50%), and the Caribbean Plains (10%). In the highlands, the main departments producing common bean are Matagalpa, Jinotega, Estelí, Madriz, and Nueva Segovia.

B. tabaci first became a pest of cotton in the 1970s, which together with adverse marketing circumstances reduced the area planted to cotton to a mere 2520 ha in 1993. Interestingly, *B. tabaci* was not an insect of economic significance in the early years of cotton production in Nicaragua. The emergence of this whitefly species as a major pest of cotton followed the introduction and intensive use of pesticides on this crop in the 1960s (Gill, 1994).

BGYMV was first observed affecting common bean in the Pacific Coast of Nicaragua, around 1971. BGYMV is particularly severe in Estelí, Nueva Segovia, León, Chinandega, and the lowlands of Matagalpa (Llano *et al*., 1997). Large populations of *B. tabaci* have been reported on cotton in the Pacific Coast of Nicaragua since 1952, and from tomato in the Atlantic Coast as early as 1953 (Hidalgo *et al*., 1975). This whitefly species was the third most important pest problem in cotton in Nicaragua in the late 1970s (Kramer, 1966). The whitefly problem became so serious that, in 1975, Nicaragua created a special commission to study *B. tabaci* and make recommendations on research and whitefly management (Hidalgo *et al*., 1975).

Mid-altitude valleys such as Sébaco (Matagalpa Department) witnessed the emergence of whitefly-transmitted viruses in 1986. By 1992, yield losses reached 100% in several tomato fields affected by what growers referred to as "*crespo*" (leaf curl). In 1991, large populations of whiteflies attacked common bean and melon, but only common bean was affected by begomoviruses. Whitefly-transmitted begomoviruses also attacked pepper and tomato plantings in the Central Highlands. Tomato plantings were affected in the region of Boaco in 1991. The same year, *B. tabaci* attacked tobacco plantings in the departments of Nueva Segovia, Jinotega, Estelí, Chinandega, Masaya, and Rivas. The melon industry in the departments of León, Rivas, Managua, and Matagalpa was first affected by whiteflies in 1991 but the incidence of viruses was low (Sediles, 1998).

No records exist of serological identification of BGYMV in Nicaragua prior to 1995. In 1993, a few common bean and tomato samples were collected near the town of Santa Lucia in the Boaco Valley. The common bean samples were taken from plants showing dwarfing symptoms, rather than mosaic. These samples reacted positively with the MAB-BS that detects whitefly-transmitted begomoviruses in general (MAB-BS). The virus was later identified as *Bean dwarf mosaic virus* (BDMV) by nucleic acid hybridization methods (Zamora, 1996). At that time, and from the same locality, samples collected from tomato plants affected by *crespo* disease also gave positive results in serological assays with the same MAB. The *crespo* disease of tomato was first

observed in Nicaragua in 1986. An in-depth investigation conducted in 2000 showed that tomato in Nicaragua is infected by at least four distinct begomoviruses (Rojas *et al*., 2000).

In 1995, three BGYMV-affected common bean samples from the northwestern department of Estelí were tested with the MAB used to detect the original Middle American isolates of BGYMV-Guatemala (MAB-GA), available at the Centro Internacional de Agricultura Tropical (CIAT). The three common bean samples reacted with the MAB-BS but only one common bean sample was recognized by the specific MAB-GA. A survey in the departments of Estelí (Condega), Matagalpa (Apompua and Sébaco), and Managua (Managua and Pochocoape) was undertaken to collect plant samples for virus assay. The presence of begomoviruses in *pipián* (*Cucurbita argyrosperma*) is interesting, considering that this crop had not been recorded previously as an important host/reservoir of begomoviruses. However, the same observation was made in El Salvador and, furthermore, this cucurbit was also doubly infected with aphid-transmitted potyviruses in both countries.

Because of the rainy conditions that affected Nicaragua in 1998 and 1999, very few whitefly samples could be taken for analyses. Although representing only a limited sample, results are taken from one of the main horticultural valleys of Nicaragua and, consequently, are interesting in that they show a predominance of the original biotype A.

Horticultural crops such as tomato and pepper have been cultivated in Nicaragua mainly to meet local demand. The area planted to tomato has doubled in the past 30 years from 350 to 750 ha, whereas in Guatemala, tomato production grew from 5000 ha in 1960 to 12,000 ha in 1970. In 1986, tomato plantings in the Sébaco Valley, Matagalpa Department, suffered unusual infestations of the whitefly *B. tabaci* and, soon after, the emergence of viral diseases associated with the whitefly outbreak. By 1991, tomato production in the Sébaco Valley had been reduced 20–50% because of yield losses ranging between 30% and 100% (Sediles, 1998). A similar situation was observed for pepper plantings, which in 1991–1992 reported yield losses between 30% and 50%. Melon, another nontraditional crop grown mostly for export, was beginning to experience whitefly-related problems. However, there was no significant incidence of begomoviruses in this crop.

In **Costa Rica**, the Central Plateau, around the capital city of San Jose, is one of the most intensively cultivated areas in the country. North and south of this region, the broken and volcanic topography of mountain ranges expands all the way to the northern border with Nicaragua and the southern border with Panama. East and west of the northern mountain ranges lie the Caribbean and Pacific lowlands,

the least developed and inhabited regions of Costa Rica. Southeastern Costa Rica receives over 1500 mm of rain annually, which prompted the development of a thriving banana production industry toward 1880. Coffee was introduced into Costa Rica in the 1830s and soon became a source of wealth for many farmers in the Central Plateau. Among the basic food staples, maize and common bean are important components of the Costa Rican diet, together with crops such as potato and plantain. Cotton is produced only to a limited extent in the Pacific lowlands of the province of Guanacaste (Pastor, 1988; West and Augelli, 1977). The Central Plateau is undoubtedly one of the most affected areas in terms of whitefly-related damage.

