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An RNA Tertiary Structure in the 3′ Untranslated Region of Enteroviruses Is Necessary for Efficient Replication

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RNA tertiary structures, such as pseudoknots, are known to be biologically significant in a number of virus systems. The 3′ untranslated regions of the RNA genomes of all members of the *Enterovirus* genus of *Picornaviridae* exhibit a potential, pseudoknot-like, tertiary structure interaction of an unusual type. This is formed by base pairing between loop regions of two secondary structure domains. It is distinct from a potential, conventional pseudoknot, studied previously in poliovirus, which is less conserved phylogenetically. We have analyzed the tertiary structure feature in one enterovirus, coxsackievirus A9, using specific mutagenesis. A double mutant in which the potential interaction was destroyed was nonviable, and viability was restored by introducing compensating mutations, predicted to allow the interaction to reform. Phenotypic pseudorevertants of virus mutants, having mutations designed to disrupt the interaction, were all found to have acquired nucleotide changes which restored the potential interaction. Analysis of one mutant containing a single-base mutation indicated a greatly increased temperature sensitivity due to a step early in replication. The results show that, in addition to secondary structures, tertiary RNA structural interactions can play an important role in the biology of picornaviruses.

Enteroviruses make up one genus of the *Picornaviridae*, a diverse family of small viruses including several of clinical or economic importance such as polioviruses, hepatitis A virus, rhinoviruses (the major cause of the common cold), and foot-and-mouth disease virus of cattle (22, 27). They have a positive-sense, single-stranded RNA genome, 7,100 to 8,500 nucleotides long, which is 3′ polyadenylated. The genome encodes one polyprotein which is cleaved by virus-encoded proteases to yield several proteins required for replication and assembly of new virus particles. RNA replication proceeds via the synthesis of negative-sense copies of the virus genome, which then serve as templates for the synthesis of new positive strands. The single open reading frame is preceeded by a long (ca. 750 nucleotides in enteroviruses) 5′ untranslated region (5′UTR) which is followed by a much shorter (70 to 100 nucleotides in enteroviruses) 3′UTR and the poly(A) tract (22). The 5′UTR has two major structural and functional domains (20). The 5′-most 90 nucleotides fold into a cloverleaf configuration essential for positive-strand synthesis, while much of the rest of the 5′UTR comprises the internal ribosome entry site (IRES), which is involved in cap-independent translation initiation (1, 14). IRES function depends on specific sequences and its complex secondary structure (14).

Despite the fact that the 3′UTR probably plays a role in RNA replication, since it must be in close proximity to the site of initiation of negative-strand synthesis, it has been comparatively little studied. The 3′UTR of all enteroviruses appears to fold into the same core structure consisting of two stem-loop domains, X and Y (10, 16). An additional domain, Z, is seen in two of the 5 enterovirus genetic subgroups which have been described previously (18, 19). Covariance between enterovirus sequences, maintaining domains X and Y, is strong evidence that these are significant, and there is direct support from structure-probing experiments (16). It has been pointed out that the loop regions of domains X and Y have the potential to participate in a phylogenetically conserved tertiary structure interaction, involving Watson-Crick base pairing, which is similar to a pseudoknot (16). This is a less common structural motif than a typical pseudoknot, and its further study is thus of general importance to an understanding of RNA tertiary structures. In addition, an alternative, but less phylogenetically conserved, typical pseudoknot has been proposed to occur in the 3′UTR of one enterovirus, poliovirus (11). An understanding of the structure and function of the enterovirus 3′UTR requires the determination of which, if either, of these alternative structures is correct. We describe here experiments on the enterovirus coxsackievirus A9 (CAV-9) which provide genetic evidence that the phylogenetically conserved tertiary structure plays a critical role in the replication of enteroviruses.

MATERIALS AND METHODS

Virus cDNA. All mutants were produced from pCAV-9, a full-length cDNA representing the complete CAV-9 genome, contained within the vector pBS (3, 9).

Oligonucleotides. A PCR mutagenesis procedure was used to make most of the mutants. Two overlapping regions of pCAV-9 were amplified with one universal, outer primer (OL256, based on the genomic sense sequence at positions 7099 to 7118 or OL419, complementary to the CAV-9 poly(A) tract and containing a *Mlu*I site and an *Xho*I site) and one of a pair of specific, inner primers. The latter were complementary and contained the specific mutation to be introduced. The oligonucleotides used were:

General	OL256	5′ GAGCAGATGAACAATACCCC 3′
	OL419	5′ GAGAGACTCGAGACGCG(T)16 3′
dm	OL365	5′ CTGTACTAA(A,C)(A,C)GAACTAGATAACG 3′
	OL366	5′ CGTTATCTAGTTC(A,C)(A,C)TTAGTACAG 3′
y4C	OL581	5′ CTGTACTAACC(C,A,T)AACTAGATAACG 3′
	OL582	5′ CGTTATCTAGTT(G,T,A)GGTTAGTACAG 3′
y3C and	OL534	5′ CTGTACTAAC(A,G,C)GAACTAGATAACG 3′
y3A	OL535	5′ CGTTATCTAGTTC(A,C,T)GTTAGTACAG 3′
XA and	OL623	5′ GCAGTAGGGGTAAATTCT(C,A)AGCATTCGGTGCGG 3′
XAA	OL624	5′ CCGCACCGAATGCT(T,G)AGAATTTACCCC 3′

