Mutational analysis of the 3′-terminal extra-cistronic region of poliovirus RNA: secondary structure is not the only requirement for minus strand RNA replication

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Received 6 July 1995; revised version received 19 September 1995

Abstract A series of mutations were introduced in the 3′-terminal untranslated region (3′-UTR) of full-length infectious poliovirus cDNA clones, and following transfection of COS-1 cells the ability of these constructs to generate viable viral particles and/or to support viral RNA synthesis was assayed. Substitution of the 3′-UTR of poliovirus RNA with the equivalent sequences of HAV RNA abrogated viral RNA replication, whereas the introduction of extended 'foreign' sequences between the open reading frame and the 3′-UTR was well tolerated. Point mutation that either destabilized the stem-and-loop structure or altered the sequence of the loop in domain 'Y' (nomenclature as per Pilipenko et al., [Nucleic Acids Res. 20 (1992) 1739–1745]) abolished both the infectivity and viral RNA synthesis. These were not restored by compensatory mutation that reconstructed the native secondary structure of this domain, suggesting that the secondary/tertiary folding of the 3′-UTR is not the only determinant for template recognition at initiation of RNA synthesis, but rather that a specific primary sequence is indeed required.

Key words: Transcription; Template interaction; Secondary structure; Mutational analysis; 3′-Untranslated region; Picornavirus

1. Introduction

The genome of poliovirus, a member of the *Picornaviridae*, is an RNA molecule about 7500 nucleotides in length which can serve either as mRNA or as template during the process of viral RNA replication. A small viral protein, VPg, is covalently attached to its 5′ end, and a genetically encoded poly(A) tract is present at its 3′ end [1,2].

A long extra-cistronic region precedes a single open reading frame (ORF) with a maximum coding capacity for a polyprotein of 200 kDa. A short untranslated region, 65 nucleotides in length, extends between the termination codon (nts 7373–75 in poliovirus type 1) and the 3′ terminal poly(A) tract.

Replication of viral RNA is a biphasic process: the genomic (plus) strand is used as a template to synthesize a complementary minus strand, which in turn is used as template during plus strand RNA synthesis. This two step transcription is carried out by a viral polymerase identified as the 52 kDa polypeptide, $3D^{pol}$ [3]. The replication apparatus must initiate de novo synthesis using two very different termini, i.e. the 3′ end of the plus strand, with a poly(A) extension, and the 3′ end of the minus strand where the heteropolymeric sequence ends in just two adenines [4].

Little is known about the mechanism(s) that secure the recognition of the template RNA by the viral replication complex. Since cellular mRNAs contain a poly(A) sequence at their 3′ end and they are not replicated by the virus replicase in vivo, it follows that other elements (primary sequence or secondary structures) must be present at (or in close vicinity of) the 3′ end to allow proper initiation of transcription.

Recently, Andino and collaborators [5] have proposed a model for the initiation of plus strand RNA synthesis in which a complex consisting of 3CD, a cellular factor, and a putative cloverleaf structure at the 5′ end of the plus strand RNA interact to catalyze in *trans* the initiation of the synthesis of new genomic RNA. This ribonucleoprotein complex appears not to be involved in negative strand RNA synthesis, suggesting that the latter is carried out in a completely different fashion [6,7]. The existence of two different mechanism of initiation of plus and minus RNA strand synthesis may also account for the asymmetry of RNA replication during the viral cycle [1].

The predicted secondary structure [8] of the 3′-terminal extracistronic region of different piconarviruses was recently tested with single and double-strand specific enzymatic and chemical probes [9,10]. These analyses revealed that tertiary structures can be generated, too: such higher order structures at the 3′ end were expected to constitute important signals for viral RNA replication.

The relative role of each element (primary sequence, secondary folding, etc.) had not been ascertained, and it was not clear whether the short 3′-UTR of poliovirus RNA acted as a single, unique module or else, whether instead functional domains could be identified. In this context, it was also important to determine whether, despite extensive nucleotide sequence divergence, signals other than primary structure had been conserved throughout the picornavirus family, allowing in this case the polymerase complex of one virus to use the RNA of another one as a suitable template to initiate transcription. To address this issue, we exchanged defined portions of 3′-UTR of poliovirus with the corresponding sequences of hepatitis A virus cDNA (a slow replicating picornavirus of the hepatovirus group) in a full-length, infectious poliovirus cDNA clone. To investigate whether the secondary structure is part of the *cis*-acting element required for specific template recognition, we engineered insertions, deletions and point mutations in the 3′-UTR of poliovirus mRNA and we tested the ability of such constructions to generate infectious virions upon transfection of COS-1 cells.

2. Materials and methods

2.1. Plasmids and reagents

Plasmid pGEM-2 and pGEM-4 were from Promega; the eukaryotic

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SSDI 0014-5793(95)01127-7

expression vector pSV-L was obtained from Pharmacia, Uppsala. Unless otherwise indicated, restriction and modifying enzymes were used according to the specifications of the manufacturer. All DNA manipulations were performed following standard procedures [12].

