Binding of a cellular factor to the 3′ untranslated region of the RNA genomes of entero- and rhinoviruses plays a role in virus replication

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The presence of cellular factors that bind to the 3′ untranslated region (UTR) of picornaviruses was investigated by electrophoretic mobility shift assays (EMSAs). A cellular factor(s) that binds specifically the 3′ UTR of polio-, coxsackie- and rhinoviruses was detected. Furthermore, this factor(s) is distinct from those which bind to the 5′ terminal 88 nt (the ‘cloverleaf’) of poliovirus. Mutations within the 3′ UTR which decrease the affinity of the RNA for the cellular factor in EMSAs decrease RNA replication and virus viability. Revertants of these mutants display changes which are predicted to stabilize the RNA secondary structure of the 3′ UTR. These results indicate that binding of a cellular factor to the UTR plays a role in virus replication and that RNA secondary structure is important for this function.

Introduction

The entero- and rhinoviruses are closely related members of the family Picornaviridae. They possess single-stranded messenger-sense RNA genomes of approximately 7500 nt and have a common genetic organization consisting of a 5′ untranslated region (UTR) of approximately 750 nt, a single open reading frame (ORF) occupying about 90% of the genome, a 3′ UTR of approximately 50–90 nt, and a 3′-terminal poly(A) tract (Kitamura et al., 1981; Racaniello & Baltimore, 1981; Stanway et al., 1983). The single ORF encodes a polyprotein which is processed in the infected cell to produce virus structural and non-structural proteins (Wimmer et al., 1993). The roles of a number of these proteins in virus replication are known, but our understanding of the precise mechanism of virus replication and the function of all the viral and cellular proteins involved remains incomplete.

The 5′ and 3′ UTRs of the genomes have important cis-acting functions in the replication of the RNAs. Evidence to date suggests that the 5′ UTR contains elements which are involved in RNA replication. In particular, a ‘cloverleaf’ structure (CL) of poliovirus formed by the 5′-terminal 88 nt of the RNA has been shown to bind viral proteins 3AB and 3CD, and a host protein of 36 kDa, originally identified as the C-terminal fragment of the eukaryotic elongation factor, EF-1α (Harris et al., 1994). In addition, proteins of molecular mass 36 kDa that bind to the poliovirus CL and to stem-loop IV of the 5′ UTR have been identified as the poly(rC) binding proteins (PCBP1 and PCBP2) (Blyn et al., 1995; Gamarnik & Andino, 1997). Recently, a role for these cellular polypeptides in poliovirus translation has been demonstrated (Blyn et al., 1997). The bulk of the remainder of the 5′ UTR constitutes an internal ribosome entry site which allows cap-independent translation, allowing viral protein synthesis in infected cells where cellular translation is shut-off via the activity of the viral protease 2A (for review see Jackson, 1988; Jackson & Kaminski, 1995).

In contrast to our knowledge of the 5′ UTR, comparatively little is known about the role of the 3′ UTR in virus replication. We have previously shown that the 3′ UTR plays a role in the replication of enteroviruses since mutations predicted to destroy stem–loop structures in this region have a deleterious effect on virus replication (Rohll et al., 1995). Others have found evidence of tertiary structural interactions that seem to be essential for viability (Mirmomeni et al., 1997; Pilipenko et al., 1996). Determination of the precise structures required for virus function, however, is complicated by the fact that it is possible to construct viable chimeras of poliovirus containing 3′ UTRs from related viruses with very different primary and predicted secondary structures. Moreover, a virus containing a deletion encompassing most of the 3′ UTR of human rhinovirus 14 (HRV14) is viable, although it replicates poorly (Todd & Semler, 1996).
Evidence has also accumulated that the 3’ UTR of poliovirus binds viral proteins 3AB and 3CD, and a host protein(s) of 34–36 kDa (Harris et al., 1994; Todd et al., 1995). Here, we report an interaction between the 3’ UTRs of poliovirus type 3 (PV3), HRV14 and coxsackievirus B4 (CB4) with a cellular factor(s) as detected by electrophoretic mobility shift assay (EMSA). Mutations which reduce the binding to the cellular factor(s) have a deleterious effect on virus replication suggesting that the interaction plays an important role in the virus replication cycle, possibly at the level of negative-strand RNA synthesis.

