The RNA-dependent RNA polymerases of different members of the family Flaviviridae exhibit similar properties in vitro

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The virus-encoded RNA-dependent RNA polymerase (RdRp), which is required for replication of the positive-strand RNA genome, is a key enzyme of members of the virus family Flaviviridae. By using heterologously expressed proteins, we demonstrate that the 77 kDa NS5B protein of two pestiviruses, bovine viral diarrhoea virus and classical swine fever virus, and the 100 kDa NS5 protein of the West Nile flavivirus possess RdRp activity in vitro. As originally shown for the RdRp of hepatitis C virus, RNA synthesis catalysed by the pestivirus and flavivirus enzymes is strictly primer-dependent in vitro. Accordingly, initiation of RNA polymerization on homopolymeric RNAs and heteropolymeric templates, the latter with a blocked 3'-hydroxyl group, was found to be dependent on the presence of complementary oligonucleotide primer molecules. On unblocked heteropolymeric templates, including authentic viral RNAs, the RdRps were shown to initiate RNA synthesis via intramolecular priming at the 3'-hydroxyl group of the template and ‘copy-back’ transcription, thus yielding RNase-resistant hairpin molecules. Taken together, the RdRps of different members of the Flaviviridae were demonstrated to exhibit a common reactivity profile in vitro, typical of nucleic acid-polymerizing enzymes.

Introduction

The virus family Flaviviridae comprises the genera Flavivirus, Pestivirus and Hepacivirus (hepatitis C virus [HCV] and recent HCV-related isolates) and includes numerous important human and animal pathogens (reviewed in Francki et al., 1991; Houghton, 1996; Monath & Heinz, 1996; Thiel et al., 1996). The small, enveloped virions of the different members of the Flaviviridae contain a single-stranded, positive-strand RNA genome of about 9.5–12.5 kb. The genome consists of a single, long open reading frame (ORF), which is flanked by untranslated regions (UTRs) at the 5' and 3' ends. Whereas the 5' terminus of the flavivirus genome is capped by a type I cap structure, initiation of translation of the pestivirus and HCV genomes relies on an internal ribosomal entry site (IRES) preceding the ORF (reviewed by Rice, 1996). Translation of the ORF leads to synthesis of a single polyprotein that is both co- and post-translationally processed by cellular and viral proteases, thus giving rise to a number of structural and nonstructural (NS) viral polypeptides (Rice, 1996; see also Fig. 1).

Concomitant with the translation process, replication of the virus genome takes place in the cytoplasm of the infected cell. Initially, full-length complementary negative-strand RNA molecules are synthesized, which then serve as templates for the transcription of progeny positive-strand RNA genomes (Gong et al., 1996; Westaway, 1987). Recent studies on subgenomic pestivirus and flavivirus RNA replicons have revealed that the NS proteins, which are encoded by the C-terminal part of the polyprotein (see Fig. 1), play a crucial role during catalysis of the diverse steps that underlie RNA replication (Behrens et al., 1998; Khromykh & Westaway, 1997). Accordingly, these viral proteins are assumed to form replication complexes in conjunction with the genomic RNA and hypothetical host cellular factors.

Studies in vitro based on heterologously expressed proteins have yielded much data concerning the functions of certain NS proteins. The viral protease complex responsible for most of the cleavages that generate the mature NS proteins (Fig. 1) was shown to consist of NS3 and NS2B or of NS3 and NS4A for flaviviruses and pestiviruses/HCV, respectively (reviewed in...
and classical swine fever virus (CSFV) strain Alfort (aa 3181–3898; Meyers et al., 1996) and the NS5 protein from West Nile virus (WNV) (aa 2559–3462; Castle et al., 1986) in insect cells.

DNA fragments were obtained by PCR from cDNA constructs of BVDV, CSFV and WNV that were kindly provided by N. Tautz (Giessen), G. Meyers (Tübingen) and G. Wengler (Giessen), respectively. To allow initiation of transcription, additional ATG codons were engineered upstream of the coding sequences of each DNA fragment by using oligonucleotide primers. Translation termination occurred at the termination codons of the virus polyproteins (Fig. 1). Virus genes were cloned initially into pGEM-T (Promega) and, after verifying the nucleotide sequences, they were subsequently cloned into pBlueBac 4.5 (Invitrogen). The restriction sites used are indicated in Fig. 1.