In each case, the altered nucleotide is underlined and mixed nucleotides introduced at certain positions are indicated in parenthesis. The overlapping amplicons were then subjected to a second PCR amplification to produce a continuous fragment of DNA. This fragment was cleaved with *Bam*HI (at a site within

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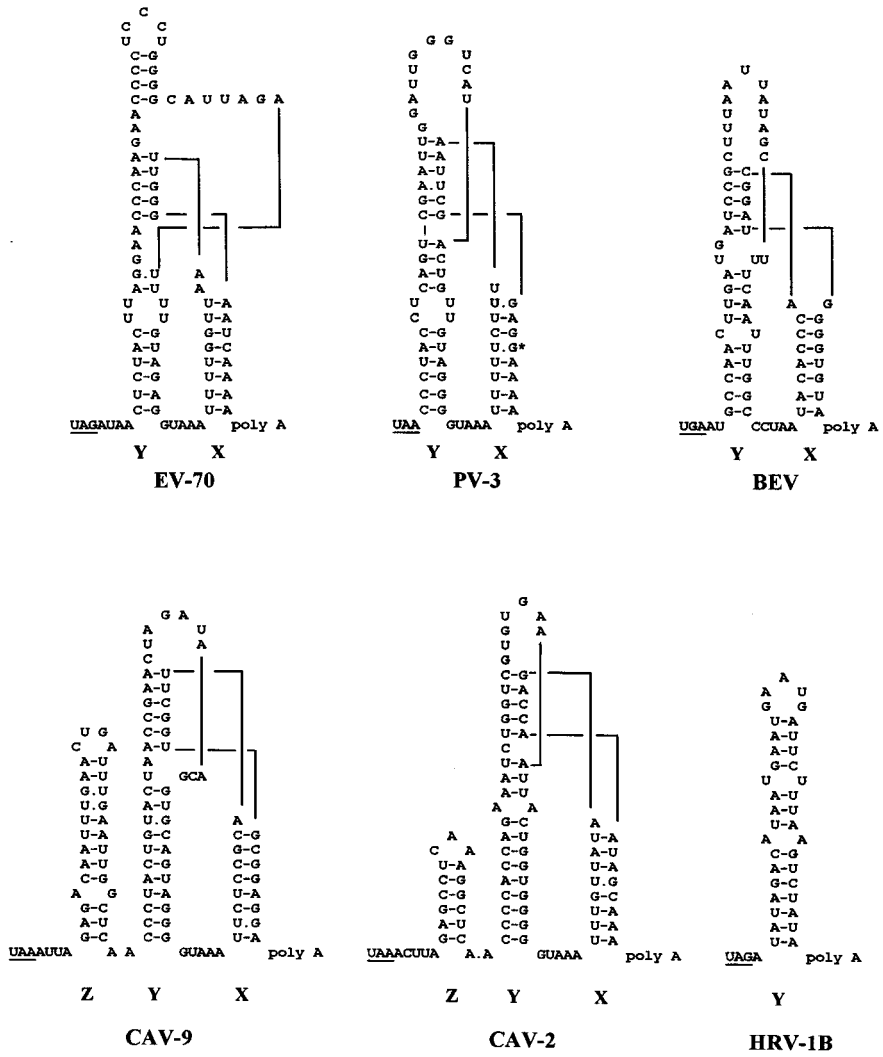

FIG. 1. Schematic representation of the proposed secondary and tertiary structures of the 3′UTR of viruses exemplifying the five genetic groups identified among enteroviruses, together with HRV-1B (15, 17, 18). Abbreviations: PV-3, poliovirus type 3; EV-70, enterovirus 70; BEV, bovine enterovirus. The asterisk in the PV-3 structure marks a nucleotide difference seen between the Leon (A) and Sabin (G) strains of this virus serotype (25).

CAV-9 cDNA at position 7178) and *Xho*I (introduced into the PCR product) and cloned into these sites in the vector pUBS. The mutated fragment was completely sequenced to ensure the presence of only the required mutation and then removed from the vector using *Bam*HI and *Xho*I and inserted into pCAV-9, which had been digested with *Xho*I and partially digested with *Bam*HI. The sequence of this region in the mutated pCAV-9 was then reconfirmed.

Some of the mutants were produced by an alternative strategy which made use of a *Bsm*I site close to the 3′ terminus of CAV-9 cDNA (position 7435). A *Bam*HI-*Xho*I subclone was cleaved with either *Bsm*I and *Xho*I or *Bsm*I and *Cla*I, and pairs of annealed, complementary oligonucleotides were introduced by ligation. The manipulated subclone was then introduced into pCAV-9. The oligonucleotides used in this strategy were:

dmrep	OL471	5′ CATTC(A,G)(C,G)TGCGGAGG(A)15CGCG 3′
	OL472	5′ TCGACGCG(T)15CCTCCGCA(C,G)(C,T)GAATGCG 3′
x4A and	OL455	5′ CATT(A,G,T)GGTGCGGAGG(A)15T 3′
x4G	OL456	5′ CGA(T)15CCTCCGCACC(A,C,T)AATGCG 3′

Virus generation and propagation. All transfections and experiments based on virus propagation were performed in 25 cm^2 tissue culture flasks, containing monolayers of GMK cells. These were maintained in 7 ml of minimal Eagle's medium (MEM) containing 10% heat inactivated fetal bovine serum, 1% MEM amino acids, and gentamycin (100 mg/liter). RNA transcription from the mutated derivatives of pCAV-9, using T7 polymerase followed by transfection into GMK cells was as described previously (9). Well-separated plaques were picked and passaged once in GMK cells. Viruses were liberated from infected cells by freeze-thawing three times, and the resultant virus-containing suspension was used directly for analysis.