The lack of suitable reproductive hosts for the whitefly *B. tabaci*, such as cotton or soybean, together with the high rainfall over much of the Costa Rican territory, protected the Central Plateau's agricultural region against the early arrival of this pest. The first records of the presence of *B. tabaci* in the country originated in the 1970s, precisely in the drier lowlands of the Pacific province of Guanacaste, where an incipient cotton industry was developed in the 1960s. The first record of *B. tabaci* as a pest and vector of BGYMV in common bean fields in the Central Valley, occurred in 1987. BGYMV had already been observed in the country in the 1960s (Gámez, 1970), albeit at a very low incidence. In 1988, *B. tabaci* moved on to tomato in the Central Valley. Coincidentally, the attack to common bean and tomato took place in the western areas of the Central Valley, which is the closest region to the cotton-growing areas of Costa Rica. *B. tabaci* is currently a pest and vector of viruses in common bean and tomato fields in most of the horticultural provinces of the country.

A BGYMV isolate from Alajuela (Central Valley) had been characterized serologically at CIAT in 1993 as a member of the Central American/Caribbean group of BGYMV isolates. However, different begomoviruses isolated during the course of this project from BGYMV-affected bean plants in different regions of Costa Rica (Puriscal, Alajuela, and Los Chiles) were shown to be serologically distinct from the original Middle American BGYMV isolates characterized in the early 1990s using MABs (Cancino *et al.*, 1995).

A begomovirus of tomato had been isolated previously in the locality of Turrialba (Castillo, 1997) and tentatively identified as *Tomato yellow mosaic virus* (ToYMV). This virus was later shown to be a different species referred to as Tomato yellow mottle virus (ToYMoV). Another begomovirus, isolated from diseased tomato plants in Turrialba, was shown in 1998 to be related to a tomato begomovirus tentatively designated as Tomato leaf curl Sinaloa virus, originally isolated in

northwestern Mexico (Idris *et al.*, 1999). However, *Tomato leaf curl virus* (ToLCV) is an Old World begomovirus that does not exist in the Americas, and, consequently, this name has to be changed.

The Tropical Whitefly Project financed a survey of *B. tabaci* biotypes in Costa Rica, conducted under the guidance of Dr. Luko Hilje of the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Turrialba. The survey confirmed the presence of *B. tabaci* in various crops of socioeconomic importance and showed the penetration of biotype B of *B. tabaci* in different regions of Costa Rica. However, at the time of the survey, the original A biotype still predominated in the country. Some of the most heavily colonized crops were cucurbits but, unlike the case of other Central American countries, *B. tabaci* was found to colonize pepper (*Capsicum* spp.) in Costa Rica.

Whiteflies and begomoviruses in Costa Rica mainly affect common bean and tomato crops, particularly in the Central Valley, where most of the horticultural crops are grown. Another area where the whitefly problem is gaining momentum is the "Pacifico Seco" (Dry Pacific Region) in Guanacaste Province. Rainfall in this region ranges from 1220 to 2000 mm annually, with an average temperature of 20–25°C. These are not optimal conditions for *B. tabaci* but the occurrence of a prolonged dry period from December through April favors the buildup of whitefly populations and transmission of viruses to susceptible horticultural crops. Fortunately, most of the Costa Rican territory receives over 1500 mm of annual rainfall, which maintains whitefly populations depressed most of the year. These climatic factors and various IPM measures implemented in Costa Rica, including the use of resistant varieties in the case of common bean, have contributed significantly to the attenuation of the whitefly/begomovirus problems in this country.

Panama receives tropical rains in excess of 2500 mm/year, a climatic factor known to drastically reduce whitefly populations. Nevertheless, during the first workshop on whiteflies in Central America and the Caribbean, held in Costa Rica in 1992, Panama was already present (Zachrisson and Poveda, 1992). According to the report presented by the Panamanian delegate at that meeting, the emergence of whiteflies as agricultural pests in Panama was associated with the expansion of nontraditional crops, mainly melon (*Cucumis melo* L.), for export to the United States. Three horticultural areas were developed in the late 1980s: the Pacific Coast of the province of Panama; the central provinces of Coclé, Herrera, and Los Santos (the largest); and the western province of Chiriquí, near the Costa Rican border (Delgado, 1994; Zachrisson and Poveda, 1992). These regions have in common the

presence of a well-defined dry period and below-average precipitation during the year.

The presence of *B. tabaci* on tomato was first observed in the Azuero Peninsula in 1983. However, it was not until 1991, when unusually dry climatic conditions favored the reproduction of *B. tabaci*, that this whitefly species became a pest in the central provinces of Panama. *B. tabaci* has been observed to reproduce in eggplant, sweet pepper, and chili pepper. In 1992, the silver-leaf symptom was observed on squash attacked by *B. tabaci*, which suggested the presence of biotype B in Panama. The western horticultural zone, located in Chiriquí Province, is at a higher elevation and receives more rainfall than the other two horticultural zones of Panama. The problem pest in this area is not so much *B. tabaci* as *T. vaporariorum* (Westwood), a whitefly species that attacks horticultural crops at altitudes above 1000 m (Zachrisson and Poveda, 1992).

In Panama, common bean is cultivated in Chiriquí Province, at altitudes between 550 and 1000 m. BGYMV was observed prior to 1970 (Gámez, 1970) but this disease never has been a major production problem of common bean in Panama. A whitefly-borne begomovirus affecting tomato was described in Panama in 1998 (Engel *et al.*, 1998). The virus, named *Tomato leaf curl virus*, was shown in this project to have a high degree of nucleotide sequence similarity when compared to ToYMV from Venezuela. The virus had been described in Panama as a strain of *Potato yellow mosaic virus*, a misnomer for ToYMV. Although this virus may be a new species, the name "*Tomato leaf curl virus*" is not appropriate because it is already given to a distinct begomovirus species that only exists in the Old World (Asia).

A molecular characterization of *B. tabaci* biotypes from the central horticultural zone of Azuero was conducted at CIAT, with samples provided by Ing. Agr. José Angel Guerra of the Instituto de Investigación Agropecuária de Panamá (IDIAP). Results demonstrate the coexistence of biotypes A and B of *B. tabaci* in the central provinces of the Azuero Peninsula. This is an interesting situation that needs to be monitored in order to study the outcome of the interaction between these two biotypes in a horticultural zone characterized by mixed cropping systems.