Generation and selection of the recombinant baculoviruses BacB5B (expressing BVDV NS5B), BacC5B (CSFV NS5B) and BacW5 (WNV NS5) were performed in Sf21 cells by using the Invitrogen recombination kit as recommended by the manufacturer.

![Diagram of recombinant baculoviruses](https://via.placeholder.com/150)

**Fig. 1.** Schematic drawing of the recombinant baculovirus constructs used in this study. The upper part of each diagram reflects the organization of the coding region of the ORFs of flaviviruses, pestiviruses and HCV. Individual polypeptides are depicted as differently shaded regions. The lower part of each diagram shows the regions cloned in this study. PCR products were cloned by using the indicated restriction sites. Pph, Polyhedrin promoter.

### Methods

**Construction of recombinant baculoviruses expressing flavivirus polypeptides.** Recombinant baculoviruses were constructed that, by analogy with the heterologous expression of HCV NS5B described previously (Behrens et al., 1996) (referred to here as BacH5B), should express enzymatically active NS5B/NS5 polypeptides. Baculoviruses were constructed to express NS5B polypeptides from BVDV strain CP7 (amino acids 3189–3907; numbering from Tautz et al., 1996) and classical swine fever virus (CSFV) strain Alfort (aa 3181–3898; Meyers et al., 1996) and the NS5 protein from West Nile virus (WNV) (aa 2559–3462; Castle et al., 1986) in insect cells.

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**Authenticity of expressed polypeptides.** The authenticity of the polypeptides was verified by SDS–PAGE and Western blotting of total protein from cytoplasmic extracts as described by Mondelli et al. (1994). Antibodies used to detect the various polypeptides were an anti-WNV NS5 antibody (kindly provided by G. Wengler, Giessen), a rabbit antiserum directed against the C terminus of the CSFV polyprotein (aa 3520–3761) (kindly provided by R. Stark, Giessen) and an anti-HCV NS5B antiserum (Tomei et al., 1993).

**Templates for RdRp assay.** Templates for assay of RdRp activity (as outlined in Fig. 3) were generated as follows. D-RNA represents the mRNA of the liver-specific transcription factor DCoH and was obtained by transcription from BglIII-linearized plasmid pT7DCoH (Behrens et al., 1996). The IRES RNA was obtained by in vitro transcription of EcoRI-linearized plasmid pCITE2A (Novagen). The BVDV 3'-end RNA was a transcript comprising the C terminus of the BVDV CP7 ORF and the authentic 3' UTR (Yu et al., 1999). The BVDV replicon RNA was obtained from plasmid pA/BVDV/D9 as described previously (Behrens et al., 1998). The BVDV minigenome RNA was transcribed from a pA/BVDV/D9 derivative, in which the BstEII site at position 421 had been fused to the EcoRV site at position 11888.

**Assay for RdRp activity.** Extracts were prepared in a lysis buffer containing 1% NP-40 to keep proteins in solution. The RdRp assay was performed as described previously (Behrens et al., 1996; De Francesco et al., 1996). Briefly, the reaction consisted of a suitable buffer, cytoplasmic extract, the four NTPs (including a single radiolabelled NTP, usually [α-32P]GTP), in vitro-transcribed template RNA and actinomycin D to inhibit DNA-dependent RNA synthesis. After incubation at 20 °C for 2 h, nucleic acid was extracted and the incorporation of radioactivity was monitored and analysed either by electrophoresis on 5% Tris–borate acrylamide gels containing 7 M urea or by binding to DE81 filters (Schleier & Schull).

**Micrococcal nuclease (MN) treatment of cytoplasmic extracts.** Potential nucleic acid primers were removed from cytoplasmic extracts of baculovirus-infected Sf21 cells by treatment with 10 U MN Plus (New England BioLabs) and 10 U Micrococcal nuclease (MN) (New England BioLabs) at 30 °C for 2 h in 1× Transcription Buffer (New England BioLabs). The RNA products were then precipitated using 2 volumes of ethanol and rinsed with 70% ethanol (v/v) and resuspended in 5 µl of DEPC-treated water.

