Sequence Requirements for Viral RNA Replication and VPg Uridylylation Directed by the Internal cis-Acting Replication Element (cre) of Human Rhinovirus Type 14

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Until recently, the cis-acting signals required for replication of picornaviral RNAs were believed to be restricted to the 5′ and 3′ noncoding regions of the genome. However, an RNA stem-loop in the VP1-coding sequence of human rhinovirus type 14 (HRV-14) is essential for viral minus-strand RNA synthesis (K. L. McKnight and S. M. Lemon, RNA 4:1569-1584, 1998). The nucleotide sequence of the apical loop of this internal cis-acting replication element (cre) was critical for RNA synthesis, while secondary RNA structure, but not primary sequence, was shown to be important within the duplex stem. Similar cre sequences have since been identified in other picornaviral genomes. These RNA segments appear to serve as template for the uridylylation of the genome-linked protein, VPg, providing the VPg-pUpU primer required for viral RNA transcription (A. V. Paul et al., J. Virol. 74:10359-10370, 2000). Here, we show that the minimal functional HRV-14 cre resides within a 33-nucleotide (nt) RNA segment that is predicted to form a simple stem-loop with a 14-nt loop sequence. An extensive mutational analysis involving every possible base substitution at each position within the loop segment defined the sequence that is required within this loop for efficient replication of subgenomic HRV-14 replicon RNAs. These results indicate that three consecutive adenosine residues (nt 2367 to 2369) within the 5′ half of this loop are critically important for cre function and suggest that a common RNNNAA internal RNA stem-loop motif exists among the cre sequences of enteroviruses and rhinoviruses. We found a direct, positive correlation between the capacity of mutated cre sequences to support RNA replication and their ability to function as template in an in vitro VPg uridylylation reaction, suggesting that these functions are intimately linked. These data thus define more precisely the sequence and structural requirements of the HRV-14 cre and provide additional support for a model in which the role of the cre in RNA replication is to act as template for VPg uridylylation.

The family Picornaviridae is one of the largest groups of nonenveloped, single-stranded, positive-sense RNA viruses. It comprises nine distinct genera, of which five (the enteroviruses, rhinoviruses, aphthoviruses, cardioviruses, and hepato-
coating of the virus, the incoming positive-strand genome is transcribed into complementary minus-strand RNA by a replicase complex, the catalytic unit of which, 3Dpol, uses a uridylylated form of VPg (VPg-pUpU) as a primer (18). This minus-strand RNA then serves as a template for the production of new, plus-strand, progeny genomes. Although the basic steps of replication are well known, relatively little is understood about the details of these processes. One of the important, yet incompletely answered questions is how the viral replicase specifically selects only viral RNA species for amplification in these reactions, because the 3′-terminal poly(A) sequence of the genomic RNA is indistinguishable from the 3′-termini of cellular mRNAs.

Until recently, it was generally believed that the 5′ and 3′ NTRs of picornaviral RNA contain the cis-acting signals necessary for the initiation of viral RNA replication (28). The specificity of genome amplification was thought to result from interactions of the replicase complex with unique cis-acting signals located in the 5′ and 3′ NTRs (1, 13, 14, 21, 22, 25, 27). However, McKnight and Lemon (11, 12) demonstrated that an internal cis-acting replication element (cre) was necessary for the initiation of RNA synthesis during replication of human rhinovirus type 14 (HRV-14). Although this replication element is located in the long open reading frame, within the segment encoding the VP1 capsid protein, the role of the cre in replication is dependent upon its RNA structure and not its protein-coding capacity (12). Mutational analysis and computer folding algorithms suggested that the cre forms a complex stem-loop structure within the positive-strand RNA that is required for initiation of minus-strand RNA synthesis (Fig. 1) (12).