Between 1991 and 1994, about 6000 tons of tomato were affected by whitefly problems in Panama. During the 1997–1998 tomato planting season, 2800 tons were lost because of the effect of *B. tabaci*, both as a pest and vector of plant viruses. Whereas large-scale tomato growers absorbed most of the losses, these pests affected a number of small-scale producers in the province of Los Santos.

Haiti is a mountainous country with over 40% of its territory located at altitudes above 490 m. The mountain ranges alternate with fertile valleys, the largest being the Plaine du Nord. Annual rainfall varies from 500 mm in the northwest to 2500 mm in the eastern/southern highlands. Agriculture accounts for one-third of the gross domestic product but subsistence farming predominates. Maize, sorghum, common bean are the main staples, with rice and sweet potato also being important components of the Haitian diet. However, most of the food consumed in Haiti has to be imported.

The whitefly *B. tabaci* was first regarded as a serious pest in Haiti, in the early 1980s. Common bean, tomato, eggplant, pepper, cowpea, and lima bean are the main crops affected by this insect pest (Donis and Prophete, 1997). As a vector, *B. tabaci* already had been observed to transmit BGYMV in 1978 (Balthazar, 1978).

The main agricultural areas of Haiti were surveyed and plant samples analyzed to determine the importance of whiteflies and whitefly-borne viruses. Of whitefly-transmitted begomoviruses, BGYMV was present in common bean and TYLCV in tomato. As in the case of the Dominican Republic, the specific monoclonal antibody (MAB-GA), which recognizes BGYMV isolates from Central America and southern Mexico, detected the Haitian isolate of BGYMV.

As suggested by the serological assay using the GA-specific MAB, the Haitian BGYMV isolate assayed is similar to the Central American and Caribbean BGYMV isolates but different from the South American bean begomoviruses, BGMV and BDMV. Few of the tomato, pepper, and cowpea samples collected in Haiti contained aphid-transmitted potyviruses. All tomato samples were shown by polymerase chain reaction (PCR) to be infected by TYLCV. The composite whitefly sample collected from eggplant resulted in the detection and identification of biotype B of *B. tabaci*.

About 25% of **Cuba**'s territory is mountainous, with three distinct mountain ranges running east to west: the eastern (Sierra Maestra), central (*alturas*), and western (Cordillera de Guaniguanico) ranges. The remaining land is composed of extensive plains and basins. Cuba has a semitropical climate with two seasons: dry from November through April and rainy from May through October. The mean annual temperature is 26°C, with a 23–28°C range. The average precipitation is 1380 mm. All these conditions favor the dissemination of whiteflies, particularly during the dry season of the year.

Horticultural products have been traditional commodities in Cuba, particularly those referred to as *viandas* and basic grains. These crops include tomato, squash, cucumber, cabbage, sweet potato, eggplant, and

common bean. Vegetable production has become increasingly important to support a rapidly expanding tourist industry in Cuba.

The emergence of *B. tabaci* as a pest is probably linked to the advent and intensive use of agricultural pesticides soon after World War II. *B. tabaci* was reported as a pest of tobacco and a vector of plant viruses of common bean in the mid-1970s (Blanco and Bencomo, 1978). But it was not until 1989 that *B. tabaci* became a major production problem of tomato and common bean, as a vector of begomoviruses (Murguido *et al.*, 1997). Currently, this whitefly species attacks tomato, common bean, squash, cucumber, melon, cabbage, and eggplant throughout Cuba.

By 1990, the new B biotype of *B. tabaci* already had been introduced into Cuba and surrounding Caribbean islands (Brown, 1994). Between 1991 and 1993, the silver-leaf syndrome induced by this new biotype was observed on squash (Murguido *et al.*, 1997). Another major production problem for Cuba and the Caribbean region occurred when an Old World virus, TYLCV, was introduced from Israel into the Caribbean region, including Cuba (Polston and Anderson, 1997). This exotic virus has caused crop losses worth millions of dollars in the Caribbean region, and it is now known to occur in southern United States and the Peninsula of Yucatán, Mexico.

A main objective of the research undertaken in Cuba was to determine the composition of the whitefly population. Ninety-nine samples were collected from tomato plants in the localities of Quivicán and Alquizar, Havana Province. The results of the RAPD assay showed that 100% of the whitefly samples tested corresponded to biotype B of *B. tabaci*. Additionally, seven whitefly samples from potato (*S. tuberosum* L.) plants grown in Havana Province were also shown to consist of *B. tabaci* biotype B. These findings suggest that biotype B has adapted well and now predominates over biotype A of *B. tabaci* in Havana Province.

BGYMV has been an important disease of common bean in Cuba since the early 1970s (Blanco and Bencomo, 1978). The epicenter of the problem was the locality of Velasco in the western province of Holguín, and it is now widely disseminated in the provinces of Holguín, Las Tunas, Guantanamo, and Havana. In recent years, the incidence of BGYMV has increased in the provinces of Ciego de Avila, Holguín, Las Tunas, and Camagüey.

When the MABs to BGYMV were developed in 1990 (Cancino *et al.*, 1995), the Cuban isolate of BGYMV reacted with an MAB (MAB 2G5) produced to a GA isolate of BGYMV, which recognized all of the Middle American BGYMV isolates. By 1993, the Cuban isolates of BGYMV were not reacting with the specific MAB 2G5. In fact, none of the

BGYMV samples from three different provinces of Cuba reacted with the specific MAB-GA (2G5), including a BGYMV isolate from lima bean (*P. lunatus* L.). The homologous control, BGYMV-GA, reacted with MAB 2G5 as expected. However, all of the diseased plant samples that were assayed reacted with the MAB-BS (3F7) to BGYMV, which recognizes bipartite begomoviruses. These tests were repeated in 1997, including some BGYMV-infected common bean plants from the Dominican Republic, a neighboring Caribbean island. The results showed that the Cuban BGYMV isolates had changed their antigenic properties, whereas the Dominican BGYMV isolates had not.

With respect to tomato, one of the most affected crops in Cuba, three random samples were take in the locality of Quivicán, Havana Province, and tested for different pathogens. The MAB-BS used to detect bipartite begomoviruses did not detect any begomovirus in the tomato samples, although the observation of two of the samples in the electron microscope revealed the presence of begomovirus-like particles. This result suggested the need to test for the presence of TYLCV, a monopartite begomovirus reported to attack tomato in Cuba (Polston and Anderson, 1997). This introduced virus is best detected by PCR, using specific primers, kindly provided by D. P. Maxwell and M. K. Nakhla, of the Department of Plant Pathology, University of Wisconsin. The PCR assay detected the presence of TYLCV in two of the tomato samples from Quivican, Cuba.