Sequence analysis of mutant and revertant viruses. RNA isolation (from 100 μl of the virus suspension), reverse transcription, and PCR were as described previously (6). The PCR product was purified by agarose gel electrophoresis and sequenced by means of a commercial cycle sequencing kit (Gibco BRL), using the primer OL441 (5′ CAGGAGCGTCCCAGTTGG 3′), complementary to positions 7271 to 7292 of CAV-9 cDNA.

Growth curve and temperature shift experiment. To derive growth curves, equal quantities (10^5 PFU) of C9wt or C9y3G in 0.5 ml of growth medium were adsorbed to several GMK monolayers at 20°C for 60 min. Unbound viruses were removed by washing with medium, and the monolayers were incubated at 37°C to allow virus replication, which was monitored by freeze-thawing three times, followed by plaque assay. A temperature shift experiment was performed in the same way except that after adsorption and washing, the infected monolayers were incubated at 37°C before being shifted to 39.5°C. Incubation was carried out for a total of 9 h (C9wt) or 12 h (C9y3G), and the level of replication was assessed by plaque assay.

RESULTS

3′UTR structure in enterovirus genetic groups. The predicted RNA secondary structures of the 3′UTR of representative enteroviruses and of a typical human rhinovirus (HRV-

1B) are shown in Fig. 1. This is based on previously published models for which there is considerable phylogenetic evidence and biochemical data in the case of two members of the *Enterovirus* genus (10, 11, 16). The structures represent each of the five enterovirus genetic subgroups defined recently, and within each group there is a high degree of sequence and complete secondary structure conservation (18, 19). In each case, proximal to the poly(A) tract there are two stem-loops, X and Y, and stem-loop X includes nucleotides from the poly(A) tract itself. Three of the genetic groups, exemplified by polioviruses, enterovirus 70, and bovine enteroviruses, exhibit only these features and differ in the size of stem-loop Y (enterovirus 70) or the primary sequence between the stem-loops (bovine enterovirus). Two of the genetic groups, exemplified by CAV-9 and CAV-2, have an additional stem-loop (Z), which differs in size between the two groups and is located upstream of domain Y. Rhinoviruses have a single stem-loop, possibly equivalent to Y (16, 30, 31). Members of all the enterovirus genetic groups exhibit the potential for a tertiary structure interaction, involving either 5 or 6 bp, between nucleotides in the loops of stem-loops X and Y (shown diagrammatically in Fig. 1). It is interesting that in each genetic group the sequences involved are quite different, yet the potential interaction is evident, which is strong phylogenetic evidence that it is significant. Within a genetic group, the sequences making up the interaction are conserved (data not shown).

Mutagenesis and repair of the tertiary structure interaction. In order to study the significance of the tertiary structure interaction, we have exploited the infectivity of RNA transcribed from a complete cDNA copy of the RNA genome of picornaviruses. Mutations were introduced into CAV-9 cDNA, and their effect was monitored by determining whether the corresponding RNA gave rise to infectious virus and, if so, analyzing the properties of the mutant viruses. Mutant cDNAs were produced by PCR mutagenesis, using synthetic primers to introduce specific changes. Manipulated fragments were completely sequenced to ensure the absence of undesired mutations and were reintroduced into the full-length cDNA. RNA was transcribed and transfected into the green monkey kidney cell line GMK, in which CAV-9 replicates efficiently. The mutant RNAs initially produced, together with the effect of the mutations on virus viability, are summarized in Fig. 2. For convenience, the nucleotides involved in the CAV-9 interaction are denoted y1 to y6 for those in loop Y and x1 to x6 for their complements in loop X. It can be seen that mutations which disrupt the potential interaction have a profound affect on the viability of CAV-9. In both cases tested, single mutations in the x3 position (C→A or C→G) gave rise to RNA which, despite several transfections, yielded no infectious virus. This indicates that these single nucleotide substitutions are lethal. A double mutant RNA, dm (positions y2 and y3, CC→GU), was also produced and shown to be noninfectious, again following several transfections. However, when this construct was modified by the introduction of two additional, complementary mutations (positions x2 and x3 GG→CA), the transcribed RNA, dmrep, proved to have an infectivity comparable to that of wild-type CAV-9 RNA (3×10^3 virus plaques formed per μg of RNA) and produced viruses of a wild-type plaque phenotype (Fig. 2). Two virus plaques were picked, passaged once in GMK cells, and analyzed, following reverse transcription-PCR, by cycle sequencing. In the region sequenced (position 7300 to the 3′ terminus), the virus genomes exhibited the four mutations specifically introduced (positions y2 and y3, CC→GU, and x2 and x3, GG→CA) and no other differences from the original CAV-9. The results indicate that the introduction of the two additional mutations,