Following the identification of the HRV-14 cre, similar internal replication signals were identified within the open reading frames of Theiler’s virus, a cardiomyosis virus (9), poliovirus type 1 (PV-1) (5), and, more recently, HRV-2 (4). The latter observations suggest that an internally located cre may be a common feature of the RNA replication schemes of all picornaviruses. The RNA segments comprising these putative 5′cres can be folded into relatively simple stem-loop structures, but the elements differ in terms of their primary nucleotide sequence.
as well as their location within the open reading frame. In cardioviruses, the cre is located in the VP2 region (9). In contrast, the PV-1 cre is not located in the P1 capsid region, but in the 2C (P2) region (5), while the HRV-2 cre is located within the 2A coding sequence (4). Studies by Paul et al. (17) indicate that the cre acts as the primary template for uridylylation of VPg by the 3Dpol polymerase in vitro. In addition, a recent mutational analysis of the poliovirus polymerase indicates that amino acid residues on the surface of the protein that are essential for uridylylation of VPg are also involved in the interaction of the polymerase with the membrane-bound 3AB precursor protein (10). Taken together, these data suggest that the cre plays a critical role in bringing viral RNA into the replication complex and in initiating VPg uridylylation, the first step in the process of viral RNA replication.

Here, we describe experiments aimed at better defining both the sequence and structural requirements for cre function during the replication of HRV-14 RNA. We show that the fully functional cre resides within a 33-nucleotide (nt) RNA segment that is predicted to form a simple stem-loop structure. By introducing single-base substitutions at each position within the loop sequence, we have determined which nucleotides are essential for replication and which nucleotide substitutions are tolerated without significant degradation of cre function. We further show that the ability of individual mutant cre sequences to support RNA replication is closely correlated with their ability to serve as template for the uridylylation of VPg in an in vitro reaction.

MATERIALS AND METHODS

Cells. HeLa cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco/BRL) with 5% fetal bovine serum (FBS).

HRV-14 RNA replication assay. The replication of HRV-14 RNA was monitored by measurement of luciferase expression in HeLa cells transfected with pAPLucCre RNA (12). pAPLucCre is a subgenomic RNA replicon in which most of the P1 segment of the HRV-14 genome has been replaced by an in-frame insertion of the firefly luciferase coding sequence (Fig. 1). Replicon transcripts were obtained by T7 polymerase-mediated transcription (T7 MEGAscript; Ambion) and ligated into pAPLucCre (12) as described above. All regions substituted for uridylylation of VPg by the 3Dpol polymerase in vitro. In addition, a recent mutational analysis of the poliovirus polymerase indicates that amino acid residues on the surface of the protein that are essential for uridylylation of VPg are also involved in the interaction of the polymerase with the membrane-bound 3AB precursor protein (10). Taken together, these data suggest that the cre plays a critical role in bringing viral RNA into the replication complex and in initiating VPg uridylylation, the first step in the process of viral RNA replication.

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considered to be due to the translation of the input RNAs in the absence of any RNA amplification. In contrast, the subsequent increase in luciferase activity between 3 and 24 h, which was observed only with ΔP1LucCRE and only in the absence of guanidine, reflects viral RNA replication. In support of this interpretation, the level of luciferase expression following expression of ΔP1LucCRE RNA was shown previously to closely follow its RNA replication kinetics as assessed by RNase protection assay (12).