**Methods**

**Construction of plasmids.** Plasmids based on a version of the vector pEMBL 18+ modified to contain a poly linker for cloning (designated pEMBLINK18+ ) were constructed by amplification of the desired UTR sequences by PCR. Positive-sense primers included a restriction site for cloning upstream of a T7 promoter region. A restriction endonuclease site for linearization prior to in vitro transcription and a downstream additional restriction endonuclease site for cloning were utilized in the negative-sense primers. The CL was amplified from the PV3 full-length cDNA using oligonucleotides 05-0007 and 34-0060 (Table 1). The product was digested with NotI and HindIII and cloned into the pEMBLINK18+ poly linker. This plasmid was linearized with SnaBI before being used as a template in a T7 reaction. The 3’ UTRs of PV3, CB4 and the mutants 286 and 287 were amplified from the UTR of their respective full-length cDNAs. The mutant AX sequence was amplified from its corresponding replicon (Rohll et al., 1995). The primers used were: 3’ PV3, 34-0061 and 34-0062; 3’ CB4, 34-0064 and 34-0063; 286, 34-0061 and 01-0286; 287, 34-0061 and 01-0287; and AX, 34-0061 and 01-0282 (Table 1). The mutant AY sequence was constructed by annealing oligonucleotides 01-0283 and 01-0284 and overhangs were filled in using Klenow DNA polymerase; the resultant insert was cloned into pEMBLINK18+, as above.

The rhinovirus 3’ UTR constructs were introduced into pEMBLINK18+ using PsI and HindIII restriction sites. The wild-type HRV14 3’ UTR was amplified from the full-length cDNA using primers 34-0083 and 34-0076 (Table 1). Rhinovirus 3’ mutants, mut 6 and mut 4, were amplified from the corresponding replicons using primers 34-0083 and 34-0076 for mut 6 (loop sequence CCUGC; see Rohll et al., 1995) and 01-0280 and 01-0281 for mut 4 (Table 1).

Full-length PV3 with mutant 3’ UTRs were constructed by transferring the XbaI–SalI fragment (nt 6265 to the 3’ terminus), from the relevant poliovirus replicon into the PV3 cDNA (Rohll et al., 1995).

**T7 transcription and purification of UTR RNA.** To produce RNA as a probe for EMSAs, standard T7 reactions were performed. Reaction mixes contained 10 mM DTT, 500 µM UTP, GTP and CTP, 1 µg linearized template, 12 µM ATP and 25 µCi [α-32P]ATP (3000 Ci/mM; Amersham), T7 RNA polymerase and buffer supplied by the manufacturer (Promega). A MEGAscript kit (Ambion) was used to produce large quantities of non-radioactive, competitor RNA. Both radioactive and non-radioactive RNAs were loaded onto 10% polyacrylamide/urea gels and purified as previously described (Mellits et al., 1990).

**EMSA.** Standard conditions for CL EMSAs were adapted from a previous report (Clarke et al., 1994) and performed as follows. First, 20 µg S10 cellular extract (Barton et al., 1995), 600 ng tRNA and reaction buffer Table 1. Oligonucleotides used in this study
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**Fig. 1.** Predicted RNA secondary structure of T7 RNA transcripts: (a) CL of PV3; (b) 3’ UTR of PV3; (c) 3’ UTR of CB4; and (d) 3’ UTR of HRV14. Bases which are added as a result of cloning are shown in bold, and are superimposed on the wild-type RNA secondary structure.

[final concentration, 10 mM Tris–HCl, pH 7.2, 0.1 M KCl, 3 mM Mg(OAc)₂, 1 mM EDTA] were mixed in a tube and allowed to incubate for 5 min at 22 °C. Competitor RNA and 0.1 ng radiolabelled RNA were added so that the final reaction volume was 7.5 µl and samples were placed at 30 °C for 15 min. Finally, 1 µl sterile glycerol was added and samples were fractionated for 1.5 h on 6% native polyacrylamide gels at 200 V and dried. Radiolabelled bands were quantified using a PhosphorImager (Molecular Dynamics).