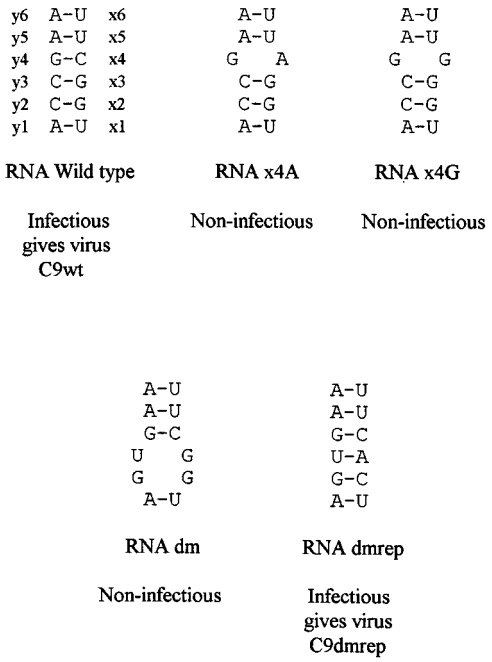

FIG. 2. Simplified version of the tertiary structure interaction between the loops of domains X and Y in CAV-9. x1 to x6 and y1 to y6 denote the nucleotides in loops X and Y, respectively, which take part in the interaction. The locations of several mutations in mutated constructs and their effects on virus viability are also indicated.

designed to repair the interaction between domains X and Y, restores the viability of the virus. This suggests that the tertiary structure interaction plays an important role in CAV-9 replication.

Analysis of CAV-9 pseudorevertants. Pseudorevertants, in which a wild-type phenotype is restored or partially restored by second-site mutations, have proved useful for the demonstration of specific RNA-RNA and RNA-protein interactions in picornaviruses. Since no pseudorevertants were obtained from the noninfectious RNAs described above, two other mutant cDNA constructs were produced as described before, and RNA was transcribed. These were y3G (position y3, C→G) and y3A (position y3, C→A), and both proved to be highly infectious. Three plaques were picked from each transfection, and the viruses were passaged once in GMK cells. Upon analysis of passaged virus, all three y3C viruses and two y3G viruses had a wild-type plaque size phenotype, while one virus derived from y3G (denoted C9y3G) had a small plaque phenotype. All the passaged viruses were sequenced from position 7300 to the 3′ terminus, and mutations were observed only in the region of the proposed tertiary structure interaction as shown in Fig. 3. The genomes of all viruses derived from y3A had an identical sequence, which differed from that of the transfected RNA at the mutated position (y3, C→A, was introduced into y3A and an A→U change was observed at this position). This mutation restores the proposed tertiary structure interaction through the formation of a U · G base pair (Fig. 3). The small plaque virus from y3G, C9y3G, had maintained the mutation introduced (position y3, C→G), and had no other mutations in the region sequenced. Both large plaque viruses from y3G were identical, unusual revertants, which had retained the mutation introduced (position y3, C→G) but exhibited an additional mutation two bases upstream (position y1, A→C). One of these, denoted C9y3Gy1C, was chosen for further study. Curiously,

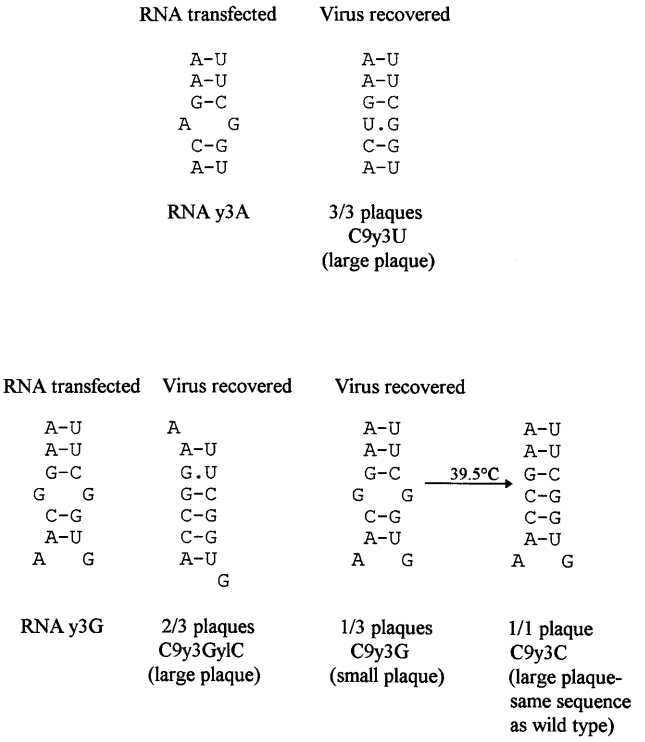

FIG. 3. Summary of the point mutations introduced into the y3 position of the tertiary structure interaction seen in CAV-9 RNA, together with the sequences of the viable viruses which resulted. The revertant C9y3C, which has the wild-type sequence, was derived from the mutant C9y3G by growth at 39.5°C.

analysis of the sequences involved indicates that they now have the potential to be involved in an alternative tertiary structure interaction, using the same loop X nucleotides but effectively shifted one nucleotide upstream with respect to loop Y (Fig. 3). The structure predicted is therefore between y0 to y5 and x1 to x6, where y0 is the nucleotide immediately preceeding the loop Y region involved in the wild-type interaction.

