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The 3′ Untranslated Region of Picornavirus RNA: Features Required for Efficient Genome Replication

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The role of the 3′ untranslated region (3′UTR) in the replication of enteroviruses has been studied with a series of mutants derived from either poliovirus type 3 (PV3) or a PV3 replicon containing the reporter gene chloramphenicol acetyltransferase. Replication was observed when the PV3 3′UTR was replaced with that of either coxsackie B4 virus, human rhinovirus 14 (HRV14), bovine enterovirus, or hepatitis A virus, despite the lack of sequence and secondary structure homology of the 3′UTRs of these viruses. The levels of replication observed for recombinants containing the 3′UTRs of hepatitis A virus and bovine enterovirus were lower than those for PV3 and the other recombinants. Extensive site-directed mutagenesis of the single stem-loop structure formed by the HRV14 3′UTR indicated the importance of (i) the loop sequence, (ii) the stability of the stem, and (iii) the location of the stem immediately upstream of the poly(A) tail. The role of a 4-bp motif at the base of the HRV14 stem, highly conserved among rhinoviruses, was examined by site-directed mutagenesis of individual base pairs. This analysis did not pinpoint a particular base pair as crucial for function. The requirement for immediate adjacent positioning of the open reading frame and the 3′UTR was examined by insertion of a 1.1-kb heterologous sequence. A replicon containing this insert replicated to about 30% of the level observed for the wild type. However, the corresponding virus consistently deleted most of the inserted fragment, suggesting that its presence was incompatible with a full replication cycle.

Poliovirus (PV), the type member of the *Picornaviridae*, has a single-stranded messenger-sense RNA genome of approximately 7,500 nucleotides (nt) composed of a 5′ untranslated region (5′UTR) of approximately 750 nt, a single open reading frame encoding a 220-kDa polyprotein, a 3′UTR of 72 nt, and a 3′-terminal poly(A) tract (13, 23, 28). The polyprotein is divided into three regions as a result of primary posttranslational proteolytic processing by virus-encoded proteases. The P1 region encodes the capsid proteins VP1 through VP4, the structures of which are known in atomic detail (10). The P2 and P3 regions encode proteins involved in protein maturation and RNA replication and include two proteases, a polymerase (13), and putative membrane-binding (7, 8) and nucleoside triphosphate-binding functions (29).

The 5′UTR contains two elements which appear to act independently: (i) a cloverleaf RNA secondary structure formed by the 5′-terminal 88 nt which is required for replication and forms a binding site for the viral proteins 3AB and 3CD and a host protein of 36 kDa, a cleavage product of elongation factor 1α (2, 3, 9, 21, 26), and (ii) a complex secondary structure, formed by nt 120 to 640, which directs translation by internal ribosome entry (17–20). In contrast to our knowledge of the 5′UTR, comparatively little is known of the role of the 3′UTR in viral replication; however, it is reasonable to assume that it plays an important role in virus replication, a view that is supported by a number of observations. Firstly, the primary and secondary structures of all three virulent and attenuated strains of PV are highly conserved. Moreover, certain motifs present in the PV 3′UTR are well conserved amongst the enteroviruses. Secondly, an 8-nt insertion at nt 7387 in poliovirus type 1 (PV1) which affects the loop region of the 5′-proximal stem-loop confers a temperature-sensitive phenotype (27) and a mutation in the 3′UTR has been implicated as a determinant of thermosensitivity (14a). Thirdly, recently presented biochemical evidence shows that the 3′UTR is a binding site for 3AB and 3CD of PV (9) and for $3D^{pol}$ of encephalomyocarditis virus (5). In addition, by UV cross-linking it has been shown that the human rhinovirus 14 (HRV14) and PV1 3′UTRs bind to a complex of host proteins with molecular masses of 34 to 38 kDa and that the latter is induced during viral replication (30).

In this report we have examined the role of the 3′UTR in replication by substitution of the PV3 3′UTR with that of other picornaviruses and by extensive site-directed mutagenesis (SDM) of the 3′UTRs of PV3 and HRV14. The influence of these alterations on replication has been monitored following substitution into a full-length PV3 genome and/or a PV3 replicon in which the N-terminal region of the capsid coding region has been replaced with the chloramphenicol acetyltransferase (CAT) reporter gene (21).

MATERIALS AND METHODS

Construction of plasmids. Leon.3′CB4 was constructed by modifying the P3/Leon (PV3) and coxsackie B4 virus (CB4) cDNA clones to contain an *Xba*I site spanning the translation termination codon. The 3′UTR of CB4 was then exchanged for the PV3 3′UTR by utilizing this site and the *Sal*I site located to the 3′ of the poly(A) tract. Virus was recovered by transfection of Hep2c cells with DNA. pREP.3′PV3/*Stu*I, pREP.3′PV3ΔX, and pREP.3′PV3ΔY were derived from pT7FLC/REP (21) by SDM of the *Xba*I-*Sal*I fragment (nt 6265 to the 3′ terminus) cloned into M13mp18 according to the method of Kunkel (14) with oligonucleotides 34-0001, 34-0014, and 34-0015, respectively (Table 1). To construct pREP.3′HRV14pre, nt 5342 to the 3′ terminus of HRV14 were amplified by PCR with a plus-sense primer which bound 5′ to the *Eco*RI site at position 7073 and a minus-sense primer, TOFF, which binds to the poly(A) tract and introduces a *Sal*I site at the 3′ end of the PCR product. The product was digested with *Eco*RI, and the overlapping termini were filled in with T4 DNA polymerase,