3’ UTR EMSAs were performed as above except that cellular extract, tRNA (calf thymus; Boehringer Mannheim) and the probe were titrated so that 80% of the probe was in a bound form. Thus, 6 µg extract and 150 ng tRNA were added for PV3, 4–6 µg extract and 120 ng tRNA for CB4, and 10 µg extract and 250 ng tRNA for HRV14. This ensured comparable molar quantities for the different probes for a given amount of cellular extract.

**In vitro transcription and transfection of recombinant viruses.** In vitro transcription of full-length constructs was carried out as described previously (Van der Werf et al., 1986). Samples of the transcription reaction mixtures were fractionated by agarose gel electrophoresis to analyse and quantify yields. Similar quantities of RNA from each reaction were used, typically 2–4 µg from a reaction containing 1 µg template DNA. Tenfold serial dilutions of RNA were transfected to obtain plaques (Evans & Minor, 1991), with the modification that the transfection buffer was HBSS (10× stock contains 50 g HEPES, 80 g NaCl, 3.7 g KCl and 1.25 g/l Na₂HPO₄, 2H₂O, pH 7.05), plus final concentrations of 1 g/l glucose and 0.5 g/l DEAE dextran.

**Sequencing of recovered viruses.** Viruses were plaque-purified and RNA-extracted as described previously (Evans & Minor, 1991). The RNA sequence of the 3’ UTR of recovered viruses was assessed by direct sequencing of PCR products obtained from reverse-transcribed PCRs. The primer used for reverse transcription and negative-sense synthesis was 06-0051, that for positive-sense synthesis was 34-0086, and that for sequencing was 34-0003 (Table 1).

**Results**

The 3’ UTRs of entero- and rhinovirus interact specifically with a distinct cellular factor

Previous studies on the 3’ UTR and the extreme 5’ 88 nt of the 5’ UTR of poliovirus (the CL structure) have provided evidence that both these regions interact with viral proteins 3AB and 3CD (Harris et al., 1994; Xiang et al., 1995). The CL has also been shown to bind to a cellular factor of 36 kDa (p36), which was observed to promote binding of the viral protein 3CD (Andino et al., 1993). Recently, UV cross-linking experiments have suggested that a cellular factor(s) of a similar size (approximately 34–36 kDa) binds to the 3’ UTR and is found at increased levels in infected cells (Roehl & Semler, 1995). Whether the 3’ and 5’ UTRs bind the same factor or whether there are distinct factors of similar molecular masses is not clear.

To study the interaction between cellular factors and the CL of PV3 and the 3’ UTRs of PV3, CB4 and HRV14, we first cloned the UTRs together with a promoter for T7 RNA
polymerase into the vector pEMBLINK18+ (see Methods). The predicted RNA secondary structures of the resultant transcripts as predicted by the program RNAFOLD (Zucker & Stiegler, 1981) are shown in Fig. 1. We looked for evidence of factor(s) binding to either the 5’ or 3’ UTRs using EMSAs on S10 extracts prepared from uninfected HeLa cells. We observed that the extracts contained factors that bound to both the CL of PV3 and the 3’ UTRs of PV3, HRV14 and CB4, as shown by a retardation of the electrophoretic mobility of the probe in the presence of HeLa cell extract (Fig. 2a, b and c, lanes 1, 2). The complex formation with the CL has been examined in detail. The complex is sensitive to the addition of protease K, SDS and RNase A (data not shown). The addition of 33-, 100- and 333-fold molar excess of unlabelled tRNA does not disrupt complex formation, whereas the addition of corresponding molar excesses of unlabelled CL significantly reduces the amount of radiolabelled probe in the complex (Fig. 2a, lanes 3–8). The addition of a similar molar excess of PV3, CB4 or HRV14 3’ UTRs does not compete for radiolabelled CL in the complex (Fig. 2a, lanes 9–14; and data not shown).