2.2. Cells and bacteria

COS-1 cells and Vero cells were routinely maintained as monolayers in Eagle's Minimum Essential Medium (MEM) supplemented with 5% or 10% Foetal Bovine Serum (FBS), respectively. Cultures of *E. coli* TG-1 were transformed following treatment with calcium chloride with minimal modifications.

2.3. Plasmid construction

2.3.1. Gap-filling mutagenesis. An *Xho*I site (CTCGAG) was inserted in the 3′-UTR of both poliovirus and HAV genome by gap-filling mutagenesis [13]. Mutant plasmids were checked by DNA sequencing before using them.

2.3.2. Polio-hepatitis A chimera. The construction in Fig. 1B was obtained by digestion of the plasmid pHAV-21 (containing a cDNA representation of the sequence of hepatitis A RNA encompassing bases 7016 through 7478, nucleotide numbering according to reference [11]) with *Dra*I and *Eco*RI (which cut at position 7412 of the insert, and in the MCS of GEM-4 vector, respectively), and the 60 bp fragment so generated was inserted into the linearized plasmid pSPol-Mut digested with *Xho*I (blunted) and *Eco*RI (which cut in poliovirus cDNA at position 7376, and in the MCS of the vector 3′ distal to the poly(A) tract of poliovirus insert, respectively).

2.3.3. Cassette exchange. Pairs of complementary oligonucleotides representing the 3′-terminal 23 nt of poliovirus 3′-UTR plus the poly(A) tract were synthesized so that upon hybridization the double-stranded fragment so generated presented a blunt end in the vicinity of the poly(A) stretch and a *Xho*I overhang at the opposite extremity. The double-stranded fragment was then ligated to the *Xho*I/*Nae*I digested plasmid, pSPol Mut to generate pAPSV (Fig. 1D) and to the *Xho*I/*Nae*I digested plasmid pALP (Fig. 1B) to generate pAESV (Fig. 1E). In a similar manner four other pairs of complementary oligonucleotides were synthesized that introduced 12 bp deletions and four point mutations either in the stem or in the loop of putative secondary structure of polio 3′-UTR. All of them have protruding *Xho*I ends both at 5′ and at 3′ ends. Each pair was ligated to CIP treated pAPSV to create plasmids pADSV, pASSV, pALSV and pACSV respectively (Fig. 1F,G,H,I). Each 'chimeric' or mutated cDNA was first obtained in pGEM-2 or in pGEM-4 and, after checking the mutants by sequencing, inserted into the pSLV vector.

2.4. Transfection

250.000 COS-1 cells in 10 cm diameter plastic Petri dishes were transfected with 10 μg of each plasmid DNA, according to the DEAE-Dextran/chloroquine procedure as described [15]. Cells were incubated at 37°C for 5 days or until any cytopathic effect (CPE) was visible. Amplification was performed by infecting monolayers of Vero cells with clarified supernatants obtained after three cycles of freezing and thawing the transfected cells. Typically, cytopathic effect developed one day after infection. The clarified supernatants served as a seed to prepare the initial viral stocks. Titration of recombinant viruses by plaque formation on Vero cells was simultaneously performed at 33°C, 37°C and 39°C.

2.5. Viral RNA purification

Stocks of mutated virus derived from plasmid transfection were grown on Vero cells. The clarified supernatants obtained as described above, were subjected to two sequential precipitations with 6% polyethyleneglycol, and viral RNA was phenol/chloroform extracted. To confirm the presence of the mutation, RNA sequencing was performed by the di-deoxy termination method [14] in a reaction driven by reverse transcriptase.

2.6. Determination of RNA synthesis by dot-blot analysis

Triplicate cultures of COS-1 cells in 25-mm diameter dishes were transfected, and the time for sampling was defined as follows: T_0 is 1 h after the end of transfection, T_1 and T_2 are 1 and 2 days thereafter. In some experiments, Actinomycin D was added as a specific inhibitor of cellular RNA transcription. At proper times, each plate was carefully washed with ice-cold PBS, and cell monolayers were scraped off. Cell pellets were lysed and RNAs were extracted three times with hot phenol (56°C). When appropriate, half of each sample was digested with RNase A before binding to nitrocellulose as described [16]. Strand specific RNA probes were synthesized from the pQI plasmid containing a cDNA tract of poliovirus genome extending from nt. 2200 through 3400. Plus and minus strand RNAs were synthesized using *Sal*I and *Hind*III digested pQI DNA, in reactions driven by T7 and SP6 polymerase. 10^6 CPM/ml of each probe were added to the hybridization solutions and incubated overnight at 42°C. Following two washings each of 2 × SSC-0.1% SDS at room temperature and one washing of 0.1 × SSC-0.1% SDS at 56°C, Kodak X-Omat films were exposed to dried membranes for different times. Quantification of the radioactivity present in each spot was done with an Instant Imaging Device (Packard).