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence(s)	Function or description
34-0001	CGACTGAGGTAGGCCTACTAAAATGAG	To introduce *Stu*I site
34-0003	GCTTTGTTGCTCCCAGAGTACTC	Sequencing primer for 3′UTR
34-0012	CATCGGTCGACTTTTTTTTTTTTTTTTTTTTTTTTTGTTCCTACTAAAATGAGTCAAGCC	Deletion of HRV14 stem-loop
34-0014	CTCCGAATTAAAGAAAAATTACTAAAATGAGTCAAGCC	Deletion of 5′ stem-loop of PV3 3′UTR
34-0015	TTTTTTTTTTTTTTTTTTTTCCCTACAACAGTATGACCC	Deletion of 3′ stem-loop of PV3 3′UTR
34-0016	CTCCTACTTCTACTCCTATATTCTCACACTTTTGTTCCTACTAAAATGAG	REP.3′HRV14/dis2
34-0017	TTTTTTTTTTTTTTTTTTTTTGCAGTAGTCGAGCTGCTACTCGCAGCTCGACTACTGCTTGTTCCTACTAAAATGAG	REP.3′HRV14/syn
34-0018	TTTATAAACTCCTACTTC(A/G/C/)(G/C/T)(G/T/A)(A/G/C)(G/T/C)AAATTAAGTGTCTATATTG	HRV14 loop mutants
34-0019	TTTTTTTTTTTTTTTTTTTTTCCTACTTCTACTCAAATTAAGTTGTTCCTACTAAAATGAG	REP.3′HRV14/short
34-0020/21	TTTTTTTTTTTTTTTTTTTTTGGTACGTATAAACTCCTACTTCTA and CTCAAATTAAGTGTCTATACGTACCTTGTTCCTACTAAAATGAG	REP.3′HRV14/long
34-0022	TTTATAAACTCCTACTTCTATCAAAATTAAGTGTCTATATTG	To introduce the RV2 loop sequence into HRV14
34-0024	TTTTTTTTTTTTTATAAACT(C/A)CTCCTTCTACTCAAAT(T/G)AATTGTCTATATTGTTCC	REP.3′HRV14/dis1 and 3′HRV14/res
34-0026/27	CTCAAATTAAGTGTAACAAGTGTTCCTACTAAAATGAGTC and TTTTTTTTTTTTTTTTTTTTATTACTCCTACTTCTA	mut4
34-0029	CTCAAATTAAGTGTAACAAGCTATATTGTTCCTACTAAAATGAGTC	mut3
34-0032	CTCAAATTAAGTGTTCACAGTGTTCCTACTAAAATGAGTC	mut1
34-0033	TTTTTTTTTTTTTTTTTTTTCACAAACTCCTACTTCTA	mut2
34-0043	GGCTAGCC	*Nhe*I linker
34-0044	CCTGGCTAGCACAATATAGACACTTAATTTG	To insert HRV14 3′UTR into pREP.3′PV3/*Stu*I(*Nhe*I), used in conjunction with TOFF
TOFF	TTCGCGAGGTTAACGTCGACTTTTTTTTTTTTT	3′ end minus-sense primer, includes a *Sal*I site
34-0045	GCAGTCGACTTTTTTTTTTTTTTTTTTTTTTT(T/C)(A/T)GCCTATAAACTCCTACTTC	To insert 5-bp insert after HRV14 stem
34-0046/47	CCTGGCTAGCACAA(C/A)ATAGACACTTAATTTG and GCAGTCGACTTTTTTTTTTTTTTTTTTTTTTT(C/A)ATAAACTCCTACTTC	Mutation of bottom base pair of HRV14 stem
34-0048/49	CCTGGCTAGCACAAT(T/G)TAGACACTTAATTTG and GCAGTCGACTTTTTTTTTTTTTTTTTTTTTTT(T/G)TAAACTCCTACTTC	Mutation of second base pair of HRV14 stem
34-0050/51	CCTGGCTAGCACAATA(C/A)AGACACTTAATTTG and GCAGTCGACTTTTTTTTTTTTTTTTTTTTTTTTA(C/A)AAACTCCTACTTC	Mutation of third base pair of HRV14 stem
34-0052/59	CCTGGCTAGCACAATAT(G/T)GACACTTAATTTG and GCAGTCGACTTTTTTTTTTTTTTTTTTTTTTTTAT(G/T)AACTCCTACTTC	Mutation of fourth base pair of HRV14 stem
34-0054	CCTGGCTAGCACAATTTTTTTATAGACACTTAATTTG	To insert (U_6) 5′ to HRV14 stem
34-0055	CCTGGCTAGCACAATTTTTTCG(G/A)TATAGACACTTAATTTG	To insert (U_6CGG/A) 5′ to HRV14 stem
34-0065	CAGGAGCTAGCTTTGTTTAAACAAATTTTCTTAAAA	Plus-sense PCR primer to amplify HAV 3′UTR, used with TOFF
34-0066	CAGGAGCTAGCTGAATTGCCAACTTGATGATCC	To amplify BEV 3′UTR, used with TOFF

digested with *Sal*I, and ligated into pREP.3′PV3/*Stu*I which had been digested with *Stu*I and *Sal*I. pREP.3′HRV14 was derived from pREP.3′HRV14pre by removal of nucleotides between the *Stu*I site and the *Hpa*I site in the HRV14 3′UTR at nt 7165, just upstream of the putative stem-loop structure. pREP.3′PV3/*Stu*I(*Nhe*I) was a derivative of pREP.3′PV3/*Stu*I with an *Nhe*I linker introduced into the *Stu*I site by primer 34-0043. To make pREP.3′HAV, the 3′UTR of hepatitis A virus (HAV) was amplified from the full-length clone pG3/7-P2.18f (12), obtained from S. Lemon, with 34-0065 and TOFF, digested with *Nhe*I and *Sal*I and cloned into pREP.3′PV3/*Stu*I(*Nhe*I), digested with *Nhe*I and *Sal*I. To make pREP.3′BEV, the 3′UTR of bovine enterovirus (BEV) was PCR amplified from a BEV cDNA clone, pHIL-1, obtained from P. Duprex, Queen's University of Belfast, with 34-0066 and TOFF and ligated into pREP.3′PV3/*Stu*I(*Nhe*I) as described for pREP.3′HAV. The sequence of the 3′UTR derived from pHIL-1 is the same as that of BEV strain VG-5-27 (6) except that it contains a G residue in place of the published C at nt 7367 (5a). pREP.3′PV3/*Stu*I+1kb was made by inserting the *Eco*RI-*Sst*I fragment (nt 2726 to 1653) of pXT1 (Stratagene), after filling in the termini with T4 DNA polymerase, into the *Stu*I site of pREP.3′PV3/*Stu*I. The orientation of the fragment was 5′-*Eco*RI-*Sst*I-3′. Mutants of the HRV14 3′UTR were made either by SDM of the *Xba*I-*Sal*I fragment (nt 6265 to the 3′ terminus) of pREP.3′HRV14 cloned into M13mp18 with primers denoted in Table 1, according to the method of Kunkel, or by inserting PCR products containing desired mutations between the *Nhe*I and *Sal*I sites of pREP.3′PV3/*Stu*I(*Nhe*I). The template for PCR amplification was pREP.3′HRV14, and primers are shown in Table 1. Full-length viral cDNA forms of the mutants were made by transferring the *Xba*I-*Sal*I fragment (nt 6265 to the 3′ terminus) into the full-length PV3 cDNA clone.

In vitro transcriptions and transfections. Ohio HeLa cells were grown in Eagle's modified essential medium supplemented with 10% fetal calf serum. For CAT assays, cells were grown in 60-mm-diameter dishes and used at 70 to 90% confluency. Prior to T7 RNA polymerase transcription, templates were linearized with *Sal*I, which cleaves at a unique site immediately to the 3′ side of the poly(A) tract. The poly(A) tract of all cDNAs was between 22 and 26 nt. T7 RNA polymerase was obtained from Promega and used according to their instructions. After transcription, samples of the transcription reaction mixtures were fractionated by gel electrophoresis and the relative yields of different reactions were estimated by eye, except for cases in which quantitated CAT activities are given, in which instances [8-^{14}C]adenosine 5′-triphosphate (Amersham) was included in the in vitro transcription reactions. Relative yields of each reaction were assessed by precipitating transcripts from equal aliquots of each transcription reaction, using trichloroacetic acid. Equal counts of each transcription reaction were then used for transfection. Typically, yields of 2 to 4 μg of RNA were obtained from reactions containing 1 μg of template DNA. Cells were transfected with 1 to 4 μg of transcripts essentially as described previously (26). CAT assays were carried out as described previously (21) or in quantitative experiments using a one-vial procedure which relies on partitioning of labelled reaction product into liquid scintillation counting fluor (16).