In 1998, Dr. Gloria Gonzalez (Instituto de Investigaciones de Sanidad Vegetal, INISAV), Havana, Cuba, collected samples from symptomatic potato plants in Havana Province. The begomovirus isolated from potato in Cuba is an isolate of *Tomato mottle Taino virus* (ToMoTV), a virus described in 1997 attacking tomato in Cuba (Ramos *et al.*, 1997). This virus was also closely related to BDMV from Colombia (93.3%), ToYMV from Venezuela, ToMoV from Florida, *Sida golden mosaic virus* from Costa Rica, and *Abutilon mosaic virus.*

Cuba has a unique advantage over the rest of the Latin American countries affected by whiteflies and whitefly-borne viruses, specifically, the limited use of agrochemicals. Cuba has been able to develop and successfully implement biological control methods to combat the whitefly *B. tabaci*. One of the most effective biological control agents has been the entomopathogen *Verticillium lecanii* (*Lecanicillium muscarium*). This fungus is very pathogenic to the immature stages of *B. tabaci*. The production of biological control agents in Cuba constitutes an industrial activity in at least 15 provinces of Cuba, where it is being applied on a regular basis to horticultural crops affected by *B. tabaci* (Murguido *et al.*, 1997).

Tabaquina, a concoction of tobacco leaf residues from the intensive Cuban tobacco industry, is another industrial subproduct used in Cuba to control whiteflies in a highly effective manner. Obviously, the well-planned cropping systems in Cuba also facilitate the implementation of legal measures regulating the time of planting for certain crops which act as reproductive host for the whitefly *B. tabaci* (Murguido *et al.*, 1997).

The control of BGYMV in beans, on the other hand, is effectively carried out through the use of BGYMV-resistant bean cultivars developed in collaboration with the Programa Cooperativo Regional de Fríjol para Centro América, México y el Caribe (PROFRIJOL) network and CIAT-Colombia.

The whitefly situation in Cuba worsened following the invasion of the new B biotype of *B. tabaci*. However, the relatively low use of insecticides and the production of biological control agents in Cuba should maintain the whitefly problem at a manageable level. The Cuban provinces most affected by *B. tabaci* and the viruses it transmits have been Pinar del Río and Havana in the western end of the island, and Holguín and Las Tunas in the eastern half of the country. The whitefly problem also has been severe in certain years in the provinces of Santiago and Guantánamo in the eastern end of Cuba. Other provinces affected have been Matanzas, Ciego de Avila, Granma, Camaguey, Villa Clara, Cienfuegos, and Sancti Spiritus.

The **Dominican Republic** comprises the eastern two-thirds of the island of Hispaniola. The country is generally mountainous, with the most prominent range being the Central Highlands (elevation up to 3175 m), the highest point in the West Indies. The Constanza Valley is a highly developed agricultural area located in the Central Highlands and is currently planted to high-value crops such as garlic, onion, and other horticultural crops. The Cibao Valley, in the northwest, is one of the most fertile agricultural areas in the country, where rice, maize, common bean, tobacco, and coffee are produced. The western part of the country is dry, but rivers provide irrigation. San Juan de la Maguana is the main common bean production area, and the neighboring Azua Valley is the main tomato-growing area in southwestern Dominican Republic. The annual mean temperature is 25°C and precipitation is 1346 mm. Tropical storms and hurricanes are a major threat every year (West and Augelli, 1977).

B. tabaci was first observed attacking common bean in 1975. However, the presence of BGYMV in the Dominican Republic had been noticed as early as the late 1960s (Schieber, 1970). By 1988, other crops such as tomato, melon, eggplant, cucumber, and watermelon were under attack from *B. tabaci*. In 1991, biotype B of *B. tabaci* made

its appearance in the main horticultural regions of the Dominican Republic. At the same time, the first begomovirus from the Old World, TYLCV was irresponsibly introduced into the Dominican Republic and the Americas. Following the introduction of a new and more aggressive biotype of the whitefly vector and an exotic begomovirus, crops such as tomato and melon practically disappeared, and the tomato processing plants had to close down in certain areas.

The first geminivirus of economic importance to be characterized at the molecular level in the Dominican Republic was BGYMV. This virus was isolated in the early 1990s by the TWFP, and later shown to be molecularly and serologically related to the GA isolate of BGYMV (Cancino *et al*., 1995; Faria *et al*., 1994). The BGYMV isolate from the Dominican Republic (San Juan de la Maguana) proved to be similar to BGYMV isolates from the Caribbean (Puerto Rica) and Central America (Guatemala). The BGYMV isolate from the Dominican Republic was shown to be a different species when compared with BGMV from Brazil. BGYMV affects over 20,000 ha of common bean in the Dominican Republic each year, particularly in the southwestern valley of San Juan de la Maguana. The presence of a begomovirus infecting tomatoes in the Dominican Republic was first noticed in 1992 and subsequently identified as TYLCV from Israel (Polston and Anderson, 1997; Polston *et al*., 1994). The introduction of an exotic begomovirus from the Old World created a major pandemic throughout the Caribbean at a cost to tomato growers and tomato-processing plants worth millions of US dollars in yield/export losses (Dupuy, 1998).

At the beginning of this project, in April 1997, common bean samples were collected in San Juan de la Maguana and tomato samples in Azua, in southwestern Dominican Republic. The common bean samples reacted with the MAB-BS 3F7 as well as with the MAB prepared to the GA isolate of BGYMV. However, the tomato samples did not react against the MAB-BS in these tests. These tomato samples were then assayed by PCR using two sets of primers to TYLCV provided by Dr. M. Nakhla, Plant Pathology Department of the University of Wisconsin, Madison, WI. These primers amplified two fragments of M_r 287 and 1793 pb, respectively, demonstrating the presence of TYLCV in the tomato plants sampled in Azua. In February 1998, six more tomato samples from Azua and one from the northern locality of Santiago were assayed with similar results, demonstrating the endemic nature of TYLCV in the Dominican Republic. Three tomato samples from the Centro de Investigaciones Aplicadas a Zonas Aridas (CIAZA), Azua, corresponded to TYLCV-tolerant tomato varieties (Gem-Pear, Gem-Star and UC82), which produce acceptably in this locality.