To extend the analysis, a further mutant cDNA clone (y4C) was produced which had a mutation at the y4 position (G→C). Repeated transfection of y4C gave only one plaque, the virus from which was passaged in GMK cells. Sequence analysis from position 7300 to the 3′ terminus indicated that the virus had retained the mutation introduced (position y4, G→C) but exhibited a further, complementary change at the corresponding position in the X domain (position x4, C→G), presumably allowing the tertiary structure interaction to reform (Fig. 4). This virus also had one further change (C→U at genomic position 7433), predicted to be in the stem of domain X but maintaining this structure through a U · G base pair (data not shown). Thus, although different patterns of reversion were observed, there is a correlation between efficient virus replication, in terms of plaque size, and the potential to form the tertiary structure interaction.

Growth properties of a mutant with a disrupted tertiary structure. The properties of the mutant and revertant viruses obtained are summarized in Table 1. It can be seen that, in terms of plaque size, the revertants are comparable to the unmanipulated virus, C9wt, while C9y3G has a smaller plaque size. The viruses were also analyzed with respect to temperature sensitivity by plaque assay at 34, 37, and 39.5°C. All, including C9wt, demonstrated some temperature sensitivity, since there was a significant reduction in plaque number at

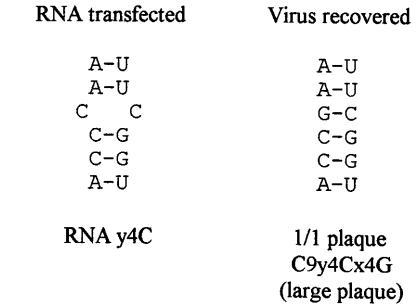

FIG. 4. Predicted structure of the noninfectious RNA produced by mutation of the y4 position of the tertiary structure interaction and of the viable pseudorevertant, C9y4Cx4G, which was recovered.

39.5°C compared to 34°C and 37°C (43-fold for C9wt at 39.5°C compared to that at 37°C). However, temperature sensitivity was extremely marked in the small plaque mutant C9y3G, which gave a 10^4-fold reduction in plaque numbers at 39.5°C compared to that at 34 and 37°C. The virus from a plaque arising during the assay of C9y3G at 39.5°C was propagated and found to have a wild-type plaque phenotype. Upon sequencing, a position y3 (G→C) change, restoring the wild-type sequence, was observed. Although giving a normal plaque size at 37°C, the revertant which had a shifted tertiary structure (C9y3Gy1C) was much more temperature sensitive than C9wt (Table 1).

To further study the effect of the single mutation in the small plaque mutant, C9y3G, a single-cycle growth experiment was performed (Fig. 5). The results show that the growth of C9y3G is greatly retarded, since infectious virus particles are not produced until 8 h postinfection compared to 4 h for C9wt. In addition, the yield of C9y3G is reduced (by a factor of 10^2), further indicating the radical effect of the single mutation in C9y3G.

The temperature-sensitive nature of the y3, C→G, mutation in C9y3G was exploited in temperature shift experiments, in order to determine the timing of the replication step affected by this mutation (Fig. 6). C9y3G or C9wt were adsorbed at 22°C to several GMK monolayers, and these were incubated at 37°C to initiate infection. At various time intervals, the infected monolayers were shifted to 39.5°C, a temperature nonpermissive for C9y3G. After a total time of 9 h for C9wt and 12 h for C9y3G, the cells were lysed and the level of virus replication was established by plaque titration. It can be seen that, although C9wt exhibits a degree of temperature sensitivity, since incubation at 39.5°C results in a reduced yield compared to that at 37°C, replication occurred in all flasks. In contrast, no replication of C9y3G occurred unless it was incubated at 37°C for at least 1 h before the temperature shift.

TABLE 1. Comparison of the infectivity ratio of wild-type and mutant CAV-9 strains at different temperatures, together with plaque sizes at 37°C

Virus	Titer at indicated temp switch		Plaque size (mm)
	34 to 39.5°C	37 to 39.5°C	
C9wt	3.5×10^2	4.3×10^1	1.4 ± 0.1
C9y3G	5.0×10^3	1.0×10^4	0.6 ± 0.2
C9y3Gy1C	1.5×10^3	1.3×10^3	1.0 ± 0.5
C9y3U	1.1×10^2	1.6×10^2	1.4 ± 0.4
C9dmrep	5.3×10^2	5.6×10^1	1.0 ± 0.5

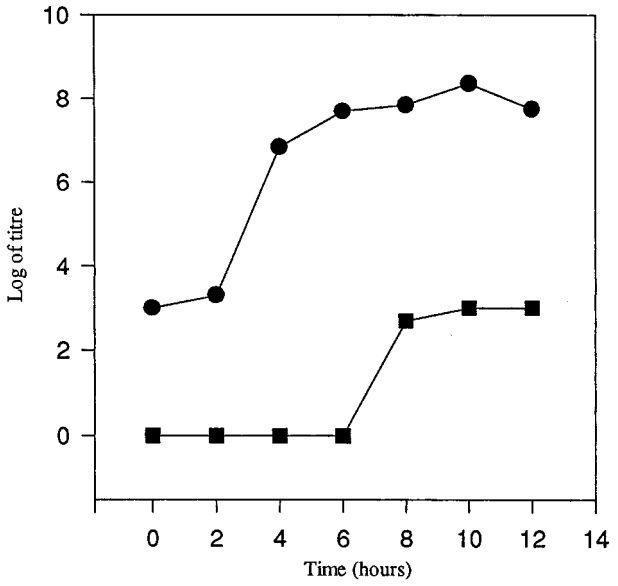

FIG. 5. Growth curves of C9wt (●) and C9y3G (■) on GMK monolayers.