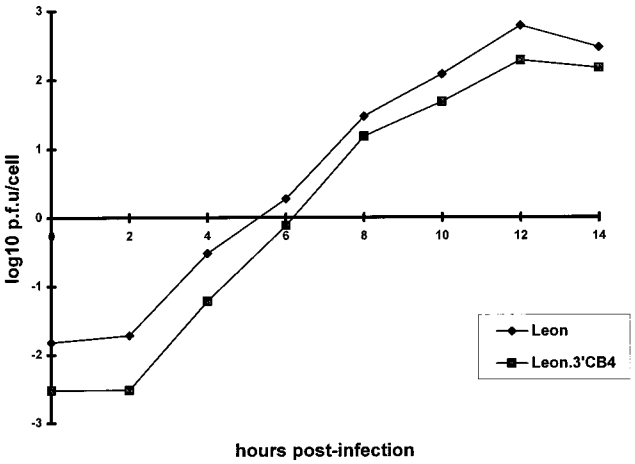


FIG. 1. Comparison of growth rates of PV3(P3/Leon) and Leon.3′CB4, a PV3 derivative in which the PV3 3′UTR is replaced by that of CB4. Hep2c cells were infected at a multiplicity of infection of 10 and incubated at 34°C. Data presented are from duplicate assays.

Analysis of recombinant viruses. The sequence of recombinant virus was assessed either by direct sequencing of PCR products (fmol sequencing kit; Promega) obtained from reverse transcription PCRs carried out on RNA extracted from virions purified as described previously (24) or by sequencing a number of clones obtained after cloning reverse transcription PCR products into pGEM-T (Promega). The primer for sequencing and plus-sense synthesis was 34-0003, and that for reverse transcription was TOFF (Table 1). The growth characteristics of virus were assessed in one-step growth curves. Prior to plaque assay, cell-associated virus was released into the tissue culture fluid by three cycles of freeze-thawing. Cell debris was then removed by centrifugation, and the supernatants were used in plaque assays.

RESULTS

Functional substitution of the PV3 3′UTR by those of other picornaviruses. In a previous series of experiments designed to examine whether the 3′UTR served as a picornavirus packaging signal, it was observed that a PV derivative in which the 3′UTR was replaced with that of CB4 was replication competent (15). Indeed, the kinetics of infection of this recombinant in Hep2c cells were very similar to those of the parental virus, P3/Leon (PV3) (Fig. 1). This result was surprising in view of the limited primary sequence homology of the PV3 and CB4 3′UTRs and the different RNA secondary structures predicted for them (Fig. 2). The PV3 and CB4 3′UTRs are predicted to have two and three stem loops, respectively, with the only strong similarity between the structures being at the lower end of the first PV3 and second CB4 stem-loops.

To examine the role of the 3′UTR in replication more fully, recombinant replicons in which the PV3 3′UTR was changed for that of HRV14, HAV, or BEV were made. These alterations were made in the PV3 replicon cDNA pT7FLC/REP, in which the N-terminal portion of the P1 capsid encoding region is replaced by a CAT reporter gene as described previously (21). These constructions involved the introduction of either a *Stu*I or an *Nhe*I restriction site just downstream of the translational terminators as described in Materials and Methods. This allowed excision of the PV3 3′UTR by digestion with either *Stu*I or *Nhe*I followed by digestion with *Sal*I, which cuts 3′ to the poly(A) tract of the cDNA. In the preliminary experiments in which the HRV14 3′UTR was introduced, a PCR fragment containing nt 7073 to the 3′ terminus [including the poly(A) tail], which includes 93 nt of the HRV14 3D coding region together with the 3′UTR, was introduced into pREP.3′PV3/*Stu*I, thereby producing plasmid pREP.3′HRV14pre. Following transfection of Ohio HeLa cells with transcripts produced in vitro, comparable levels of CAT activity for this replicon and for FLC/REP, the wild-type PV3 replicon (data not shown), were observed, suggesting that the fragment of HRV14 from nt 7073 to the poly(A) tract is functionally equivalent to the PV3 3′UTR. To establish that this property was conferred by the predicted stem-loop structure of the HRV14 3′UTR (Fig. 2), use was made of the *Hpa*I site just upstream of the HRV14 stem-loop in pREP.3′HRV14pre, to allow precise replacement of the PV3 3′UTR with the stem-loop of HRV14. In agreement with the previous result, this replicon (pREP.3′HRV14) produced CAT activity indistinguishable from that observed for the wild-type replicon (Fig. 3A). The effect of substitution of the HRV14 3′UTR on replication of the complete virus was also assessed. The result showed that the chimeric virus FLC.3′HRV14 accumulated to about 0.5 $\log_{10}$ lower levels than did the wild-type virus (Fig. 3B) and had a slightly smaller plaque size. A similar level of replication was also observed for FLC.3′PV3/*Stu*I, indicating that the introduction of the *Stu*I site had affected replication efficiency slightly, possibly because of disruption of the lowest base pair of the PV3 stem-loop (C-G) (Fig. 2). Introduction of the *Nhe*I site into the replicon had no discernible effect on the level of replication observed (data not shown).

The levels of CAT activity observed for the replicons containing the HAV 3′UTR (pREP.3′HAV) and the BEV 3′UTR (pREP.3′BEV) were significantly lower than those for the replicons discussed above (Fig. 4). This level of CAT activity was comparable to that routinely observed for REP.Δpol, a replication-defective derivative of FLC/REP in which nt 6052 to 6267 were deleted, causing an in-frame deletion in the 3D coding region. The weak CAT signal produced by REP.Δpol was shown to be due to translation of input RNA rather than to a low level of RNA replication by the fact that inclusion of 2 mM guanidine hydrochloride in the incubation medium did not reduce the CAT signal (Fig. 4). Thus the low levels of CAT activity for the pREP.3′HAV and pREP.3′BEV replicons suggest that the HAV and BEV 3′UTRs do not support efficient replication of PV3. Nevertheless, infectious virus was recovered for both of the comparable constructs when built into the infectious PV3 clone, pFLC. However, the specific infectivities of FLC.3′BEV and FLC.3′HAV transcripts were about 100-fold lower than that obtained for FLC (10^5 PFU/μg), and they produced only pinprick plaques. These observations suggest that the weak CAT signal observed for the replicons is principally from translation of input RNA rather than this low level of replication. It is also possible that mutation is required for viability of the recombinant viruses. However, the integrity of the 3′UTRs of both recombinant viruses was assessed after three passages on HeLa cells by sequencing of pGEM-T clones containing 3′UTRs obtained by reverse transcription PCR of viral RNA but no base changes were observed compared with the cDNA. Further analysis of these recombinant viruses at other positions is under way.

Separation of the coding region from the 3′UTR. The result obtained with pREP.3′HRV14pre not only indicated that the HRV14 sequences could substitute for those of PV3 but also suggested that the abutting of the translational terminator and the 3′UTR was not essential for replication. We examined this idea more fully by introducing 1,073 nt of heterologous sequence into the *Stu*I site, to produce REP.3′PV3/*Stu*I+1kb. CAT activity in cells transfected with this replicon was reduced by about 70% compared with that observed for FLC/REP (Fig. 5), indicating that wide separation of the open reading frame and 3′UTR can be tolerated but at the expense of replication efficiency. Virus was recovered from cells transfected with the