The common bean samples from the valley of San Juan de la Maguana behaved as expected for the BGYMV isolates previously detected in this locality. The presence of BGYMV in the Constanza Valley is interesting, because it is located at altitudes between 1000 and 1100 m, above the altitudinal range (0–950 m) at which *B. tabaci* is usually a problem. However, the incidence of the virus was moderate in the Constanza Valley.

The arrival of TYLCV from Israel and biotype B of *B. tabaci* brought the thriving tomato industry to a halt, causing economic losses in excess of US$30 million. This crisis was managed through the adoption of legal measures that included a 3-month crop-free period to break the cycle of the whitefly vector. Additionally, virus-resistant tomato cultivars were introduced. Following the implementation of legal measures against *B. tabaci*, the melon industry made a comeback. The case of common bean was not promising, because this crop was being displaced from the traditional bean-growing areas such as the valley of San Juan de la Maguana by other crops without whitefly problems and in higher demand, including cassava and sweet potato. In 1984, there were 63,000 ha of red-seeded bean in the country and in 1994 the area planted to common bean is only 36,000 ha, almost half of the area planted 15 years ago. As a result, the country has had to import common bean, usually grain types of little demand in the country, considering that red-seeded bean is the main food staple in the diet of the Dominicans (Fundación de Desarrollo Agropecuario, 1995).

The Dominican Republic constitutes a unique example of a country that implemented legal measures to minimize yield losses due to the activity of *B. tabaci* both as a pest and virus vector. Nevertheless, pesticide use remains high in most agricultural regions of the country, and the existing cropping systems favor the reproduction of *B. tabaci*. Currently, the situation in the lowlands and highlands remains under partial control, and some crops that had been taken out of production such as melon are being planted again. Another crop and good host to the whitefly *B. tabaci*, tobacco, also has been increasing its area in the country. A recent viral epidemic in tobacco in the Cibao region was diagnosed at CIAT as being caused by *Tobacco mosaic virus*.

In the case of tomato, the cultivation of varieties resistant to TYLCV should contribute to the recovery of the production of industrial tomato plantings. In the case of common bean, however, we have noticed a marked reduction in the area planted, because of the susceptibility of most local varieties to BGYMV. Moreover, TYLCV has been reported to attack common bean in other parts of the world (Navas-Castillo *et al.*, 1999).

D. Trialeurodes vaporariorum *as a Pest in Mixed Cropping Systems of the Andean Highlands*

The Andes encompass a wide range of latitudes and climates from the hot and tropical to snow-capped mountains, within the spectrum of "vertical geography." Usually, areas above 2000 m above sea level are known as highlands, but temperatures in the tropical Andean zone are already moderate above 1000 m. The tropical highlands of Colombia, Venezuela, Ecuador, Peru, and Bolivia include extensive agricultural areas with mean temperatures of 12–20°C; variable rainfall patterns (700–1600 mm); and relative humidity ranging from 50% to 85%. In periods of low rainfall in the tropical Andean highlands, frost becomes a threat to many crops such as maize and potatoes. In this region, small-scale farmers grow common bean and snap bean (*P. vulgaris*), tomato, potato, temperate cereals, crucifers, and other horticultural crops, both for home consumption and for sale. Crops are usually grown on steep, erosion-prone slopes, with no irrigation and little or no use of fertilizers. This is the territory of the species *T. vaporariorum*. According to Vet *et al*. (1980), this species is probably indigenous to tropical and subtropical America, but it is currently found throughout the world.

This whitefly was viewed as a minor pest until the early 1980s (Cardona, 1995), but it became a pest on common bean, snap bean, tomato, potato, and ornamentals in the mid-1980s, and is now considered the most important whitefly species in the tropical highlands of South America. Damage is due to the continuous sucking of sap from the phloem by nymphs and adults, and to the abundant excretion of honeydew that covers the leaves and fruits of their reproductive and feeding hosts, acting as a substrate for nonparasitic fungi (sooty molds) that block photosynthesis and affect the yield and quality of several crops. A series of trials in Colombia (Cardona *et al*., 1993; Prada *et al*., 1993) showed that high infestation levels (up to 450 nymphs/leaf) can reduce the yields of common bean by 38% and of snap bean by 54%. In past years, *T. vaporariorum* has shown its capacity to transmit plant viruses in the Andean region, affecting both potato and tomato crops in the Colombian, Ecuadorian, and Peruvian highlands (Morales *et al*., 2005; Salazar *et al*., 2000).

The TWFP is working in the Andean region with a view to: (1) reducing levels of pesticide use by farmers, (2) reducing health risks to farmers and consumers of contaminated produce, and (3) reestablishing the ecological equilibrium by developing integrated pest and disease management systems in areas that have been disturbed by

excessive pesticide use, and (4) reducing yield losses caused by whiteflies as pests and vectors of plant viruses. Major activities include monitoring of whitefly populations, monitoring of whitefly natural enemies, testing of nonchemical alternatives for whitefly control, development and refinement of a simplified action threshold for whitefly control, development of a sampling method for whitefly populations, monitoring of insecticide resistance levels, testing in farmers fields of different alternatives for management of whiteflies in susceptible crops, and characterization of viruses transmitted by *T. vaporariorum* in the Andean highlands.

Changes in whitefly species and biotype composition were detected in the Cauca Valley where the B biotype of *B. tabaci* is now more important than *T. vaporariorum*. In Ecuador, *T. vaporariorum* is still the only whitefly species affecting beans, potatoes, and vegetable crops in highland areas. An action threshold for rational control of *T. vaporariorum* was refined. Highest increases in yield were obtained when chemicals were applied when first instar nymphs appear and occupy less than 30% of the leaf area. Sampling methods for whitefly nymphs and adults were developed and tested. The parasitoids *Encarsia nigricephala* and *Amitus fuscipennis* were identified as the most important natural enemies of *T. vaporariorum* in the Andean zone. Resistance to insecticides in adult and nymphal populations of *T. vaporariorum* was measured in two critical locations in Colombia and in five locations in Ecuador. In general, the situation has not changed, with populations showing high levels of resistance to organophosphates, some susceptibility to pyrethroids, and susceptibility to carbamates like methomyl. With some exceptions, there is a general trend for conventional insecticides to be less efficient for control of *T. vaporariorum* nymphs in both Colombia and Ecuador. Fortunately, this whitefly pest is still susceptible to insect growth regulators and neonicotinoids.