As indicated above, previous studies by McKnight and Lemon mapped the cre to a 96-nt-long segment within the VPI coding region of HRV-14 that is capable of forming a complex stem-loop structure within the positive-strand HRV-14 RNA (12) (Fig. 1). A mutational analysis confirmed the necessity for duplex formation within the apical stem of this structure. However, the requirement for the lower stem was less clearly documented, because only one of two mutant HRV-14 replicons with a 4-nt substitution in the lower stem was significantly impaired in replication (12). These data suggest that the lower duplex structure may not be essential for cre function. Thus, to better define the 5’ and 3’ boundaries of the minimal HRV-14 cre, we created a series of in-frame deletions within the previously identified 96-nt cre segment in the background of the replication-competent D1LucCRE subgenomic replicon. As shown in Fig. 1, a series of double-deletion mutants were constructed (D1, D2, and D3), in which increasing lengths of sequences flanking the predicted apical stem-loop structure of the cre were removed from pΔP1LucCRE. In D1, the single-stranded regions 5’ and 3’ of the predicted complex stem-loop structure (nt 2318 to 2329 and 2405 to 2413) were deleted. In D2, the 5’ deletion was extended inward to include nt 2338 and the 3’ deletion to nt 2393, effectively removing most of the predicted distal duplex structure. In D3, the 5’ deletion was further extended to include nt 2353 and the 3’ deletion to nt 2387, removing the internal bulge-loops and leaving only a simple stem-loop structure with a 9-bp stem (Fig. 1).

RNA transcripts derived from pΔP1LucCRE and each of the three deletion mutants were electroporated into HeLa cells. Cell lysates were analyzed for luciferase activity at 3 h and 24 h following transfection, with an increase in luciferase expression during this period of time indicative of viral RNA replication. Transfection of the three double-deletion mutants resulted in similar levels of luciferase expression as a result of translation of the input RNA at 3 h after transfection (Fig. 2B), indicating that each RNA had undergone replication and that the minimal functional HRV-14 cre is located within the 33 nt remaining in mutant D3 (nt 2354 to 2386). In contrast, in cells transfected with pΔP1LucXN, which lacks any cre sequence (Fig. 1), luciferase activity decreased significantly between 3 and 24 h following transfection (Fig. 2B), indicating a failure of viral RNA replication. These data indicate that the minimal functional HRV-14 cre is considerably smaller than the 96-nt structure proposed previously (12).

**Nucleotide sequence required within the cre for RNA replication.** Previous studies have indicated that extensive substitutions can be made within the duplex stem of the cre without compromising replication, provided that compensatory changes are introduced to maintain base pairing within the stem (12). Several nucleotide substitutions within the loop segment, however, were found to be lethal to replication. To better define the requirements for specific nucleotide sequence within the loop, we carried out an extensive mutational analysis of this region (nt 2363 to 2376). Using site-specific mutagenesis with degenerate oligonucleotide primers, we constructed a series of pΔP1LucCRE mutants containing single-base substitutions in which every possible nucleotide substitution was created at each of the nucleotide positions within the loop (Fig. 3A). (For convenience, we refer to these nucleotide positions according to their unique last two digits [e.g., “26” rather than “2326”] in Fig. 3 and the following sections.) Four of the intended substitutions, 63U, 66U, 70U, and 75U, introduced...
stop codons into the reading frame of ΔP1LucCRE, and hence could not be evaluated for their effect on replication as single-base substitutions. Thus, a total of 38 mutants were evaluated, each containing a point mutation at one of the 14 bases in the loop (Fig. 3A). These mutated cre sequences were analyzed by the MFOLD program to predict whether the single-nucleotide substitutions would lead to potential secondary structure alterations. Only three of them—69U, 76U, and 76C—were suggested by MFOLD to have a significant change in secondary structure from that of the wild-type cre (Fig. 3B).

Runoff transcripts representing the wild-type replicon and replicons with single-base substitutions within the cre were electroporated into HeLa cells. Replication was monitored by measuring luciferase activity at 3 and 24 h following electroporation. The efficiency of RNA replication was expressed as the fold increase in luciferase activity at 24 h over the luciferase activity at 3 h relative to that observed with the parental HRV-14 cre (ΔP1LucCRE), which was considered to be 100%. An increase in luciferase activity that was ≥15% of the parental level was considered indicative of significant RNA replication, while a fold increase in luciferase activity <15% that observed with the parent was considered to represent failure of significant replication and only the accumulation of translation products derived from input RNA. This 15% cutoff level represents approximately 3 standard deviations below the mean fold increase in luciferase expression observed between 3 and 24 h after transfection of the parental RNA, ΔP1LucCRE, in a series of four independent experiments, each done in triplicate (average fold increase, 32.9 ± 8.7). As shown in Fig. 4, most mutants replicated at levels either significantly above or below this breakpoint.