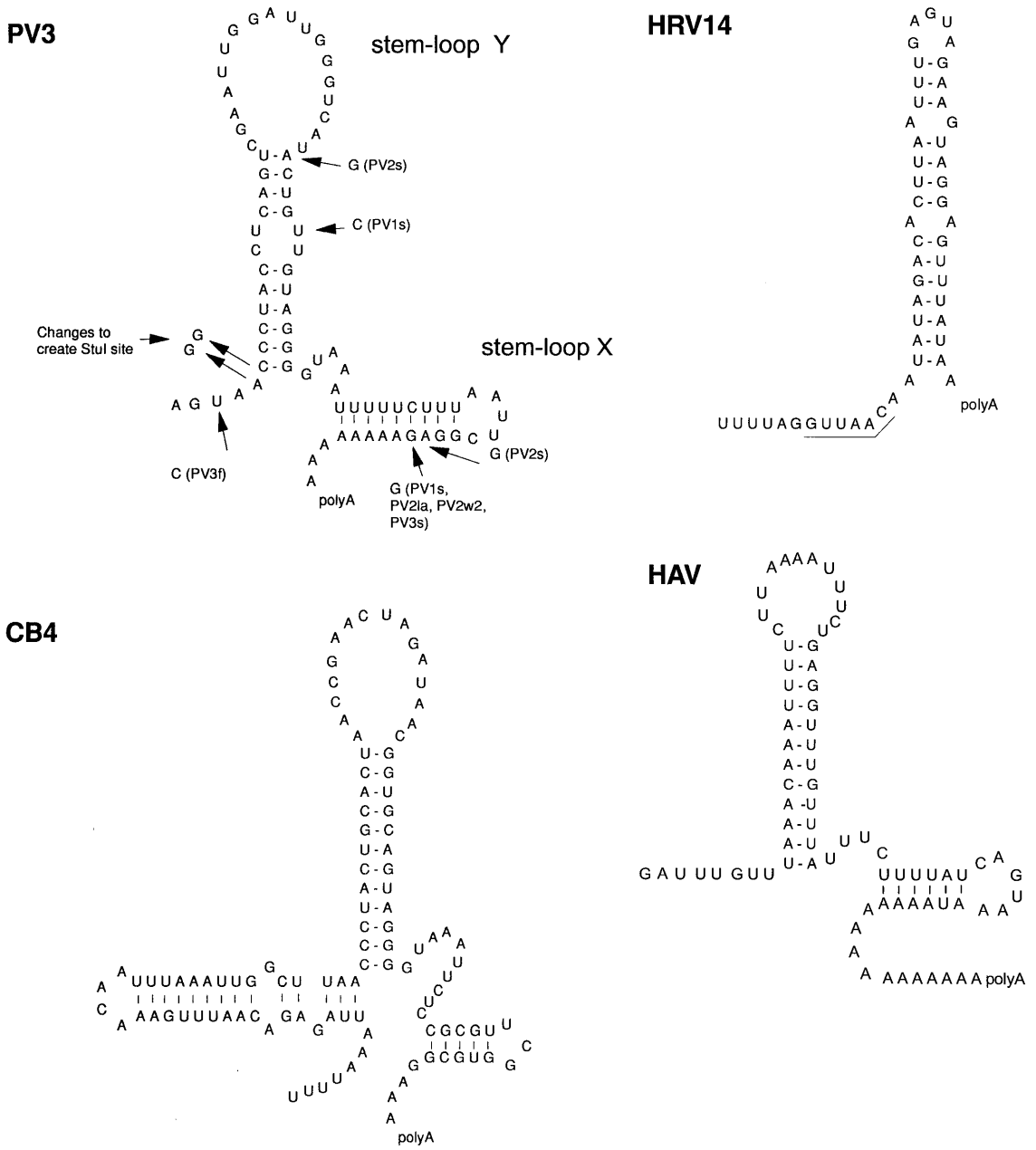


FIG. 2. Secondary structures of the 3′UTRs of various picornaviruses. Structures of PV3, CB4, and HRV14 3′UTRs are as proposed previously (22). The nucleotides marked in the PV3 structure were altered as a result of introducing the *Stu*I site in pREP.3′PV3/*Stu*I. Nucleotide differences from PV3(P3/Leon) are indicated as follows: PV1s, PV2s, and PV3s, Sabin strains of PV1, PV2, and PV3, respectively; PV2la, PV2 Lansing strain; PV2w2, PV2 W-2 strain; PV3f, PV3 strain 23127 Finland/84. The *Hpa*I site located to the 5′ side of the HRV14 stem-loop structure is indicated. Data were obtained from the EMBL database. The structure of the HAV 3′UTR is the most stable structure predicted by the MFOLD RNA secondary structure prediction program ($\Delta G = -7.4$ kcal/mol).

FLC version of this mutant much less efficiently than from cells transfected with PV3 transcripts. Those viruses that were recovered contained internal deletions, with only sequences derived from the ends of the insert remaining. In view of the longer period for recovery of virus (5 days versus overnight for PV3), the CAT activity observed in cells transfected with pREP.3′PV3.*Stu*I+1kb almost certainly reflects replication of the intact replicon rather than of forms modified by deletion of the foreign sequence.

An intact stem-loop is important for the function of the HRV14 3′UTR. The results obtained with REP.3′HRV14 and FLC.3′HRV14 indicated that the HRV14 3′UTR could substitute functionally for the PV3 3′UTR. This was a remarkable observation in light of the lack of sequence and structural homology between the PV3 and HRV14 3′UTRs (Fig. 2) and suggested either that the stem-loop structures were not important for replication, and hence interchangeability could be tolerated, or that the 3′UTRs contained signals which directed replication but that the replication apparatus employed by the virus could utilize a variety of different signals.

In order to understand how such signals were operating, SDM was used to examine the role of the PV3 and HRV14

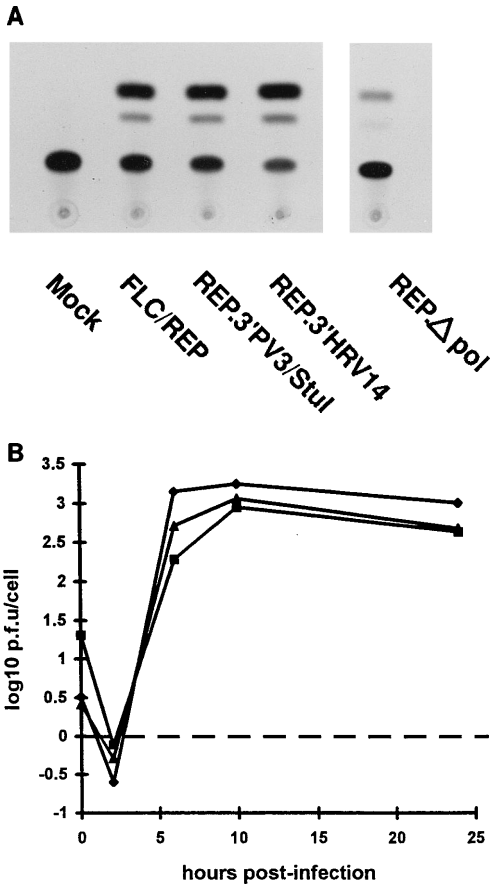


FIG. 3. Replication of 3′UTR recombinants. (A) Ohio HeLa cells were transfected and incubated for 7 h before preparation of cytoplasmic extracts and enzymatic CAT assay. (B) Growth curves of virus containing recombinant 3′UTRs. Ohio HeLa cells were infected at a multiplicity of infection of 10 and incubated at 34°C. ◆, Leon; ■, FLC.3′HRV14; ▲, FLC.3′PV3/*Stu*I.