Seed treatments and drench applications of the new generation of systemic insecticides were compared with foliar applications of conventional (less costly) products, in some cases with applications based on preestablished action thresholds. These treatments were compared with farmers' practices and, in some cases, with untreated checks. In Ecuador, where the project has entered the diffusion stage, large-scale demonstrative plots were used to conduct the trials. In Colombia, alternative management strategies resulted in yields that did not differ from those obtained by farmers with their traditional management approaches. Selective use of systemic insecticides as seed dressing or in drench application resulted in higher benefit cost ratios with a substantial reduction in the amount of applications per crop cycle and less detrimental

effect on natural enemies. Our results indicate that it is feasible to reduce the number of insecticide applications to manage *T. vaporariorum* populations.

Monitoring changes in whitefly species composition in affected areas was a continuous activity. A reliable monitoring system for the proper identification of whitefly species and biotype composition using molecular (RAPD) techniques was developed. Whiteflies (adults or pupae) were collected on different host plants in the Cauca Valley in Colombia and the Chota Valley in Ecuador. All RAPD characterizations were made by comparison with patterns obtained from existing colonies of different whiteflies maintained at CIAT. An analysis of 32 samples from the Cauca Valley showed that *T. vaporariorum* was at that time the most important species affecting snap beans, tomato, cucumber, dry beans, sweet potato, squash, and melon in the region (45% of samples taken). The B biotype, a relatively new pest in the area, was present in 30.3% of samples taken as a major pest of cucumber, snap beans, cotton, and sweet potatoes. The A biotype was not found. In the Chota Valley of Ecuador, *T. vaporariorum* was the sole species found on dry beans and potatoes.

In Colombia, the situation changed drastically in past years. Analysis of 123 samples taken in 23 locations of the Cauca Valley showed that 63% of the whiteflies collected belonged to the B biotype of *B. tabaci*, and 21% to *T. vaporariorum*. The A biotype was not found. The now predominant B biotype has caused very serious problems in agricultural areas of the Cauca Valley: uneven ripening of tomatoes, silver-leaf disorder on squash, and sooty mold on cotton. In snap bean growing areas, it has become the causal agent of a physiological disorder known as pod chlorosis, which renders the produce useless. Moreover, *B. tabaci* has become a vector of begomoviruses affecting snap bean, tomato, and pepper production (Morales *et al.*, 2002).

E. B. tabaci *as a Vector of Viruses in Mixed Cropping Systems of Southern Asia*

In Asia, India and Pakistan have been the countries most severely affected by plant viruses transmitted by *B. tabaci*. Begomoviruses such as *Acalypha yellow mosaic virus*, *Ageratum yellow vein virus*, *Chilli leaf curl virus, Cotton leaf curl virus, Croton yellow vein mosaic virus, Dolichos yellow mosaic virus*, Horsegram yellow mosaic virus, *Indian cassava mosaic virus*, Lupin leaf curl virus, *Mungbean yellow mosaic virus, Bhendi yellow vein mosaic virus*, *Okra leaf curl virus*, *Papaya leaf*

curl virus, Pepper leaf curl virus, Tobacco leaf curl virus, ToLCV, and Zinnia leaf curl virus are some of the main whitefly-borne viruses described in India and Pakistan (Jones, 2003; Muniyappa, 1980). As more virus research is conducted in other parts of Southeast Asia, begomoviruses such as *Ageratum yellow vein virus* (Taiwan, Singapore), Eggplant yellow mosaic virus (Thailand), *Malvastrum yellow vein virus* (Yunnan), *Pepper leaf curl virus* (Malaysia), Pepper yellow leaf curl virus (Thailand), *Sweet potato leaf curl virus* (Taiwan), *Tobacco curly shoot virus* (Yunnan), ToLCV (Indonesia, Laos, Malaysia, Thailand, Taiwan, Vietnam), and TYLCV (Thailand) have been described in this region.

In Phase I of the TWFP, AVRDC (Dr. Peter Hanson and Sylvia Green, tomato breeder and plant virologist, respectively) investigated the genetic diversity of the vector *B. tabaci* in seven countries selected for this study. The invasive B biotype was already widely distributed in the Philippines, and was present in samples from South Korea and Taiwan. The remaining samples belonged to local *B. tabaci* biotypes. The use of microsatellite markers showed the presence of distinct *B. tabaci* groups on cultivated (cassava, tomato, and chili) vs noncultivated hosts in Indonesia. Some of the samples from Indonesia and Sri Lanka contained mixed populations of *B. tabaci* and *T. vaporariorum*.

Begomoviruses were detected in the eight countries selected, particularly in the main target crop (tomato), and in cucurbits and legumes. Five distinct begomoviruses were found to be affecting tomato in Indonesia, Malaysia, the Philippines, Sri Lanka, and Thailand. Some of these begomoviruses were similar to those begomoviruses isolated from squash in Indonesia and Thailand (Hanson and Green, 2002).

During Phase II, after 2 years of on-station and on-farm testing in North Vietnam, two fresh market tomato hybrids, FMTT847 and TLCV15, were identified as resistant to whitefly-transmitted begomoviruses under high geminivirus pressure in farmers' fields. These hybrids yielded 30–40% more than local begomovirus-susceptible cultivars. Parental lines of the hybrids were delivered to the national program (Research Institute of Fruits and Vegetables: RIFAV) to enable seed production of the hybrid for farmers. AVRDC will make these two hybrids available to researchers in other countries for evaluation and possible release.