Surprisingly, at most positions within the loop (positions 64 to 66 and 70 to 75), any base substitution resulted in a replication-competent phenotype (Fig. 4). In contrast, any substitution of the three adenosines spanning positions 67 to 69, except for 69G, a purine transition, resulted in a failure to replicate, as evidenced by a lack of increase in the level of luciferase expression following electroporation of the RNA (Fig. 4). Since the 69U substitution causes a significant perturbation in the predicted secondary structure of the cre (Fig. 3B), it is not possible to discriminate whether the lethal effect of this mutation is related to an alteration in structure or primary sequence, or both. However the failure of 69C to replicate suggests the latter, since this substitution was not predicted to have altered the structure of the loop. These results indicate that A67, A68, and (to a lesser extent) A69 are essential for cre function in supporting RNA replication.

We created additional mutants to assess the effects of base substitutions that would result in the formation of stop codons within the cre. Since the 72U substitution creates a stop codon, we introduced another substitution at the second position of the codon, 73C. 73C by itself is replication competent, as was the multiple-substitution mutant MS2, which contains both 72U and 73C substitutions (Fig. 3C and 4). We could not use this approach to evaluate the effect of 66U, which creates a UAA stop codon, since any substitution at the relevant second base position, 67, was lethal (Fig. 4).

63U and 75U also introduce stop codons into the cre sequence. The further evaluation of these substitutions was complicated by the results of other substitutions at positions 63G and 76A, which are located at the junction of the stem and the loop in the predicted structure of the wild-type cre (Fig. 3A). Some substitutions at these bases, 63A and 76G, resulted in a replication-competent phenotype (Fig. 4). In contrast, other substitutions (63C, 76C, and 76U) were lethal or resulted in mutants with marginal replication capacity (Fig. 4). Although...
63G and 76A may form a noncanonical base pair in the wild-type structure (Fig. 3A), the two latter substitutions at position 76 have the potential to result in the formation of stronger base pair interactions at the base of the cre loop, 63G-76C or 63G-76U. Such base pairing could reduce the size of the loop from 14 nt to 12 nt or otherwise change its conformation (Fig. 3B). Thus, we suspected that the inhibition of replication by the 76C and 76U substitutions might be caused by alteration in the secondary or tertiary structure of the loop. To test this hypothesis, and at the same time to evaluate the effect of the remaining two substitutions that result in stop codons, 63U and 75U, we created the multiple-substitution mutant MS1 shown in Fig. 3C. This mutant contains 63U, 64U, 75U, and 76U substitutions and is both free of stop codons and not capable of base pairing between positions 63 and 76. Although the 4 base substitutions in MS1 do not change the predicted secondary structure, this mutated cre failed to support RNA replication (Fig. 4). Thus, it is not clear whether the negative effects of some single-nucleotide substitutions at positions 63 and 76 result from alterations of the secondary structure alteration, rather than changes in the primary sequence of the RNA (see Discussion). Similar to the situation at position 69A, it seems that only a purine transition is well tolerated at positions 63G and 76A.

In summary, no substitution of 67A or 68A was permissible. At the position immediately following these two adenosines, 69A, only a purine transition was acceptable. This was also the case with the 2 nt at the bottom of the loop, 63G and 76A, at which only purine transitions were tolerated. In contrast, any substitution was permitted at other positions in the loop without significantly impairing the cre function in replication. These conclusions are summarized in Fig. 5.