We then examined the complexes formed with the radiolabelled 3’ UTR probes of CB4, HRV14 and PV3. Retarded complex(s) were detected (Fig. 2b, lanes 1, 2; c, lanes 1–3; and data not shown) that, like those formed with the CL, were sensitive to addition of protease K, SDS and RNase A. The CB4 3’ UTR probe forms several complexes of which the slowest migrating is most prevalent and specific as it is not disrupted by 10-, 33-, 100- or 333-fold molar excess of tRNA (Fig. 2b, lanes 3–6) or 10-, 33-, 100- or 333-fold molar excess of CL (Fig. 2b, lanes 11–14), but is disrupted by addition of non-radiolabelled CB4 RNA 3’ UTR (Fig. 2b, lanes 7–10). The HRV14 3’ UTR probe forms two distinct complexes with the cellular extract; complex b is of higher affinity and is faster migrating and complex a probably represents a low-affinity binding complex, since free probe can be first competed from complex b and then from complex a (Fig. 2c; and data not shown). Complex b formation could not be dissociated by excess tRNA or excess CL RNA, but was dissociated by homologous RNA (Fig. 2c). Similar results were obtained using the 3’ UTR of PV3 as a probe. These results suggest that HeLa cells contain a cellular factor(s) that specifically recognizes the 3’ UTRs that is separate and distinct from the factor(s) which bind to the CL.

Mutations in the 3’ UTRs of entero- and rhinovirus affect host factor binding

We have previously reported the construction of a PV3/HRV14 chimera which possessed the body of the PV3 and the 3’ UTR of HRV14. This chimera is viable and its replication is similar to that of wild-type poliovirus (Rohll et al., 1995). This indicates that the 3’ UTRs of PV3 and HRV14 are functionally interchangeable and suggests that they interact with the same cellular factor. To investigate this, competition experiments were performed that were similar to those described above. We demonstrated that non-labelled poliovirus 3’ UTR competes equally as well as HRV14 3’ UTR for formation of the HRV14 complex, indicating that they do indeed interact with a common factor (Fig. 3, compare lanes 4–7 with lanes 8–11). The relevance of this interaction to virus replication was investigated by studying a series of mutants. Mutations introduced into the HRV14 sequence were designed to better define the RNA structure required for factor binding. Fig. 4(a, b) illustrates two mutants, mut 4 and mut 6, which contain perturbations in the stem and hairpin loop of the 3’ UTR, respectively. Structure modelling suggests that, although mut 4 has an altered primary sequence, its secondary structure remains intact. However, mut 6, which was designed to replace sequences in the hairpin loop, is predicted by modelling to have a rearranged RNA secondary structure (Fig. 4c). In our previous study, both of these mutations were shown to have dramatically reduced replication potential in a replicon version of the chimera (Rohll et al., 1995). We therefore analysed these replication deficient mutants for their ability to compete for binding with the host cell factor(s) in EMSAs, and quantified the results. As illustrated in Fig. 4(d), RNAs constructed from these mutants had a reduced ability to compete with wild-type probe for the cellular factor suggesting that their affinity for the factor was significantly reduced. Greater than 3-fold of the mutated RNA was required to displace 50% of the bound radiolabelled probe compared to the wild-type RNA.

We next examined the interaction of the cellular factor with the PV3 3’ UTR by analysing mutants in which one or other of the stem–loops of this double stem–loop structure (Fig. 4a, b) had been deleted (mutants ∆Y and ∆X). These mutations severely decreased the replication capacity of a PV3 replicon (Rohll et al., 1995). To determine whether these deletions affected the interaction with the cellular factor, the RNAs derived from these mutants were used as competitors in the EMSA. As can be seen from Fig. 5(c), the RNAs were much less able to compete for the binding of the cellular factor than wild-type PV3 3’ UTR. Approximately 10-fold more mutant RNA was required for a 50% displacement of the labelled probe, indicating that the mutants had reduced affinity for the host cell factor.