Incubation at 37°C for periods greater than 2 h had little further effect on the yield of C9y3G. This experiment demonstrates that the temperature-sensitive lesion affects a step early in replication, rather than a later event such as particle assembly.

Evidence for the existence of stem X. Although there is strong phylogenetic evidence for the existence of both stems X and Y, stem X is relatively weak in many enteroviruses (Fig. 1). It is therefore possible that stem X does not form and that nucleotides interacting with stem-loop Y are not themselves in

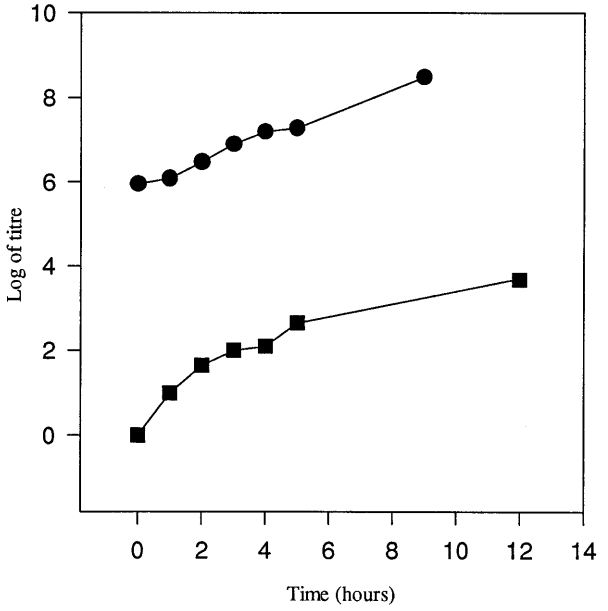

FIG. 6. Temperature shift experiment. C9wt (●) and C9y3G (■) were adsorbed to monolayers of GMK cells and then incubated at 37°C for the time indicated before being shifted to 39.5C. After a total incubation time of 9 h for C9wt and 12 h for C9y3G, the level of virus produced was monitored by plaque titration.

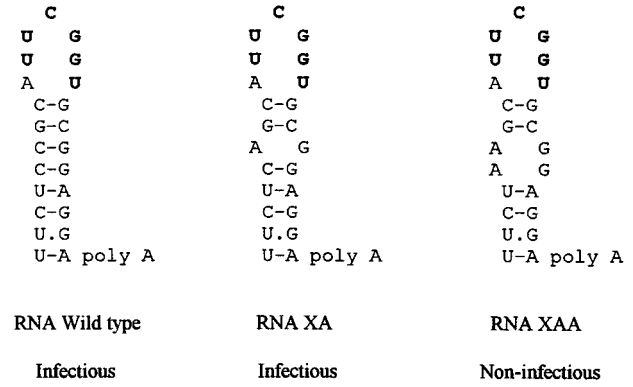

FIG. 7. Predicted structure of the stem-loop X region of wild-type CAV-9 RNA and that of single (XA) and double (XAA) mutants, designed to probe the significance of this potential secondary structure. Nucleotides which take part in the interaction with loop Y are shown in bold type.

a loop region, i.e., that the interaction is a typical pseudoknot. To determine whether stem X is functionally significant, two other constructs were produced. These had either one or two mutations designed to disrupt base pairs in stem X (Fig. 7). RNA transcribed from the single mutant (XA) was highly infectious and gave a virus which phenotypically approximated the wild type (data not shown). Several plaques were picked, and the virus was passaged once in GMK cells. All were found, by sequence analysis from position 7300 to the 3′ terminus, to have only the mutation introduced. In contrast, RNA transcribed from the double mutant (XAA) was completely noninfectious. The results imply that stem X can still form in the single mutant, despite the presence of a single mismatch, but that the structure is so weakened in the double mutant that it cannot form. The data suggest that stem X is indeed functionally significant and that the tertiary structure proposed is of a complex form, involving the loop regions of two stem-loops (16).

DISCUSSION

In many cases, RNA tertiary structure is vital to biological function (17). Frequently, non-Watson-Crick interactions stabilize these structures, a prime example being tRNA in which several such interactions maintain the compact structure (8). However, critical tertiary structures are frequently stabilized by Watson-Crick base pairs between nucleotides of a loop and residues outside that loop, giving a pseudoknot (17). This motif is important in the structure of rRNA and the control of translational regulation of ribosomal proteins in *Escherichia coli*, while a pseudoknot downstream of a slippery sequence is involved in −1 ribosomal frameshifting in several virus families (2, 17, 25, 32). 3′UTRs of plant viruses frequently harbor pseudoknots, which are required for the folding of a tRNA-like structure seen in some viruses (28). Other plant virus 3′UTRs have sequential arrays of pseudoknots which may play a role in translation or replication (17).