The characterization of begomoviruses from North and South Vietnam showed that there were at least two distinct tomato begomoviruses in Vietnam. One virus found in the north, named *Tomato leaf curl virus* Vietnam 1 (ToLCV-VN1), is a monopartite virus, and a second virus

found only in the south, called *Tomato yellow leaf curl virus* Vietnam 2 (TYLCV-VN2), is a bipartite virus. These two viruses showed only 70% DNA sequence homology in their DNA-A. Thus, FMTT847 and TLCV15 are resistant to the begomovirus in the north but susceptible to TYLCV-VN2. This suggests that these two distinct viruses are confined to certain areas in the country. Eventually however, the lack of physical barriers between North and South Vietnam that might prevent long distance movement of viruliferous whiteflies might bring these two viruses together. RIFAV desires to arrange for large-scale seed production of the two hybrids so farmers can access the seed. Parental lines of the hybrids were developed by AVRDC and small seed quantities of the parental lines have been given to RIFAV.

AVRDC also signed a subcontract with the University of Agricultural Sciences Bangalore (UASB) on November 19, 2005, as a continuation of the Crop Protection Programme (CPP) project of Drs. V. Muniyappa (UASB) and J. Colvin (NRI, UK), but within the TWFP also financed by DFID. The previous CPP project developed sustainable technologies and management practices for one of the most serious pest and disease problems of tomato in India: tomato leaf curl. Three tomato leaf curl-resistant tomato varieties, TLB111, TLB130, and TLB182, were bred in collaboration with AVRDC and evaluated extensively in on-station and participatory on-farm trials in Karnataka state, India. Yield of one of the varieties, TLB182, was not reduced significantly when 14-day-old seedlings were inoculated rigorously with ToLCV. The profit made by tomato farmers in India has been increased up to 10 times following the adoption of the virus-resistant tomato varieties promoted by the CPP project led by Dr. John Colvin with the collaboration of AVRDC and the University of Agricultural Sciences of Bangalore. The promotion of these varieties has continued this year as a joint effort of the CPP and TWFP.

Currently, new open-polinated (OP) lines are being assessed for yield, resistance, and improved horticultural characters in India. Six of the 18 tomato lines provided by AVRDC to UASB were selected for further field evaluation during the Rabi season (Nov 2005–Feb 2006). TLB-407-1 and TLB-409-1 produced round- to oblate-shaped fruits; TLB-401-1 and TLB-414-1 had oblong fruit. TLB-416-1 had very firm, square round, deep red fruit with a light green shoulder and an average weight of 111 g. Infected plants were observed in all entries, although frequency was much lower than the susceptible check. Seeds were harvested from individual, symptomless plants in selected entries TLB-409-1 (two plants), TLB-414-1 (one plant), and TLB-416-1 (one plant), for further evaluation in summer 2006.

AVRDC has selected a set of 10 tomato inbred lines resistant to begomoviruses and other diseases such as bacterial wilt (*Ralstonia solanacearum*) with good fruit set and quality. Seed will be made available to Indian seed companies and institutions for multilocation testing and the international community, subject to the conditions of the AVRDC Material Transfer Agreement.

F. Whiteflies as Pests of Cassava in South America

Approximately 11 species of whiteflies have been reported as direct-feeding pests of cassava production in Latin America. However, the main whitefly species causing yield losses in the northern region of South America (Colombia, Venezuela, and Ecuador) and certain areas of Central America is *Aleurotrachelus socialis.* On experimental fields, infestations of 6 months resulted in a 42% yield reduction, and of 11 months in a 79% reduction (Vargas and Bellotti, 1981). In recent years, the populations of *A. socialis* have increased dramatically in certain cassava growing regions of Colombia, particularly in the Departments of Cauca, Valle del Cauca, Quindio, Risaralda, and Caldas. In some of these regions, whitefly populations used to be relatively low and not suspected of causing yield losses up to about 3 years ago. The rapid increase in whitefly populations of *A. socialis* and *T. variabilis* are presently causing severe crop damage. The reason for these outbreaks is not fully understood. *Aleurotrachelus socialis* is more typically associated with lower altitudes and warmer temperatures as found in the Tolima Valley, where the pest has been endemic for many years. A study conducted by the TWFP has shown that the expansion of cassava varieties such as CMC-40 and Chirosa has led to rapid populational increases of *A. socialis.* The shorter development time and the high rate of survival of *A. socialis* on Chirosa (MCol 2066) explain the high whitefly populations found on this genotype.

High levels of resistance to the neotropical whitefly pest, *A. socialis*, have been identified in cassava genotypes from South America (Arias *et al.*, 2004; Gomez and Bellotti, 2003). Considering that whitefly resistance in cultivated crops is rare, research supported by this project aimed to allow whitefly-resistant germplasm from the neotropics to be evaluated for resistance to African and Indian cassava whiteflies. To this end, four cassava genotypes from CIAT, Col 1468, MCol 2063, CG 489-34, and MEcu 72 with various degrees of resistance to South American

whitefly species, were sent by CIAT to the Natural Resources Institute, UK. Three whitefly colonies, *B. tabaci* from Namulonge (Uganda), *B. afer* from Entebbe (Uganda), and *B. tabaci* from Trivandrum (India), were collected and established on cassava in the insectary at NRI, UK. Eighteen days after the release of the African cassava *B. tabaci* onto the cassava genotypes, there were no significant differences between the populations and it was evident that all of the genotypes could be colonized. The variety with the greatest level of resistance, however, was MEcu 72. Thirty-eight days after the African cassava *B. tabaci* were released, MEcu 72 still showed the highest level of resistance and the difference was now significant (>65%).

The level of *B. afer* resistance in the varieties MEcu 72, Col 1468, and CG 489-34 did not differ significantly, as the mean populations on these varieties were similar within the time periods of 18 and 38 days after colonization. MCol 2063, however, had a significantly higher mean number of eggs and nymphs in both generations and, by the second generation, it had over four times the mean population present on Col 1468. Development of *B. afer* was also significantly faster on MCol 2063, as the number of "pupae" present was approximately five times higher on this variety (35.3 pupae) than on Col 1468 (6.2 pupae), 38 days after colony initiation.

The three whitefly populations oviposited successfully on the four cassava varieties. For the African *B. tabaci*, there was a significant cassava genotype effect and the fewest eggs were produced on MEcu 72. For the other two populations, there was no apparent cassava genotype effect. Overall, African *B. tabaci* oviposited the fewest eggs and Indian *B. tabaci* oviposited the greatest numbers of eggs.