It has been reported by others that mutations which affect tertiary interactions (‘kissing’ mutants) in the 3’ UTR of poliovirus destroy virus viability (Mirmomeni et al., 1997; Pilipenko et al., 1996). We, therefore, constructed similar mutations aimed at disrupting the tertiary interaction, to assess whether the non-viability correlated with a decreased affinity for the cellular factor in the EMSAs (Fig. 6a, b, c). We reconstructed a mutant described by Pilipenko et al. (1996) which disrupts tertiary or kissing interactions (mutant 286) (Fig. 6b) as well as a more drastic mutation further destroying the kissing interaction (mutant 287) (Fig. 6c). Quantification of the EMSAs shows that non-labelled RNAs prepared from these mutants were again less able than wild-type RNA to
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Fig. 2. EMSAs comparing the ability of 5' and 3' UTRs to compete for each other. (a) Radiolabelled CL incubated alone (lane 1), in the presence of HeLa cell S10 extract (lane 2), or in the presence of HeLa S10 extract and 33-, 100- and 333-fold molar excess, relative to probe, of tRNA (lanes 3–5), CL (lanes 6–8), and the 3' UTRs of PV3 and CB4 (lanes 9–11 and 12–14, respectively). (b) Radiolabelled CB4 3' UTR incubated alone (lane 1), in the presence of HeLa cell S10 extract (lane 2), or in the presence of HeLa S10 extract and 10-, 33-, 100- and 333-fold molar excess, relative to probe, of tRNA (lanes 3–6), CB4 3' UTR (lanes 7–10) and CL (lanes 11–14). (c) Radiolabelled HRV14 incubated alone (lane 1), in the presence of HeLa cell S10 extract (lanes 2 and 3) or in the presence of HeLa S10 extract and 10-, 33-, 100- and 333-fold molar excess, relative to probe, of tRNA (lanes 4–7), HRV14 3' UTR (lanes 8–11) and CL (lanes 12–15).

Taken together, the experiments discussed above suggest that mutations in the 3' UTR that alter the RNA secondary and tertiary structure affect the affinity for the cellular factor in the EMSAs and decrease replication potential for the virus replicons.

Mutations which alter binding to the cellular factor also affect plaque viability

To assess the effects of 3' UTR mutations on virus viability, the debilitated mutants ΔY and ΔX of poliovirus and mut 6 of rhinovirus were reconstructed into a full-length infectious clone of PV3. Transfection of RNA from the mutants ΔY and ΔX produced virus that caused pinprick-size plaques on HeLa cells indicating that these mutations, consistent with their effects on the replicons, had a deleterious effect on virus replication (Fig. 5, compare d with e and f). Similarly, mut 6, which caused a perturbation of the loop of HRV14 3' UTR, had a drastic effect on virus viability. The infectivity of the RNA was much reduced compared with wild-type T7 RNA with plaque titre/µg of RNA being approximately 10^{-3}-fold lower, suggesting that reversion mutations had occurred. In this case, however, the plaques were almost wild-type size. Two
independent plaques from the transfection experiment were therefore isolated and sequenced. Both recovered viruses displayed single U → C transitions in the apical stem of the HRV14 structure and, in both cases, the predicted effect of reversion is to stabilize the RNA, forming G–C rather than G–U pairs. This has the overall effect of reforming the wild-type RNA structure, albeit using different sequences (Fig. 4e).

Discussion

The objectives of this study were to obtain further information on the role of the 3’ UTR in the replication of entero- and rhinoviruses. In particular, we wished to determine whether cellular factors known to bind the CL were distinct from those which bind to the 3’ UTRs of these viruses and, if so, whether they play a role in virus replication. Proteins binding to the terminal 5’ and 3’ regions are likely to be involved in positive- and negative-strand RNA synthesis, respectively, and the viral polymerase precursor, 3CD, along with the viral protein 3AB have been shown to complex with both regions. The data presented here suggest strongly that the factor(s) which binds the 3’ UTR is distinct from those that bind the CL. This was indicated by EMSA competition reactions (Fig. 2) which established that excess 3’ UTR RNA was unable to compete for binding to cellular the factor(s) bound to CL; reciprocally, excess CL did not compete for factors bound to radiolabelled 3’ UTR probes.