The secondary structure of the picornavirus 5′UTR plays an essential role in replication (24). Although conserved tertiary structural features have also been reported, as yet there is little experimental evidence that they are functionally significant (13). The 3′UTR has been less studied, but phylogenetic conservation of secondary and tertiary structures between all enteroviruses suggests that these structures are important to the life cycle of these viruses (16). The conserved features are two

stem-loops (X and Y) and a tertiary structure interaction, similar to a pseudoknot, between the loops of these domains. We have shown here that in CAV-9, mutations in sequences involved in the tertiary structure are lethal or have a radical effect on replication ability, particularly at elevated temperatures. However, the effects of a double mutation in loop Y can be suppressed by directed mutagenesis of loop X, designed to restore the tertiary structural interaction (Fig. 2). Furthermore, in some cases, pseudorevertants of loop Y mutants could be isolated and were analyzed by sequencing. In each, the reverting mutation was consistent with the recreation of the tertiary structure interaction (Fig. 3 and 4). The data therefore provide compelling evidence that the proposed tertiary structure interaction is critical for the ability of CAV-9 to replicate. Phylogenetic conservation makes it likely that this interaction plays an important role in all enteroviruses. Indeed, the introduction of four nucleotide changes into the loop Y domain has been shown to be lethal for poliovirus (15). Although no attempts were made to repair the mutant and no pseudorevertants were obtained, this effect is likely to be due to disruption of the tertiary structure interaction.

In the present work four different classes of revertant were identified: a direct back mutation (observed upon growth of C9y3G at 39.5°C), a second-site mutation in loop X compensating a mutation in loop Y, an A→U change which restores the interaction through a U · G base pair, an additional mutation which allows a new interaction shifted one base upstream (Fig. 2 to 4). In the latter two groups of mutants, the interaction is weaker than that of the wild-type virus, since they contain U · G base pairs (Table 1). This probably accounts for their increased temperature sensitivity, relative to C9wt, which is particularly evident for C9y3Gy1C. Both result from transversions, and it is not clear why these revertants appear frequently. One possibility is that a weaker loop-loop interaction may be advantageous in the experimental system employed. The lack of any viable mutants or pseudorevertants from RNA mutated at the x4 position and the generation of only a single pseudorevertant from RNA mutated at the y4 position is striking, since y3-mutated RNAs were highly infectious, producing several revertants and one viable mutant C9y3G. This suggests that disruption of the y4-x4 base pair completely prevents RNA replication and so does not allow the possibility of revertants or pseudorevertants arising (Fig. 2 and 4). In contrast, replication of RNA with a disrupted x3-y3 interaction can take place, possibly indicating that the interaction can still occur (albeit inefficiently since C9y3G grows poorly and is highly temperature sensitive) by virtue of the other base pairs in this structure (Fig. 3). Both y3-x3 and y4-x4 base pairs are near the center of the interaction, but y4-x4 may be more critical as it is adjacent to two A · U base pairs, which may not form without the strong C · G y4-x4 base pairing (Fig. 2). The quadruple mutant, C9dmrep, and the slipped interaction pseudorevertant, C9y3Gy1C, both have different loop Y sequences interacting with the loop X residues than the wild type, suggesting that tertiary structure, not primary sequence, is the critical feature involved. This is consistent with the variation in the sequence making up this structure in the five enterovirus genetic subgroups (Fig. 1).

There is strong phylogenetic support for the proposed enterovirus 3′UTR structure (Fig. 1). However, the relative weakness of stem X makes it possible that this structure does not occur and, therefore, that the region interacting with loop Y is not itself part of a loop. The mutation of nucleotides predicted to form stem X suggests that this structure is important, since a double mutant (XAA) in which two potential base pairs are removed is nonviable (Fig. 7). Using the program FOLD, the stability of stem X in the wild type, single mutant XA, and double mutant XAA is −10.0, −4.3, and −0.2 kcal/mol, respectively, showing the marked effect of the mutations introduced (33). Further evidence for the occurrence of stem X in enteroviruses, is the sequence variability seen at the 3′ terminus of some poliovirus strains. For instance, the Sabin vaccine strain of poliovirus type 3 has an A→G change relative to its parental strain Leon immediately preceding the poly(A) tract (26). In both these strains, base pairing can occur at this position through U-A or U · G interactions (Fig. 1). Other direct experimental evidence for the importance of stem-loops X and Y is the abrogation of RNA replication in poliovirus replicons when either stem-loop is precisely deleted (21). Mutations introduced into the base of stem-loop Y also had a lethal effect on poliovirus replication, indicating, in agreement with the strong phylogenetic conservation, that this stem-loop is also an important feature of the 3′UTR (15).

Evidence has been presented that in poliovirus, some of the nucleotides corresponding to both the stem and loop of Y interact with an upstream sequence located within the coding region, forming a conventional pseudoknot (11). This is based on a temperature-sensitive mutant with an 8-nucleotide insertion which reverts to a wild-type phenotype by deletion of all or part of the insertion (11, 23). Coincidentally, the original insertion is located immediately between the nucleotides making up the left hand side of the Y stem and the loop Y nucleotides which we suggest are involved in the inter-domain interaction (see the poliovirus structure shown in Fig. 1). This insertion disrupts completely the proposed conventional pseudoknot but affects only one extremity of the loop X-loop Y tertiary structure interaction and both potential structures are restored in the revertants (data not shown). The poliovirus revertant data presented do not therefore argue against the conserved interdomain tertiary structure. The conventional pseudoknot described in poliovirus has less phylogenetic support, because no analogous structure is observed in some of the enterovirus genetic groups, although a similar structure was proposed in coxsackievirus B1 (11). Furthermore, the analysis of the CAV-9 pseudorevertants described here gives no support to the conventional pseudoknot, since no second-site mutations were observed in other regions proximal to the 3′UTR and all the results can be explained in terms of the loop X-loop Y tertiary structure. The observed effect on RNA amplification in the poliovirus mutants may therefore be due to the disturbance of the interloop tertiary structure rather than disruption of the conventional pseudoknot (11).