Capacity of mutant cre RNAs to support VPg uridylylation in vitro. The HRV-14 cre has been shown to be an effective template for the uridylylation of the VPg proteins of either HRV-14 or PV-1 type 1 by the PV-1 3Dpol enzyme (17). This prompted us to investigate whether the capacity of the cre mutants to support replication in vivo would correlate with their ability to function in an in vitro VPg uridylylation reaction. VPg uridylylation reactions were carried out with synthetic PV-1 VPg, and recombinant PV-1 3Dpol and 3CDpro, as described previously (17). RNA transcripts representing each of the HRV-14 cre mutants were added to individual reaction mixtures. The efficiency of the reaction was measured by PhosphorImager quantification of the yield of uridylylated VPg, VPg-pU, and VPg-pUpU, as shown in Fig. 6. In each experiment, the efficiency of uridylylation was compared to that of a reaction mixture containing the wild-type HRV-14 cre RNA.

FIG. 4. Luciferase expression as a measure of replication of HRV-14 cre mutants in transfected HeLa cells. At 3 and 24 h following transfection, cell lysates were harvested and assayed for luciferase activity as described in Materials and Methods. Each column represents the fold increase in luciferase activity observed with a single RNA construct. The efficiency of RNA amplification was calculated as the ratio of luciferase expression at 24 h relative to luciferase expression at 3 h and compared with that observed with the parent ΔPLucCRE (set as 100%). The dashed line indicates a fold increase in luciferase activity equivalent to 15% of that observed with the parent RNA. Greater fold increases in luciferase activity were considered indicative of significant RNA amplification, while lesser increases were considered to be due primarily to translation from input RNA (see text). wt, wild type.

FIG. 5. Summary of the effect of nucleotide substitutions within the HRV-14 cre loop on RNA replication. Only the loop and the proximal 4 bp within the stem are shown in this figure. (A) Replication-competent HRV-14 cre mutants. (B) Mutations with a lethal effect on HRV-14 replication that represent those contributing critically to cre function (see legend to Fig. 4). Underlined substitutions are those predicted by MFOLD to introduce changes in the secondary structure of the RNA. The nucleotides in triangles are those for which no substitution was permissible, while the nucleotides in the squares are those for which some substitutions were permissible. At the other loop positions, any nucleotide substitution was permissible, provided that it did not introduce a stop codon.
As shown in Fig. 6A, the PV-1 3D\textsuperscript{pol} enzyme efficiently directed the uridylylation of the PV-1 VPg protein in the presence of the wild-type HRV-14 cre RNA. Those mutant cre sequences that retained the ability to support RNA replication also remained active in the VPg uridylylation reaction, although with various levels of efficiency producing various amounts of VPg-pU and VPg-pUpU. A few mutants produced yields of uridylylated VPg that were comparable to or higher than that produced with the wild-type cre, such as 64C, 66C, or 66G (Fig. 6A). However, most of the mutant cre RNAs produced lower yields of the uridylylated protein (15 to 80% of the wild-type level), such as 63A, 64G, 64U, 69G, 76G, and all substitutions at positions 65 and 70 to 75 (Fig. 6).

As shown previously, each of the nucleotide substitutions at positions 67 and 68 abolished replicon amplification (Fig. 4). Importantly, these substitutions also failed to produce detectable uridylylation products (Fig. 6). The 63C, 69C, and 76C substitutions, as well as the multiple mutant MS1, each of which was also deficient in RNA replication (Fig. 4), resulted in yields of uridylylated VPg that were no more than the background level (Fig. 6). The marginally replicating mutant 76U and a nonreplicating mutant 69U (Fig. 4) produced yields of uridylylated VPg that were less than 10% of the wild-type level. There was thus a good correlation between the capacity of each mutant cre to support RNA replication and its ability to function in the in vitro uridylylation reaction, as shown schematically in Fig. 7.