**Results**

The organization of the viral genome, in particular the region encoding NS3 to NS5, is virtually colinear in pestiviruses and HCV. Accordingly, the C-terminal part of the...
polyprotein, the NS5 region, is processed by the NS3/NS4A protease complex to give rise to two viral polypeptides, namely NS5A and NS5B (Rice, 1996). In contrast, the NS5 region of the flavivirus ORF yields a single protein (Fig. 1). Predictions based on the analysis of amino acid sequences (Argos, 1988; Miller & Purcell, 1990) and previous experimental evidence suggested that RdRp activity should be a function of HCV NS5B, BVDV NS5B and dengue virus NS5 proteins (Behrens et al., 1996; Zhong et al., 1998; Tan et al., 1996).

To allow a comparative analysis of the RdRps encoded by representatives of each of the three genera of *Flaviviridae*, recombinant baculoviruses were constructed that should be capable of producing enzymatically active NS5B polypeptides from BVDV strain CP7 and CSFV strain Alfort and NS5 from WNV in insect cells.

DNA fragments were obtained by PCR. Additional ATG codons were engineered upstream of the respective coding sequences of each DNA fragment; translation termination occurred at the termination codons of the viral polyproteins (Fig. 1). A certain quantity of the recombinant proteins was expected to differ from the authentic viral polypeptides because of the presence of an additional methionine residue at the N terminus. However, as a result of the activity of cellular methionine aminopeptidases, which remove N-terminal methionine residues co-translationally (Tsunasawa et al., 1985) when they are adjacent to small amino acids such as glycine (WNV) or serine (BVDV, CSFV and HCV), a considerable amount of heterologously expressed protein was expected to contain the authentic N terminus.

As shown in Fig. 2, infection of SF21 cells with the different recombinant baculoviruses led to the expression of each of the viral polypeptides. The authenticity of the expressed proteins was verified by SDS–PAGE and Western blotting. As expected, bands corresponding to proteins of 65 kDa (HCV NS5B), 77 kDa (BVDV NS5B, CSFV NS5B) and 100 kDa (WNV NS5) were detected specifically by antisera that had previously been raised against the respective proteins.

To test the heterologously expressed viral proteins for RdRp activity, cytoplasmic extracts were prepared from the infected SF21 cells. The assay, which is based on the detection of incorporated labelled nucleotides, was carried out by following a protocol used successfully during characterization of the HCV-encoded NS5B RdRp (Behrens et al., 1996; De Francesco et al., 1996).

Initially, we employed a non-viral 399 nucleotide mRNA (D-RNA) as the template in the reaction (Fig. 3) because this RNA was observed previously to be used efficiently as a substrate by HCV RdRp (Behrens et al., 1996). As shown in Fig. 4, all experiments including the negative control, the latter carried out with cytoplasmic extract of either uninfected or wild-type baculovirus-infected SF21 cells, revealed labelling of the input template RNA (as determined by silver staining side by side with the input RNA transcript; data not shown). This observation is in accordance with previous data (Behrens et al., 1996) and was explained by the activity of cellular terminal nucleotidyl transferases present in the cytoplasmic extracts of SF21 cells. Accordingly, the addition of a small quantity of a single-strand-specific ribonuclease such as RNase T1 (5 U in 40 µl reaction volume) to the assay mixture in the presence of 500 mM NaCl (high ionic strength) led to the disappearance of
Assay of RdRps on heteropolymeric templates. Cytoplasmic extracts of Sf21 cells that had been infected with the recombinant baculovirus constructs served as the sources of RdRp. Extract and template were incubated in the presence of nucleotides. The resulting RNA products were extracted and separated by electrophoresis. The results shown summarize the results of four independent experiments. RNA products are shown schematically to the right of the autoradiograms; dark-shaded bars indicate the positive-strand RNA template and light-shaded bars indicate newly synthesized negative-strand RNA. (a) RdRp assays using D-RNA as a template. Lanes: 1, RNA products derived from an assay with cytoplasmic extract of BacB5B-infected cells (BVDV NS5B); 2, as in lane 1 but the products were treated under high-salt conditions with a low concentration of RNase T1; 3–10, as in lanes 1 and 2, but with extracts of BacCS5B-infected cells (lanes 3, 4), BacHS5B-infected cells (5, 6), BacWS5-infected cells (7, 8) or non-infected cells (negative control) (9, 10). Lanes 11–13 show results of RdRp assays with RNA templates and BVDV 3′-end RNA. Lanes 1–5 show results of RdRp assays with D-RNA and the following extracts: BVDV NS5B (lane 1); CSFV NS5B (2); HCV NS5B (3); WNV NS5 (4); and non-infected cells (5). Lanes 6–10 show results of experiments identical to those shown in lanes 1–5, but the RNA products were treated with a high concentration of RNase A and RNase T1 under high-salt conditions. Lane 11, RdRp assay with BVDV NS5B and BVDV 3′-end RNA; 12, as lane 11 but RNA products were treated with a high concentration of RNase A and RNase T1 under high-salt conditions; 13, as lane 11 but carried out with 3′-oxidized BVDV 3′-end RNA.