The 3′UTR tertiary structure resembles a pseudoknot, although there is discussion whether interactions between two loop regions should be so described (17, 28). It has been suggested that the interaction allows the 3′UTR to fold into an L-shaped, tRNA-like structure (16). This would occur through stacking of the pseudoknot-like unit with either stem X or stem Y, giving a long quasicontinuous helix. The involvement of two loop regions means that the interdomain interaction is of an unusual form. The extra topological constraints may require an unstructured region near the tertiary structure and this may explain why in loop Y, downstream of y1 to y6, there are a number of unpaired nucleotides in all enteroviruses (Fig. 1).

In view of the relatively close overall structural relationship between enteroviruses and human rhinoviruses (HRVs), it is surprising that the latter have a much shorter, A/U-rich 3′UTR which is predicted to have a single stem-loop, apparently equivalent to Y (16, 31). The absence of domain X precludes a tertiary interaction. The difference may be related to distinct cell tropism and temperature optima between enteroviruses and HRVs, but its final explanation must await a fuller under-

standing of the tertiary structure in enterovirus replication. The recent construction of recombinants between poliovirus type 1 and HRV-14 is a further puzzling aspect, since it was possible to replace the complete 3′UTR of poliovirus with that from HRV-14, yielding infectious virus (21). This apparently contrasts with our results on CAV-9, in which in some cases the introduction of a single base substitution was lethal. However, mutations within the HRV-14-derived 3′UTR of this construct adversely affected virus replication (21). Taken together, the results indicate that the 3′UTRs of enteroviruses and rhinoviruses have diverged into distinct structural entities, while maintaining functional identity. They can thus be considered as modules, constrained in terms of individual sequences and structures, but functionally interchangeable. This implies that the specificity of the interaction between 3′UTRs and virus proteins is low, since the poliovirus polymerase must be able to recognize the HRV-14 3′UTR. Although superficially distinct structurally, one possible resolution of the apparent paradox raised by the CAV-9 and poliovirus-HRV work, may be stacking of stem Y and the duplex produced by interaction between loops X and Y. This may give a long helical structure, which is effectively similar to that achieved in HRVs by having a continuous stem.

Work on HRV-14 itself has indicated that the requirements for RNA structural determinants may be less stringent than are suggested by the high degree of structural conservation between HRV 3′UTRs (30, 31). It was reported that viable virus mutants with large deletions could be constructed, including one which lacks 37 of the 44 3′UTR nucleotides. All the deletion mutants had defective growth phenotypes, suggesting that even if the rhinovirus 3′UTR stem-loop is not essential, it is necessary for efficient replication. Furthermore, it should be noted that a large 3′UTR deletion abolished RNA replication in the poliovirus-HRV-14 replicon and two point mutations, which disrupted the predicted stem-loop, had a similar effect (21). These observations strongly support a functional role for the HRV stem-loop.

Our results indicate that the tertiary structure interaction plays a central role in the CAV-9 life cycle. The temperature shift experiment, using the viable but temperature-sensitive mutant C9y3G, shows that the step involving the interaction occurs early in the replication process (Fig. 6). Roles in both translation and RNA synthesis have been suggested for conventional pseudoknots identified in plant viruses and the present experiments do not formally exclude the latter possibility (5, 12). However, translation proceeds throughout most of the replication cycle in picornaviruses and hence is unlikely to be the temperature-sensitive step in the mutant C9y3G, since this can be overcome by incubation at 37°C for only 1 to 2 h prior to the 39.5°C shift. Wild-type CAV-9 cDNA and C9y3G were transcribed/translated with apparently similar efficiency in a coupled in vitro system, suggesting that the mutation has little effect on translation (data not shown). The proximity of the 3′UTR to the likely origin of negative-strand synthesis makes it probable that the tertiary structure has a role in this process. The low efficiency of growth of CAV-9, however, makes it difficult to study RNA synthesis, and this will require the generation of suitable replicons, containing efficient reporter genes, such as have been produced for poliovirus (1, 20, 21).

The present experiments do not indicate what the role of the pseudoknot might be. Binding of both cellular and virus proteins to the 3′UTR of enteroviruses, HRVs, and another picornavirus, encephalomyocarditis virus, has been demonstrated, and since pseudoknots are frequently recognized by proteins, the tertiary structure may be involved in an obligatory RNA-protein interaction (4, 7, 17, 30). Alternatively, the tertiary structure element may be important for stability of enterovirus RNA in the infected cell. Tertiary RNA structures have also been implicated in the function of catalytic RNA molecules such as group 1 introns and hepatitis delta virus (17, 28). The lack of a corresponding tertiary structure interaction in HRVs would appear to argue against a fundamental role, such as involvement in a catalytic RNA function. However, it has been reported that the 22-amino-acid peptide VPg is covalently attached to the 5′ terminus of negative- and positive-strand RNA in poliovirus by an apparently autocatalytic mechanism, and it is conceivable that the loop-loop tertiary structure interaction could be involved in such a process (29). In view of the evident importance of the tertiary structure in enterovirus replication and the unusual loop-loop interaction involved, these aspects are worthy of further study.

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