**DISCUSSION**

To date, cres have been identified within the protein-coding sequences of viruses representing three genera of picornaviruses: rhinoviruses, enteroviruses, and cardioviruses (4, 5, 9, 11, 12). These cres are similar in that they are all located within protein-coding sequence, but function at the level of RNA. Each is predicted to form a stem-loop structure about 30 to 65 nt in length, generally involving nucleotide tracts with low P-num values supporting the likelihood that the structure is conserved and thus biologically significant (16). The available evidence suggests that these RNA structures are required within the positive strand of the RNA for efficient initiation of minus-strand RNA synthesis (12) and that they are likely to be common to the RNA replication scheme of most if not all picornaviruses.

However, there are differences in these cres that are also remarkable. First, they are located in different regions (VP1,
VP2, 2A, or 2C coding region) of the genome, suggesting that cre function is not dependent on a specific location and that different viruses have evolved cress at different sites within the genome in ways conducive to their dual roles as both replication element and protein-coding sequence. Another surprising difference, considering their common roles in RNA replication, is the extent of the diversity that is evident in their primary sequences as well as predicted secondary structures. The sequence differences present in the HRV-14, PV-1, and HRV-2 cress (Fig. 8A) are even more surprising, since the HRV-14 cre is capable of substituting for the PV-1 and HRV-2 cress in VPg uridylylation reactions with PV-1 and HRV-2 enzymes (4, 17). This suggests that the PV-1 and rhinovirus enzymes involved recognize some common sequence and/or structural features in these cress. Our primary aim in this study was to identify the sequences that are critical for HRV-14 cre function and, by comparing these with other known or predicted cress, to identify those sequences and structural features that are important for RNA replication.

Since previous results suggested that the minimal functional HRV-14 cre (12) was significantly larger (96 nt) than the cress identified subsequently in other picornaviruses (4, 5), we created a series of deletion mutants in which the sequences flanking the apical loop of the structure predicted for the HRV-14 cre were progressively deleted (Fig. 1). The ability of each of these mutants to support the replication of HRV-14 RNA (Fig. 2B) indicated that the minimal functional cre resides within a 33-nt sequence that is predicted to form a simple stem-loop (nt 2354 to 2386) (Fig. 1). The HRV-14 cre is thus no larger than the cress identified in other picornaviruses.

These results indicate that only the top stem and loop se-

FIG. 7. Least-squares-fit plot showing the correlation between the efficiencies of each mutant cre to support in vitro VPg uridylylation and to function in RNA replication, based on the data presented in Fig. 4 and 6. The dotted lines represent values for replication capacity and uridylylation activity equal to 15% of the wild-type cre. The $R^2$ value obtained in a transformed regression model was 0.7685.

FIG. 8. Comparison of the sequences and predicted structures of the cress of human rhinoviruses and enteroviruses. (A) Alignments of the sequence of the HRV-14 cre with the PV-1-type 1 cre sequence (left) and the HRV-2 cre sequence (right). The brackets depict the nucleotide segments predicted by MFOLD to form the terminal loops of the cre hairpins. (B) MFOLD predictions of the structures of the cress of HRV-14, PV-1 (5), HRV-2 (4), and the predicted cress of HRV-1b and -16 (4). The conserved critical bases identified by mutational analysis of the HRV-14 cre are shown in boldface. The proposed equivalent structures in the PV-1 and HRV-2 cress are enclosed in dashed boxes. At the far right is shown the proposed common structure for HRV and enterovirus cress. R = A/G; W = A/U; M = A/C; N = any nucleotide.
quences of the originally described 96-nt cre structure (12) are required for replication of HRV-14 RNA and that the large internal loop and lower duplex stem (see ΔP1LucCRE structure in Fig. 1) are not essential. Despite this, McKnight and Lemon (12) found that some mutations in the lower stem were lethal to replication of HRV-14 RNA. Since the sequences forming the lower stem are located outside of the region, we have shown here to contain the minimal functional cre (Fig. 1), these results are best explained by changes in the folded structure of the top loop and stem of the cre mediated by mutations in the lower stem. If this interpretation is correct, the tertiary structure of the cre loop is critically important to its ability to function in RNA replication and probably also uridylylation of VPg. In general, however, it appears that a stem of 8 to 10 bp, similar to what we have determined for the minimal HRV-14 cre, is sufficient to support cre function. Disruption of the lower part of the PV-1 cre stem (leaving 9 bp at the base of the loop) had little effect on cre function (24). In addition, the predicted cardiovirus cre structures have stems formed by 8 to 10 bp (9).