The factors that bind to CL have been characterized previously in some detail. The eukaryotic elongation factor EF-1α was identified by microsequencing after being isolated from a complex with the CL (Harris et al., 1994). Other studies have identified two closely related cellular poly(rC) binding proteins, PCBP1 and PCBP2, which were originally identified as binding to stem–loop IV of poliovirus 5’ UTR, as also being capable of binding the CL (Blyn et al., 1995; Gamarnik & Andino, 1997). These proteins also play a role in regulation of translation during poliovirus translation (Blyn et al., 1997).

UV cross-linking studies have previously identified a presumed cellular factor of approximately 34–36 kDa that binds to the 3’ UTR of rhinovirus and poliovirus (Todd et al., 1995). Competition experiments suggest that the same factor binds the 3’ UTRs of both rhinovirus and poliovirus. Using identical extracts to those used in our EMSA, we also detected complexes by UV cross-linking with the 3’ UTRs of any of...
PV3, CB4 and HRV14, suggesting that these RNAs all bind to the same factor. We additionally can detect complexes of 60 and 70 kDa, which also bind to PV3, CB4 and HRV14 3′ UTRs (unpublished results). It is intriguing that 3′ UTRs of apparently such different structures and sequences can bind the same factor and substitute for each other in chimeric genomes, given that mutations in either of these can adversely affect cellular factor binding and virus viability.

Our experiments additionally suggest that the binding of cellular factors to the 3′ UTR is essential for virus replication. This conclusion is based on an analysis of mutations engineered in both the poliovirus and rhinovirus 3′ UTRs. We observed that mutations which debilitated replicative capacity from a poliovirus replicon or a poliovirus/rhinovirus recombinant replicon, also severely affected plaque phenotype when built into the corresponding full-length infectious cDNA clone (Rohll et al., 1995) (Fig. 5 d, e, f). When used as competitor RNAs, mutant 3′ UTRs were less effective than the wild-type in competing for binding with radiolabelled 3′ UTR probe (Fig. 4 d; Fig. 5 c). This suggests that structures which retain the capacity to bind effectively to the cellular factor are important for virus viability and that the mutant RNAs, which had a lower affinity for the cellular factor than wild-type, had impaired virus replication. This was a consistent finding among mutants previously described from our laboratory and known to debilitate virus replication, and among kissing mutants described by others (Pilipenko et al., 1996) (Fig. 6) that alter regions of the structure believed to be important for tertiary RNA interactions. Thus, it is likely that the 3′ secondary and tertiary structure is required for efficient binding to the cellular factor. Our analysis of one of the HRV14 mutants (mut 6) showed that plaques could be recovered at approximately $10^{-5}$-fold of the wild-type efficiency. Analysis of two of these plaques showed reversion mutations had occurred in the apical stem of the HRV14 3′ UTR. Predicted RNA folds on the revertant sequence suggested structures in which the overall stem–loop architecture had been restored (Fig. 4 e). Our results suggest that a minimal stem–loop is required for efficient entero- and rhinovirus replication. These results contrast with those of Todd & Semler (1996), who suggest that the 3′-
terminal GTTTTAT(Aₙ) is the minimal sequence required for negative-strand synthesis.

The fact that the 5' and 3' UTRs bind similar viral proteins but apparently different cellular factors suggests that the cellular factors may modulate the activity of the viral polymerase. This could play a role for example in the regulation of positive versus negative RNA synthesis during virus replication. The identity of the 3' binding cellular factor and its precise role in virus replication is currently under investigation.

We acknowledge the excellent technical assistance of Moy Robson, Daniel Bailey, Yasmin Chaudhry, Emma Boulton and Thomas Lamb. This project was funded by the Medical Research Council programme grant no. G9006199.

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Received 6 November 1997; Accepted 20 March 1998