This peculiar properties have been shown previously (Behrens et al., 1996) to be due to the unusual features of the reaction product; it concerns a duplex RNA ‘hairpin’ molecule consisting of the sense (template) strand, to which an antisense strand of similar length has been attached covalently (Fig. 4b). The duplex nature of each of the products obtained was confirmed by electrophoresis under highly denaturing conditions (Behrens et al., 1996; data not shown). Such a structure, which is caused by intramolecular priming of RNA polymerization at the 3′-hydroxyl group of the input template (see below) and synthesis of a complementary RNA strand by a turn or ‘copy-back’ mechanism, explains the unusual electrophoretic mobility as well as the limited susceptibility to nucleases. Since at high ionic strength, which favours the annealing of complementary double-stranded RNA structures, only the turn-around loop ought to be accessible to nucleases (Fig. 4b), hydrolysis of the covalent link between sense and antisense strand generates a double-stranded molecule that, in contrast to the original hairpin molecule, is also denatured in 7 M urea under normal denaturing electrophoresis conditions. The radioactively labelled band that remains detectable after RNase treatment consequently corresponds to a newly synthesized antisense strand that has the same length as the original template (Fig. 4b, lanes 6–10).

A D-RNA hairpin product was obtained with cytoplasmic extracts containing NS5B from BVDV, CSFV and HCV and NS5 from WNV, and RNA synthesis was found to be dependent on the addition of an external RNA substrate, ribonucleotides and magnesium ions (not shown). We therefore concluded that NS5B from BVDV and CSFV and NS5 from WNV exhibited essentially the same activity as HCV NS5B; in other words, they functioned as RdRps.

Generation of dimer-sized RNA hairpin molecules via initiation of RNA polymerization at the 3′-hydroxyl group of the template implies intramolecular priming, which is probably enabled by RNA structures near the 3′ terminus of the RNA. This indicates that initiation of RNA polymerization, as catalysed by each of the different Flaviviridae RdRps, represents...
a primer-dependent mechanism. To address this in more detail, we next tested the different polymerases on a poly(C) template, i.e. a homopolymeric RNA that should be incapable of forming internal structures. In order to deplete the assay mixture of nucleic acid molecules that might conceivably serve as unspecific primers, the cytoplasmic extracts of baculovirus-infected Sf21 cells were treated with MN before determining RdRp activity. After inactivation of the MN, the RdRp assay was carried out either in the absence or presence of an oligo(dG)$_{18}$ primer. Incorporation of [³²P]GTP was monitored by filter-binding. As shown in Fig. 5, experiments with the different RdRps revealed that, in all cases, RNA polymerization occurred exclusively in the presence of oligodeoxynucleotide primer molecules. These data thus show that the RNA-dependent RNA polymerization process of the different Flaviviridae RdRps is strictly primer-dependent. Comparable results were obtained with heteropolymeric substrates such as D-RNA that had previously been 3'-oxidized (oxidation was carried out with sodium metaperiodate, as described by Behrens et al., 1996). In this case, RNA polymerization was detectable only in the presence of the respective antisense oligodeoxynucleotide primers (data not shown).