We also carried out an extensive mutational analysis of the loop of the HRV-14 cre, because previous work points to its importance in RNA replication (12). The HRV-14 cre presents a unique opportunity for such a mutational analysis, since unlike the PV-1 or HRV-2 cre, it is located within the P1 region that encodes capsid proteins that are not necessary for RNA replication (12). Thus, mutations in the HRV-14 cre do not alter the amino acid sequence of proteins that contribute to the replicate. This distinguishes the cre mutants we have studied here from many of the mutations that have been studied previously in the PV-1 or HRV-2 cres (4, 17) and has also allowed us to do a more complete analysis. The fact that we found a strong correlation between the effects of these HRV-14 mutations on VPg uridylylation in vitro and on RNA replication in vivo (Fig. 7) provides additional indirect, but nonetheless strong, evidence that the ability of the cre to function as a template for VPg uridylylation is essential for viral RNA synthesis.

We found that an AAA triplet (nt 2367 to 2369, or 67A, 68A, and 69A) located in the 5′ half of the loop and 2 nt, 63G and 76A, at the bottom of the loop, are important for cre function, while there are no requirements for specific bases at other positions (Fig. 5). Any substitution within the AAA triplet abrogated RNA replication, except for the substitution of 69A with 69G. These results are reminiscent of previous studies of the PV-1 cre, in which the mutation of either of the first two A’s of the AAA triplet within the 5′ half of the loop in the PV-1 element abolished infectivity (24). Mutation of these adenosines in the HRV-2 cre also abrogated uridylylation of VPg and replication of the virus (4). This AAA triplet is part of the AAACA motif identified by Rieder et al. (24) in the PV-1 cre loop and considered to be a common feature of the cre. The mutational analysis of the HRV-14 cre shown in Fig. 4 and 6, however, demonstrates that the CA dinucleotide within the AAACA motif is not necessary for replication or uridylylation. We also found that 63G and 76A, located at the junction of the cre loop and stem, were critically important for cre activity. As with 69A, only purine transition mutations were permissible at these bases.

Alignments of the sequences around the AAA triplet in the HRV-14, PV-1, and HRV-2 cres indicate that the critical base residues we have identified in the HRV-14 cre are perfectly conserved in these other cres (Fig. 8A, boldface type). This includes the 63G and 76A bases at the junction of the HRV-14 cre loop and stem. However, in contrast to the HRV-14 loop sequence, which is predicted by MFOLD to be 14 nt in length, the homologous sequences in the PV-1 and HRV-2 cres are predicted to contain several base pairs and an internal loop and to fold differently from HRV-14 as well as each other (Fig. 8B). These structure predictions have not been tested by any physical means, however, nor by appropriate mutational analyses, so their validity is unknown. Despite the differences in the computer-predicted secondary structures, we suspect that these sequences are similarly structured in all three cres. Such structural homology would make the observation that these cres are functionally exchangeable with each other in uridylylation reactions understandable (4, 17). Alternatively, it is possible that the binding of proteins such as 3CD, 3D, and VPg to the PV-1 and HRV-2 elements in the earliest steps of the uridylylation reaction might cause a change in the conformation of the RNA, opening up internal base pairs so that the PV-1 and HRV-2 structures resemble that of the HRV-14 cre.

Gerber et al. (4) identified potential cres in two rhinoviruses that are closely related to HRV-2, HRV-16 and HRV-1b. These proposed cre sequences were located at essentially the same position in the genome as the HRV-2 cre. Again, the bases that we found to be essential for HRV-14 cre activity are conserved in these putative cres, including 63G and 76A at the junction of the loop and the stem. It is interesting to note, however, that in the proposed HRV-16 cre, the AAA triplet is replaced with AAG. We found this substitution to be functional in the HRV-14 background, both for replication and for uridylylation (Fig. 4 and 6).