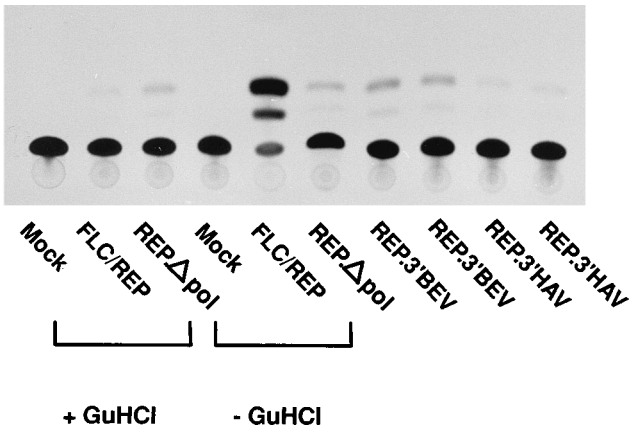


FIG. 4. CAT activities from recombinant replicons in which the PV3 3′UTR was replaced with the 3′UTRs of either BEV or HAV and demonstration that CAT activity observed with REP.Δpol is not due to replication are shown. The activities of two independently derived clones of each recombinant replicon were assessed as described in the legend to Fig. 3A. To show that CAT activity could be observed in the absence of replication, CAT activities obtained in cells incubated in unsupplemented medium (−GuHCl) or medium supplemented with 2 mM guanidine hydrochloride (+GuHCl) were compared for FLC/REP and REP.Δpol.

stem-loops. First we tested if either of the PV3 stem-loops was functionally equivalent to the single stem-loop of the HRV14 3′UTR by removing each of the stems individually. Very low levels of CAT activity were observed for replicons in which only one of the stem-loops was present (REP.3′PV3ΔX and ΔY), indicating that neither of the PV3 stems was fully equivalent to the HRV14 stem-loop (Fig. 6) and suggesting that the complete PV3 3′UTR formed a functional unit. In view of the relative simplicity of the HRV14 stem-loop, further experiments concentrated on examining its essential features. Mutants REP.3′HRV14/dis1 and REP.3′HRV14/res, in which the middle segments of the stems are destabilized and restored, respectively (Fig. 6), provide strong evidence for both the existence of the stem and its importance for replication and also suggest that the primary sequence is not important in this region of the stem. The importance of the stem is also indicated by the lack of replication observed for REP.3′HRV14Δ, in which the stem was removed completely (Fig. 6), and for REP.3′HRV/dis2 (Fig. 7), in which the stem was completely disrupted but the distance between the termination codon and the start of the poly(A) tract was maintained. Replicons in which the stem but not the loop sequence was replaced with an extremely stable stem (REP.3′HRV14/syn [−32 kcal/mol; 1 kcal = 4.184 kJ]), shortened by removal of the lower portion of the stem (REP.3′HRV/short [0.9 kcal/mol]), or extended by introducing 6 bp immediately at the base of the stem (REP.3′HRV14/long [−23.8 kcal/mol]) also failed to replicate (Fig. 7). The free energy of the stem-loop for the wild type is calculated to be −7.1 kcal/mol (calculated as described in reference 1). It is interesting that for these mutants the CAT signal was consistently weaker than that observed for the replication-defective REP.Δpol, suggesting that their translatability or stability might also be impaired. This possibility is under further investigation.

Influence of the HRV14 loop sequence on replication. Comparison of the secondary structures formed by the 3′UTRs of HRV types for which sequences were available showed that there was no highly conserved loop sequence, suggesting that the loop sequence might not play an important role in replication (Fig. 8). This idea was tested with loop mutants generated by SDM with a degenerate primer. In transfection experiments with these mutants, input RNA and CAT activity were quantified as described in Materials and Methods. The results show that alteration of the loop sequence has a significant

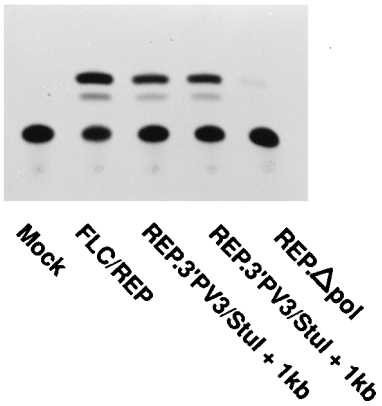


FIG. 5. CAT activities from a PV3 replicon in which the open reading frame and the 3′UTR are separated. Heterologous RNA (1,073 nt) was inserted into the *Stu*I site of pREP.3′PV3/*Stu*I. Two independently derived clones containing the insert were analyzed. The experimental procedure was as described in the legend to Fig. 3A, except that incubation was for 15 h.

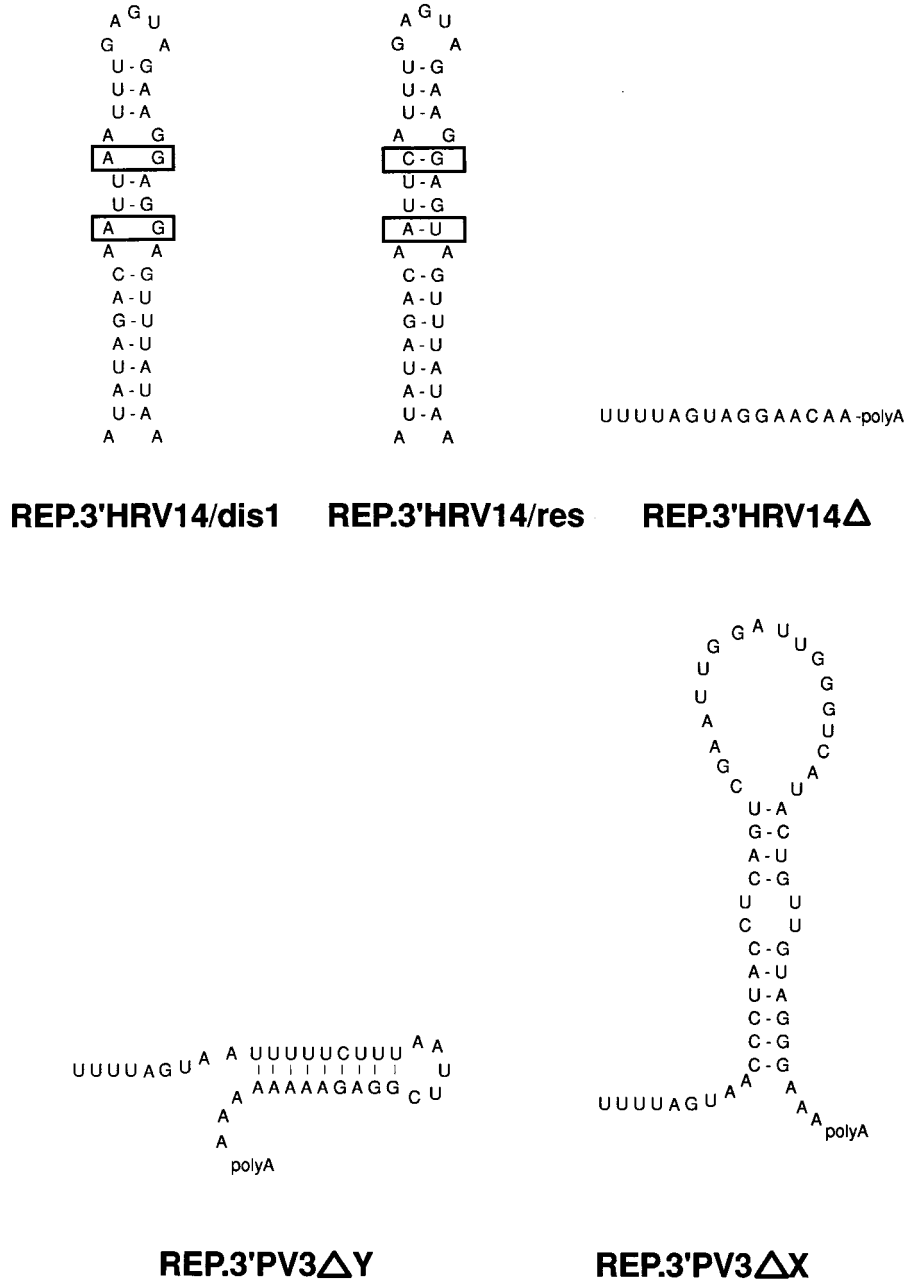
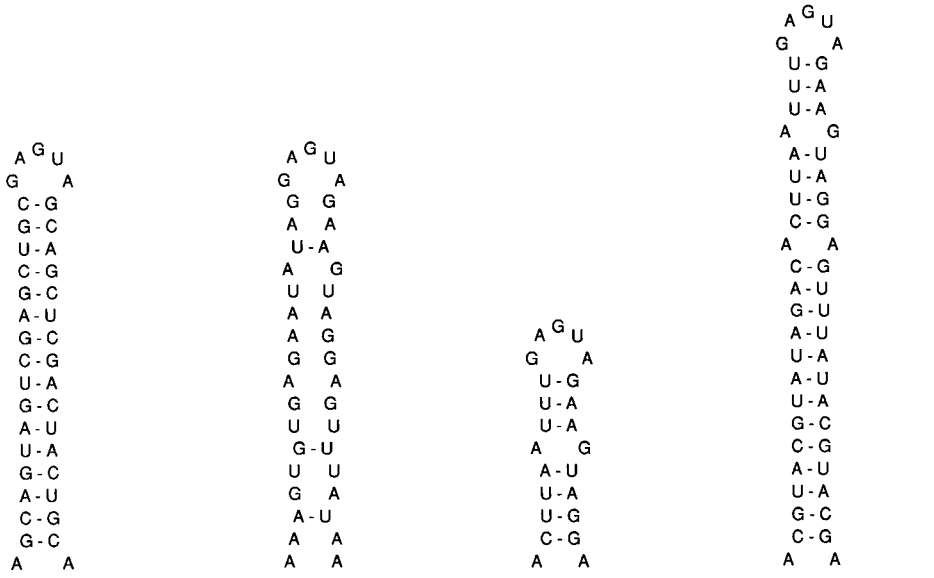