Thus far, we had demonstrated that the RNA polymerization activity of each of the different Flaviviridae RdRps was a primer-dependent reaction when acting on unspecific RNA templates. Next, we were interested to test the respective enzymes with RNAs that, for example, contained the authentic virus genomic 3' end, i.e. the presumed specific starting point (‘minus-strand promoter’) of the RNA replication pathway (see above, Fig. 3). As shown for an experiment with BVDV NS5B on the BVDV 3'-end RNA (Fig. 3), this assay gave rise to a pattern of radioactively labelled bands that differed significantly from that obtained with the D-RNA transcript. In this case, not only a single product but a series of RNA molecules both shorter and longer than the input template were detected (Fig. 4b, lane 11). After RNase treatment at high ionic strength, the pattern changed, yielding another series of differently migrating RNA fragments (Fig. 4b, lane 12). In line with experimental data obtained with other positive-strand RNA virus systems, specific initiation of RNA replication is suspected to occur by priming mechanisms that are different from initiation of RNA polymerization on the 3'-hydroxyl group of the template (see below). To test whether the observed pattern of products might be explained by events different from intramolecular priming, the experiment was repeated with the same template containing an oxidized 3'-hydroxyl group (see above). As shown in Fig. 4(b) (lane 13), oxidation of the viral RNA transcript resulted in a complete blockage of the RNA polymerization process. Congruent data were obtained if the BVDV RdRp was tested either on RNA transcripts containing the 3' as well as the 5' UTR (BVDV minigenome) or with templates corresponding to replication-competent subgenomic-ribozyme BVDV D19c RNA (Fig. 3 and data not shown; see also Zhong et al., 1998). Interestingly, a nearly identical product profile was obtained if the RdRp assay was performed on an RNA template encoding the IRES of encephalomyocarditis virus (data not shown). The HCV and WNV RdRps, if tested on the respective viral RNAs or IRES-containing templates, also yielded similar reaction profiles (not shown; see also Lohmann et al., 1997).

Taken together, we conclude that intramolecular priming represents the preferred mechanism for initiation of RNA polymerization by the different Flaviviridae polymerases on heteropolymeric RNA templates in vitro. ‘Template priming and copy-back’ apparently takes place irrespective of whether or not the templates contain cis-encoded signals that should be essential for specific initiation of the virus replication process. Like IRES-encoding transcripts, the viral RNAs, in particular the UTRs, contain numerous pronounced RNA structural elements (Blight & Rice, 1997; Brinton et al., 1986; Wengler & Castle, 1986; Yu et al., 1999). We therefore reasoned that the large number of different RNase-resistant products that were detected during experiments employing the viral RNAs as templates were caused by a significantly lower ability of the enzyme to perform a continuous (processive) polymerization reaction like that carried out on other, less structured, heteropolymeric templates such as the D-RNA. Most likely, RNA polymerization on highly structured templates initially yields RNA hairpin molecules containing antisense strands of variable lengths. These may then be recognized again by the RdRp, which may perform further polymerization cycles, thus leading to a broad spectrum of different RNase-resistant products.

**Discussion**

The aim of this study was to compare the properties of the RdRps of different members of the virus family Flaviviridae. According to previous studies (Behrens et al., 1996; Tan et al.,
1996; Zhong et al., 1998), the proteins NS5B of the pestiviruses BVDV and CSFV and NS5 of the flavivirus WNV were suspected to represent the RdRp of these viruses. Hence, we decided to express the respective viral genes in insect cells by means of recombinant baculoviruses. Heterologous expression of the respective polypeptides appeared to be appropriate for two reasons. (i) An efficient in vitro infection system for HCV is still lacking; accordingly, our entire knowledge of HCV-encoded enzymatic activities obtained so far has been derived from experiments based on the expression of parts or the entire ORF (reviewed by Houghton, 1996). In order to allow direct comparison of the HCV RdRp with the pestivirus and flavivirus enzymes, we chose identical procedures for producing the protein and assaying it for activity. (ii) Surprisingly, RdRp assays performed on cytoplasmic extracts of cells that had been either infected with BVDV or transfected with entire genomic or subgenomic BVDV replicon cRNA (Behrens et al., 1996; Fig. 3) failed to detect a significant RNA polymerization activity, even if the homologous viral RNA was used as a template (data not shown). This may be explained by a rather low level of expression or stability of the NS5B protein in comparison to other viral proteins (e.g. NS3), as observed by Western blotting of cellular extracts after infection or transfection (data not shown). Alternatively, partially or fully assembled replication complexes, which are supposedly present in the extracts as membrane-associated subcellular compartments (Bienz et al., 1992), may be largely inaccessible to externally added RNA templates. Studies to examine this aspect further are currently in progress.