Based on our mutational analysis of the HRV-14 cre and comparisons of the HRV-14 cre sequence with the cres of other viruses, we propose a common motif for the loop segment of rhinovirus and enterovirus cres that likely defines a common structure (Fig. 8B): R<sup>3</sup>NNNAAR<sup>2</sup>NNNNNNR<sup>3</sup>.

We predict that an AAR triplet will be present in the 5′ half of the loop sequence in all these replication elements. At the third base position of this triplet (R<sup>2</sup>), the base may be either A or G, but there is clearly a preference for A. This is also the case for R<sup>3</sup>, while a G is preferable at R<sup>1</sup>. R<sup>1</sup> and R<sup>2</sup> are located at the extreme ends of the loop and seem likely to be involved in a non-Watson-Crick base pair interaction (see below). At the remaining positions, A, C, G, or U appears to be equivalently acceptable for both replication and uridylylation, and the specific base present is likely to be determined more by the requirement for coding specific amino acids than by a requirement for preservation of cre function. However, a mutation involving the substitution of 4 contiguous bases within the loop sequence (69A through 72A) was found previously by McKnight and Lemon (12) to be lethal for replication, even though we have shown here that each of the individual base substitutions in this mutant was permissive for replication and uridylylation. It is likely that the failure of the multiple-substitution mutant to function as a cre was due to perturbation of the structure of the loop following substitution of such a large proportion of the bases within it.

All of the known cardiovirus cre sequences also possess an AAA motif that is predicted to be at least partially single...
stranded in these viruses. A mutation at the second A of this motif in the Theiler’s virus cre was shown to be lethal for RNA replication (9). However, the cardiovascular cre appears to have a very different structure from that of the PV-1 or rhinovirus cre, because the homologous AAA triplet in Theiler’s virus appears to be located within a small bulge-loop that is part of a larger hairpin structure (9). This difference in structure is consistent with the failure of the HRV-14 cre to substitute for the cardiovascular cre in supporting viral replication (9), as well as the greater phylogenetic distance between the cardiovascular and these other picornaviral genera.

Studies by Paul et al. (17) and Gerber et al. (4) have shown that the PV-1 cre acts as the primary template for VPg uridylylation in vitro, a reaction that requires only synthetic VTP, UTP, purified PV-1 RNA, PV-1 RNA polymerase 3Dpol, and Mg2+. Mutations that abolish the ability of the PV-1 or HRV-2 cre to act as template for VPg uridylylation in vitro also eliminate their ability to support viral RNA replication in vivo (4, 17). Substitution of the AAA triplet in the HRV-2 cre with CAA also led to a change in the specificity of the nucleotidylation reaction, with the covalent addition of guanine to VPg leading to VPg-pG (4). These observations suggest that the AAA triplet functions as a template for the nucleotidylation of VPg by using a slide-back mechanism in which the most 5′ adenosine of the AAA triplet templates the nucleotide to be linked to VPg. This indicates that the conserved AAR2 residues within the loop of the rhinovirus and enterovirus cre are likely to be located on the surface of the folded RNA structure. The conserved 63G and 76A residues (Fig. 5B) have the potential to form a non-Watson-Crick closing pair at the base of the loop. Recent studies of synthetic RNA aptomers suggest that the presence of a GA closing pair significantly influences the structure of the adjacent RNA loop and may have a critical role in determining the ability of the loop to form stable loop-loop interactions (2). While there is no evidence that the cre loop is involved in a loop-loop interaction, we speculate that the conserved 63G and 76A residues form such a closing pair and that the presence of this closing pair contributes in an important way to the structure of the cre loop that is required for proper presentation of the AAR2 triplet as the template for uridylylation.

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