FIG. 6. Predicted secondary structures of the 3′UTRs of the mutants (top) and CAT activities from PV3 replicons containing mutated PV3 and HRV14 3′UTRs (bottom) are shown. Boxed nucleotides in 3′HRV14/dis1 and 3′HRV14/res indicate changes from the wild-type HRV14 stem-loop. The experimental procedure was as described in the legend to Fig. 3A.

effect on replication, with none of the mutants producing more than 50% of the activity observed for the wild-type replicon (Fig. 9), indicating that the loop region does play an important role in replication. Particularly low levels of activity were observed for mutants with loop sequences of GCCCU, CCCCC, and CCUGC, suggesting that cytosine residues are not favored. Interestingly, the mutant with the HRV2 loop sequence also showed a reduced level of replication, indicating that sequences of the loop alone do not confer efficient replication.

Identification of residues important for activity of the HRV14 stem-loop. The lack of replication observed for REP.3′HRV14/long, in which the lower segment of the stem is elongated (Fig. 7), suggested that either the stability of the stem or the presence of a motif within the lower part of the stem was important



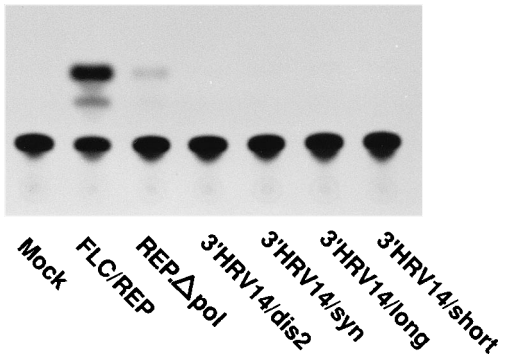


FIG. 7. Predicted secondary structures of the 3′UTRs of the mutants (top) and CAT activities from PV3 replicons containing mutated HRV14 3′UTRs (bottom) are shown. The experimental procedure was as described in the legend to Fig. 3A.

for replication. In REP.3′HRV14/long, such a motif might be nonfunctional because of its sequestration in the middle of the elongated stem. Alternatively, the lack of replication of REP.3′ HRV14/long might have been due simply to the addition of six residues between the stem and the poly(A) tract. To test these ideas we assessed the replication competence of mutants in which either U_6, U_6CGA, or U_6CGG was placed immediately 5′ to the base of the stem, reasoning that in REP.3′HRV14 (U_6) the stem would be elongated because of base pairing of the U_6 tract and poly(A) tail and that in REP.3′HRV14 (U_6CGA) and REP.3′HRV14(U_6CGG) a 3-nt bulge would be introduced between the bottom portion of the genuine HRV14 stem and a U_6-A_6 stem, thereby repositioning the bottom of the HRV14 stem adjacent to a non-base-paired region. In addition, we also constructed a mutant containing a GGCUG insertion immediately 3′ to the stem [REP.3′HRV14(GGC UG)] to examine if replication required positioning of the stem immediately upstream of the poly(A) tract. When REP.3′ HRV14(U6) was transfected into cells, a relatively low level of replication was observed; however, nearly wild-type levels of replication were observed for REP.3′HRV14(U_6CGA) and REP.3′HRV14(U_6CGG) (Fig. 10), supporting the idea that a motif located near the base of the HRV14 stem played an important role in replication. Poor replication was also observed for mutant REP.3′HRV14(GGCUG), indicating that positioning of the stem immediately upstream of the poly(A) tract was required for efficient replication.

Support for the existence of a motif important for HRV14 replication and located at the base of the stem also comes from the observation that the bottom 4 bp of the stems formed by HRV1B, 2, 9, 14, 16, and 89 are completely conserved (Fig. 8). We attempted to examine the significance of this by mutating the sequences of either the left- or right-hand side of the stem in this region while maintaining the secondary structure, thereby examining the contribution of the primary sequence on each side of the stem. This approach is somewhat problematical in view of the need to introduce G-U base pairs at some positions, these having a marked influence on the stability of the stem because of the unfavorable stacking interactions between G-U and adjacent base pairs.

No replication was observed for either mut1 or mut2 in which either the right- or left-hand side of the stem is maintained (Fig. 11). The free energy values for these stems are estimated as −3.5 and −2.6 kcal/mol, respectively, showing that they are significantly destabilized compared with the wild-type stem (−7.1 kcal/mol).

The most informative conclusions can be drawn from the experiment with mut4, which is predicted to have only a slightly lower stability than that of the wild-type stem (Fig. 11). The fact that this mutant is replication defective provides strong evidence for the importance of the lower part of the stem in replication. mut3 has an identical stem to that of mut4; however, the conserved (A)UAUA(G) sequence which forms the left-hand side of the stem was reintroduced to the 5′ side of the stem. No replication was observed for this mutant, suggesting that the (A)UAUA(G) motif, if important for replication, must be located in a secondary structure for activity.