In a separate study, an African cassava genotype, Nase 9, showed a high level of resistance to *B. tabaci* in the field (Omongo, 2003), and was found to have a similar level of resistance to MEcu 72 under laboratory conditions using African *B. tabaci*. However, MEcu 72 clearly showed the highest level of resistance to whiteflies from Africa and India, and it is consequently a promising parental material to reduce the impact of *B. tabaci* as a pest and virus vector. In Africa, *B. afer* has been reported to cause losses to cassava, and preliminary data suggests that it is the vector of cassava brown streak (Maruthi *et al*., 2004). Differences in the acceptability of cassava varieties to whiteflies have been recognized for some time (Maruthi *et al*., 2001; Nair and Daniel, 1983), which suggests that host-plant resistance may provide an alternative solution to the whitefly pest and virus problems of cassava.

III. Conclusions

The whitefly species *B. tabaci* is undoubtedly one of the most important insect pests the world of agriculture has ever experienced. The original biotypes of this species gained notoriety as vectors of economically important viruses such as ACMV in East Africa, and BGMV in South America. The intensive use of pesticides used to control other insect pests after World War II elevated *B. tabaci* to a pest status in crops such as cotton. The emergence of a new biotype B in the 1990s further complicated the management of a whitefly pest that had already developed resistance to most of the insecticides used in attempts to control the original biotypes. The new biotype proved to be more polyphagous, fertile, and adaptable to a wider range of climatic conditions and crops. This development brought as a consequence a notable increase in the number of begomoviruses capable of infecting both traditional and nontraditional food and industrial crops in Latin America, Africa, and Asia.

In Latin America, *B. tabaci* has benefited from the diversification of crops and the intensive use of insecticides that eliminate the natural enemies of *B. tabaci*, associated with the production of high-value crops, such as tomato, peppers, and melons. In sub-Saharan Africa, different viruses capable of inducing cassava mosaic disease have arisen as a result of the movement and exchange of infected cassava planting material (cuttings) and continuous use of ACMD-susceptible cassava varieties. In East Africa, the diversification of crops has created begomovirus problems in horticultural crops, similar to those described above for Latin America. In India, ACMD and many other diseases of horticultural crops are caused by different begomoviruses transmitted by *B. tabaci*. Tomato leaf curl and tomato yellow leaf curl are the most important diseases affecting tomato production in Asia.

Regarding the management of these whitefly-associated problems, the TWFP has confirmed the fact that genetic resistance is the most effective and environment friendly method of control for whitefly pests (as in the case of whitefly-resistant cassava in South America), and whitefly vectors of plant viruses (as in the case of the begomovirus-resistant common bean, cassava, and tomato varieties developed in Latin America, Africa, and Asia, respectively). However, improved varieties must meet the various agronomic and quality criteria expected by farmers and markets for a commercial variety to be adopted. Otherwise, farmers will continue to grow susceptible cultivars, which will perpetuate and further aggravate whitefly and begomovirus problems in the near future. Unfortunately, traditional plant breeders are being rapidly

replaced by molecular plant breeders that are usually more interested in elucidating the genomic nature of these virus/pest problems than in solving them in the field. Molecular marker-assisted plant breeding may expedite the selection of plant genotypes possessing certain genes of interest, but often fails to fulfill the expectations of farmers and consumers in general.

Pesticide abuse is the second most important factor driving whitefly and begomovirus epidemics. This undesirable practice is the consequence of the lack of technical assistance to farmers, induced by the substantial reduction of funds available for this purpose and crop production-oriented research in general, as a result of the precarious economic situation of most developing countries and the preferential funding of environmental and social research by international aid agencies. Pesticide abuse increases crop production costs (and thus hunger and poverty), environmental contamination, and human health problems in urban and rural communities exposed to highly noxious pesticides both in the environment and the food products they consume on a daily basis. As long as these negative conditions persist, "integrated pest management" will remain an interesting and potentially useful approach to control whitefly pests and whitefly-transmitted viruses, but nothing else. Biological control agents are not effective in highly disturbed agroecosystems, and most other IPM practices fail to prevent the significant damage inflicted by high whitefly populations in the absence of resistant cultivars and a rational use of chemical and cultural pest and disease control practices. Fortunately, in view of the severe damage caused by these pests, some Governments are adopting legal measures that are also highly complementary to the implementation of effective and sustainable IPM practices.

The socioeconomic surveys conducted by the TWFP in Phase I clearly demonstrate that most farmers are not informed about the nature of these phytosanitary problems. Under these circumstances, it is very difficult to make recommendations to farmers on integrated pest management practices that require a complex analysis of the different factors that contribute to the occurrence of whitefly-related crop production problems. Farmer participatory research is an effective mechanism to educate farmers on the nature and management of these pests, but given the global extent of the whitefly problem and limited resources available, these kinds of participatory activities are not possible to scale out at the moment. As a complementary approach, the TWFP is currently producing a considerable number of educational and training materials written or produced for professionals, technicians, and farmers on

the most effective and sustainable methods for whitefly and virus management available to date. The TWFP is confident that the substantial amount of information collected in the last decade by the project should make a substantial contribution to alleviating the crop losses inflicted by whitefly pests and whitefly-borne viruses in developing countries, and thus contribute to improving the livelihood of resource-poor farmers in the Tropics.

Acnowledgments

The information contained in this chapter has been compiled by the author based on research conducted for the TWFP by subproject leaders of the participating international agricultural research centers; and collaborating national agricultural research institutions, universities, and advanced research institutions. The TWFP has been mainly supported by the DFID of the United Kingdom, but the project is also indebted to the DANIDA, the USDA and USAID, the ACIAR; and the New Zealand Agency for International Development (NZAID) for financial support of specific activities conducted during phases II and III of the TWFP. Special thanks are also due to the support provided by the participating Internacional centers: CIAT, the IITA, the CIP, the AVRDC, the ICIPE, the National Resources Institute (NRI-UK), the Systemwide IPM Programme, and the many professionals and workers who have made possible the implementation and development of the TWFP in the past decade.

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