As shown in Figs 4 and 5, RNA synthesis as catalysed by each of the different viral proteins expressed was easily detectable by employing an assay system that had been established initially for the HCV RdRp (Behrens et al., 1996; De Francesco et al., 1996). Interestingly, the optimal conditions determined previously for the in vitro reaction (e.g. concentration of magnesium ions, reaction temperature etc.) were found to be perfectly suitable to assay the pestivirus and flavivirus RdRps. Differences in RdRp activity, as observed for example between the BVDV NS5B and WNV NS5 proteins, correlated directly with the level of expression of the viral proteins in insect cells. Hence, in agreement with recent data (M. Collett & J. B. Flanegan, personal communication; Zhong et al., 1998), these experiments demonstrate clearly that the product of the NS5B gene of the pestiviruses BVDV and CSFV represents an RdRp. Furthermore, our data on the WNV NS5 confirm and extend previous studies in vitro on the dengue virus RdRp (Tan et al., 1996).

As with other virus RdRps studied in vitro (Neufeld et al., 1991; Sankar & Porter, 1991), the activities of the RdRps of the members of the Flaviviridae examined were found not to be restricted to the authentic viral template and, furthermore, were shown to be primer-dependent. In the case of heteropolymeric templates, the formation of stable structures by annealing nucleotides at or near the 3’ end of the RNA apparently allowed intramolecular priming at the 3’-hydroxyl group. Priming was then followed by ‘copy-back’ transcription of a complementary RNA strand, thus giving rise to dimer-sized, hairpin-like RNA molecules (Behrens et al., 1996; Fig. 4). On homopolymeric templates and 3’-terminally blocked heteropolymeric templates, RNA synthesis was consistently found to be dependent on the presence of RNA (not shown) or DNA primer molecules complementary to the template (Fig. 5). In contrast to previous experiments with unfractionated cytoplasmic extracts of baculovirus-infected SF9 cells (Behrens et al., 1996), annealed primer-template hybrids apparently remained rather stable within the MN-treated cytoplasmic extracts of SF21 cells. This may be explained by a significantly lower activity of cellular RNA helicases under the chosen experimental conditions, and turned out to be advantageous, since it enabled the measurement of primer-dependent RNA synthesis without prior purification of the viral protein. Obviously, the experimental test system chosen did not permit the additional testing of the flavivirus and pestivirus RdRps for a co-fractionating terminal nucleotidyl transferase activity, as described for the purified HCV NS5B (Behrens et al., 1996) and BVDV NS5B (Zhong et al., 1998).

Priming at the 3’-hydroxyl group of RNA templates appears to be a common property of RNA-polymerizing enzymes in vitro (Cazenave & Uhlenbeck, 1994; Konarska & Sharp, 1989). However, hairpin-like molecules are also formed in vivo during virus infection, as observed in the cases of poliovirus and encephalomyocarditis virus (Senkevich et al., 1980; Young et al., 1985). In view of these observations and the fact that data from cell culture-based RdRp assays are either not available or are difficult to compare with the conditions in vitro (Chu & Westaway, 1985; Grun & Brinton, 1986), it is not currently possible to decide whether intramolecular priming reflects the situation in vivo. If it does, one would have to postulate a ribonuclease that cleaves specifically RNA hairpins derived from the homologous viral genome. Those cases of positive-strand RNA viruses where specific initiation of RNA synthesis has been detected in vitro have all involved the presence of other virus- and/or host cell-encoded proteins besides the viral RdRp (Ball, 1995; Blumenthal & Carmichael, 1979; Hayes & Buck, 1990; Lemm et al., 1998; Wu et al., 1992). Thus, it is tempting to assume that the RdRps of members of the Flaviviridae are necessary but not sufficient to catalyse the initial step of the replication pathway. It will be important in the future to search for factors that either mediate the specificity of the virus RNA polymerization pathway as such or modulate the RdRp in a way to generate specificity for the viral template. The observation that the polymerases are significantly less processive on highly structured templates such as the natural viral RNAs may point in this direction, suggesting, for instance, an important functional role for the viral helicase, which, at least in the case of flaviviruses, was found to be associated with the NS5 RdRp (Chen et al., 1997; Kapoor et al., 1995). Future experiments will focus on such virus protein–protein inter-
actions for pestiviruses and HCV and on the reconstitution of a specific in vitro priming system, similar to that recently developed for the RdRp of poliovirus (Paul et al., 1998).

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