To probe the function of the 4-bp motif in replication, SDM was used to change each of the base pairs as indicated in Fig.

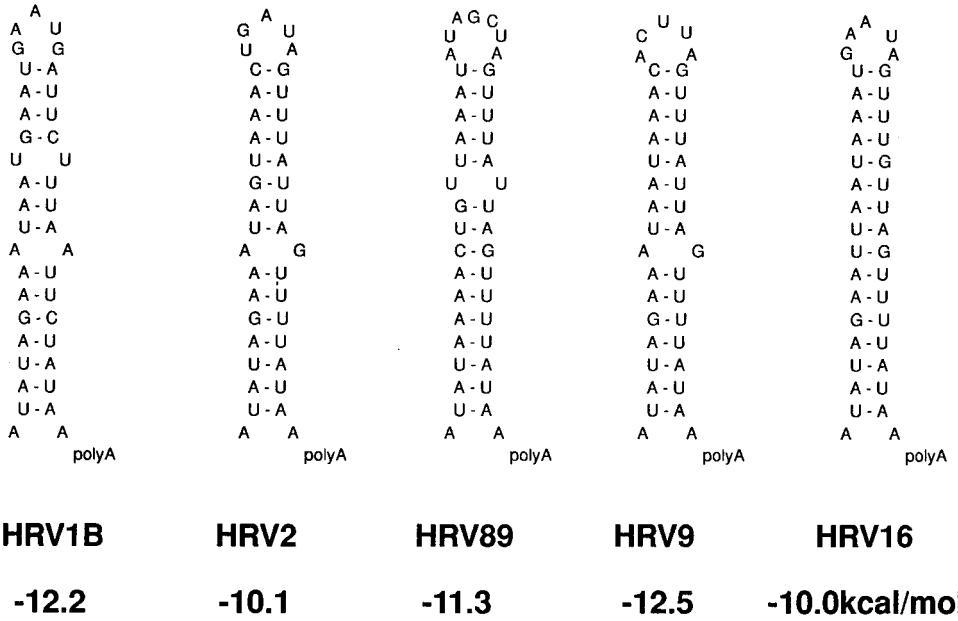


FIG. 8. Comparison of the 3′UTRs of HRVs. Sequences were obtained from the EMBL database and modelled on the HRV14 stem-loop structure shown in Fig. 2. Free energy values were calculated with values in the STAR secondary structure prediction program (1).

12. When the effect of these mutations was assessed by CAT assay in extracts of cells cultured for 14 h posttransfection, all mutants in which normal Watson-Crick base pairing was possible replicated efficiently; however, lower levels of replication were observed for all remaining mutants (data not shown). This included a mutant in which A-U is replaced by G-U at the fourth position. In this case there was probably destabilization of the stem caused by adjacent positioning of two G-U base pairs. Quantitative analysis of CAT activity from mutants in which Watson-Crick base pairing was possible was also performed in experiments terminated at 7 h posttransfection. Contrary to expectation based on the observations of mut4, these results do not identify any particular base pair as critical for replication. However, some configurations result in a 10 to 30% reduction in replication ability and multiple changes may have a cumulative effect. Moreover, single changes would probably be selected against if they occurred in nature.

DISCUSSION

The role of the 3′UTR in the replication of PV has been studied by constructing recombinant replicons and viruses in which the 3′UTR was derived from either CB4, HRV14, BEV, or HAV and by SDM of the PV3 and HRV14 3′UTRs. Each of the foreign 3′UTRs could support replication, albeit with widely different efficiencies. Viruses and replicons containing the CB4 and HRV14 3′UTRs replicated to levels only slightly lower than those with PV3, whereas those with BEV or HAV 3′UTRs replicated to significantly lower levels. That any of the recombinants replicated at all was surprising in view of the limited sequence and structural similarity of PV3 to the foreign 3′UTRs. This suggested either that the 3′UTR is a nonessential region of the genome or that it functions through relatively nonspecific interactions with the remainder of the replication apparatus. Further experiments, however, showed that extensive mutagenesis of the 3′UTR severely impaired replicon and/or virus replication, thereby indicating that certain structures are essential. For example, individual excision of either of the two stem-loops of the PV3 3′UTR destroyed replication capacity, suggesting that the entire 3′UTR functions as a unit. In addition, point mutations in certain regions of the HRV14 3′UTR seriously debilitated replication.

In contrast to that of PV3, the HRV14 3′UTR appears to form only a single stem-loop which, in secondary structure, is highly conserved amongst rhinovirus types. We therefore chose to examine which of the features of this 3′UTR are essential for replication competence. Mutations of the loop region had a marked effect on replication, suggesting that, despite the wide variety of loop primary sequences found in rhinovirus types, some sequence specificity is required for efficient repli-

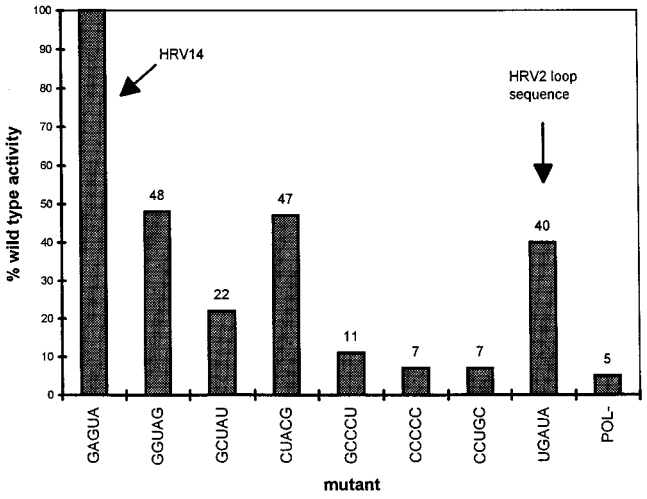


FIG. 9. CAT activities from PV3 replicons containing HRV14 3′UTRs with alterations in the loop sequence. Sequences of the putative loop regions for mutants are indicated. The sequences of the HRV14 and HRV2 loops are GAGUA and UGAUA respectively. Transcripts and CAT activities were quantitated as described in Materials and Methods, and CAT activity is expressed as a percentage of that observed for REP.3′HRV14. Ohio HeLa cells were transfected and incubated at 34°C for 7 h before preparation of cytoplasmic extracts. The mean results of two experiments are shown.

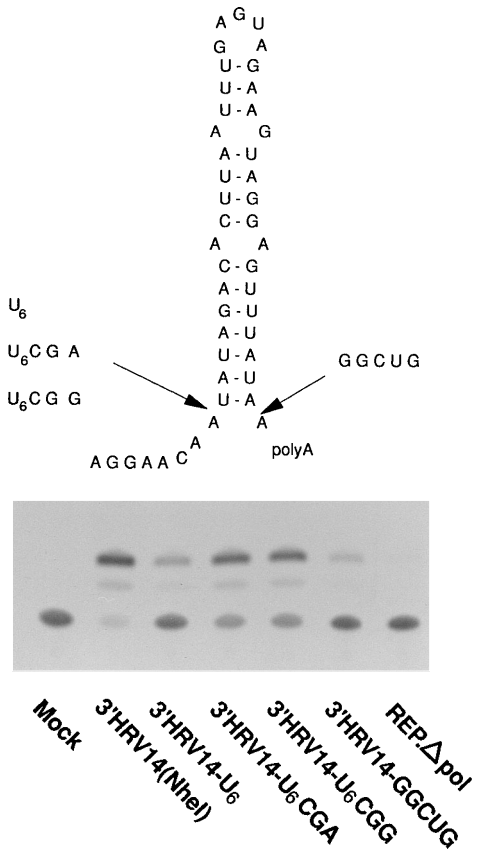


FIG. 10. Effect of insertions near the base of the HRV14 stem on replication. The locations of insertions in the HRV14 3′UTR (top) and CAT activities observed for the mutants (bottom) are shown. The experimental procedure was as described in the legend to Fig. 3A.

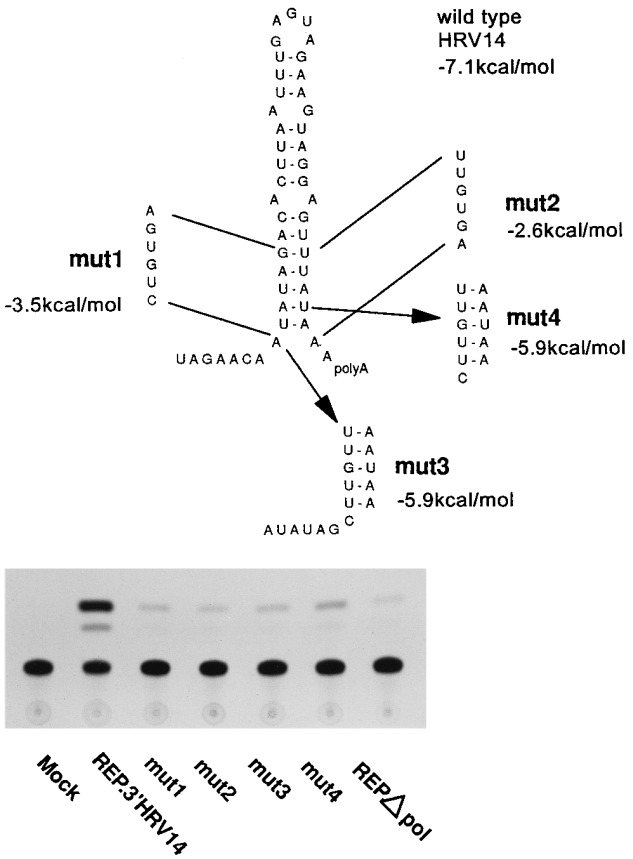


FIG. 11. Analysis of the role of the conserved motif at the base of the HRV14 stem. Structures of HRV14 mutants designed to examine the role of the conserved motif (top) and CAT activities observed for the mutants (bottom) are shown. Free energy values were calculated with values in the STAR secondary structure prediction program (1). The experimental procedure was as described in the legend to Fig. 3A.

cation. In particular, cytosine-rich sequences seemed to be detrimental for replication. A mutant which had the HRV2 loop sequence replicated to a level of only 40% of that observed for the equivalent construct with the full HRV14 stem-loop, suggesting that full replication competence requires compatibility between the stem and the loop. Mutations which altered the stability of the stem region caused reductions in replication efficiency, suggesting a requirement for a moderately stable structure, possibly because it needs to be disrupted at some point during replication. A comparison of sequences of the rhinovirus types showed that a 4-bp motif at the base of the stem is highly conserved, suggesting that it may be important. Evidence in support of this idea was provided by the mutant mut4, in which the sequence but not the predicted structure of this region was changed. This mutant had a very low level of replication. However, extensive mutagenesis failed to identify individual base pairs that were critical for replication, although some mutations, particularly those near the base of the stem, reduced replication by about 30%. It is likely that the cumulative effect of such mutations accounts for the low replicative capacity of mut4. The result obtained for mutant 3′HRV14 (GGCUG) suggests that the stem must be positioned immediately upstream of the poly(A) tract for replication competence.

The explanation for the comparatively poor replication of the recombinants possessing the 3′UTRs of BEV and HAV is unclear. Possibly, the genomes of these viruses are so far diverged from PV3 that interrecognition of 3′UTR and other components of the replication machinery is very poor compared with that possible between PV3 and HRV14 or CB4. One possibility is that longer-range interactions between the 3′UTR and other regions of the genome occur during replication. In this regard, it will be of interest to further characterize these recombinants after adaptation for growth by multiple passage. There is evidence for the formation of a pseudoknot between the 3′UTR and sequences upstream of the translational terminator in PV1 (11), and this has been reported to be important for replication. Although a similar pseudoknot interaction is possible in the PV3/CB4 recombinant, comparable structures in the PV3/HRV14 recombinant seem highly unlikely. Moreover, the recombinant REP.3′PV3/*Stu*I+1kb showed that it is possible to insert an extra kilobase of RNA between the open reading frame stop signal and the 3′UTR without

position	wild	mutants			
4th	A - U	U - A (102%)	G - U		
3rd	U - A	C - G (107%)	C.....U	A.....G	A - U (92%)
2nd	A - U	G - C (77%)	G.....A	U.....C	U - A (70%)
bottom	U - A	C - G (72%)	C.....U	A.....G	A - U (115%)

FIG. 12. SDM analysis of the conserved motif at the base of the HRV14 stem. The position and nature of mutations are shown. CAT activities observed for mutants are expressed as percentages of that observed for pREP.3′HRV14/*Nhe*I, a derivative of pREP.3′PV3/*Stu*I(*Nhe*I) in which the PV3 3′UTR is replaced by the 3′UTR of HRV14 obtained by PCR with primers 34-0044 and TOFF. The experimental procedure was as described in the legend to Fig. 9. Mutants for which no CAT activity is indicated showed only low levels of activity when assayed at 15 h posttransfection.

abolishing replication competence. This suggests that the presence of a pseudoknot is not essential for replication, although the possibility that a functional pseudoknot can be formed even in the presence of the heterologous sequence or by a combination of PV3 and heterologous sequences cannot be excluded. A further possible explanation is that the 3′UTRs are initially bound by host proteins which subsequently recruit viral proteins to form a complex capable of initiating RNA synthesis. If the different 3′UTRs are recognized by different members of a family of proteins, the abundance of individual members might therefore determine the efficiency with which a particular 3′UTR supports replication in a given cell type. Thus, the low levels of replication observed for the replicons with the 3′UTRs of HAV and BEV may be due to low levels of the cellular protein(s) which recognizes them. Such a model has already been demonstrated for the 3′UTRs of the bromo-, tymo-, and cucumoviruses, which form tRNA-like structures which bind and are charged with amino acids by cognate tRNA synthetases (4, 25). Interestingly, it has been suggested that the 3′UTRs of PV and CB4 form tRNA-like structures although the similarity to tRNAs is much less than that for the viruses noted above (22). The identity and function of host proteins which interact with picornavirus 3′UTR are at present unknown, although proteins of 34 to 38 kDa have been reported to interact with the HRV14 and PV1 3′UTRs (30). Since it is likely that a common mechanism is utilized for the initiation of minus-sense RNA synthesis of all picornaviruses, it is conceivable that these proteins are members of a family of proteins with a similar function. We are presently examining the properties of virus recovered from cells transfected with FLC forms of various mutants: mut4, 3′HRV14(U_6), 3′HRV14(CCCCC), and 3′HRV14(GCUAU). We are also investigating whether any of our recombinant viruses display relatively altered growth properties in different cell types and trying to identify proteins which interact with the different 3′UTRs.

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