3. Results

The 3′-UTR of poliovirus does not contain any properly located restriction site allowing the easy manipulation of this sequence. Accordingly, we created a *Xho*I site at position 7376 (Fig. 1A) in the poliovirus genome by gap-filling mutagenesis as described in section 2. COS-1 cells were transfected with this construct (pSPol-Mut, Fig. 1A) and the virus recovered from the supernatant was further amplified in Vero cells. The presence of the mutation was ascertained by direct sequencing of the viral RNA. The mutant virus recovered was titrated by plaque formation on Vero cells at 33°C and 39°C. There was no difference in the titer of the mutant at either temperature as compared with that of the parental *wt* Mahoney strain of poliovirus type 1. The insertion of six extra nucleotides in this position was perfectly tolerated, and had no noticeable effect on the replicating properties of the virus.

3.1. Search for conserved features in the 3′-UTR of picornavirus RNA

We first investigated whether the *cis*-acting elements responsible for template recognition (presumably present in the 3′-UTR of picornavirus RNA) had been conserved to such an extent as to allow the exchange of sequences between two unrelated members of the family. The construction depicted in Fig. 1A was used to replace the entire 3′-UTR of poliovirus with the equivalent sequence of HAV RNA. The resulting recombinant, in which the whole 3′-UTR of poliovirus had been replaced with the corresponding fragment of hepatitis A genome (Fig. 1B) was not infectious: we were unable to recover any virus upon repeated transfections of COS-1 cells and subsequent amplification, nor was it possible to detect any RNA synthesis by dot blot hybridization.

The HAV sequences introduced in the poliovirus genome between the end of its ORF and the poly(A) tract had a detrimental effect on viral replication and it was not clear whether the HAV fragment contained a putative 'poison' sequence that blocked viral replication, or whether the inhibition was due to the inability of poliovirus polymerase to recognize HAV sequences at initiation of minus strand RNA synthesis. To clarify this issue, the above construction was digested with *Xho*I/*Eco*RI (which cut in the HAV insert at position 7450, and in the MCS of the vector), and the fragment so removed was replaced by 'cassette exchange' with the entire poliovirus 3′-UTR. This generated a recombinant (Figs. 1C and 2B) containing a 30 bp fragment of HAV cDNA inserted between the poliovirus ORF and its 3′-UTR. Upon transfection and subsequent amplification, fully infectious poliovirus was recovered

carrying the extra HAV sequence. This was confirmed by direct RNA sequencing. This 'chimeric' polio-hepatitis A virus was indistinguisable from the parental poliovirus type 1 (Mahoney strain) as far as efficiency of plaque formation, length of replication cycle and plaque size are concerned, suggesting that the HAV sequences inserted upstream the 3'-UTR of poliovirus had limited (if any) effect on the replication ability of the construct.

3.2. Minimal length of the 3'-UTR

The above experiments indicated that while HAV sequences inserted immediately adjacent to the poly(A) tract were incompatible with poliovirus replication, foreign sequences were tolerated when intercalated between the ORF and the 3'-UTR (Fig. 1C), suggesting that the elements (whichever they might be) required to confer template specificity extended inside the 3'-UTR. To locate the 5'-border of these sequences we introduced limited deletions in the 3'-UTR, and assayed the effect that these changes had on both poliovirus infectivity and RNA replication. In a first approach we removed the 5'-most two-thirds of the 3'-UTR (delta 42). This was done by insertion of a synthetic double-stranded oligonucleotide of the form:

TCGAGGTAAATTTTTCTTTAATTCGGAGAAAAAAAAAAAA
CCATTTAAAAAGAAATTAAGCCTCTTTTTTTTTTTT

with a blunt end and a *Xho*I overhang, a structure that allowed its ligation into the *Xho*I/*Nae*I digested pSPol Mut (Fig. 1D). Such deletion, leaving the 23 nts immediately adjacent to the poly(A) tract intact, gave a non-infectious cDNA clone (Fig. 1D). The lack of infectivity could not be ascribed to the diminished length of the 3'-UTR: when the sequences of poliovirus removed with the above deletion were replaced with the homologous ones of HAV 3'-UTR (Fig. 1E), the length of the 3'-UTR was almost completely restored, but the 'chimeric' construct was not infectious. Moreover, dot hybridization analysis with plus and minus strand specific probes failed to detect de novo synthesis of poliovirus RNA (data not shown).

Since the 23-nt of poliovirus sequence adjacent to the poly(A) tract was not enough to secure the proper functioning of the template, a shorter deletion was introduced, aimed at removing the sequence immediately downstream of the ORF (delta 12) (Figs. 1F and 2C). This was done following the same procedure used to create the previous mutation, i.e. we inserted a double-stranded oligonucleotide of the form:

TCGAGCGAATTGGATTGGGTCATACTGTTGTAGGGC
CGCTTAACCTAACCCAGTATGACAACATCCCGAGCT

into the *Xho*I-digested plasmid shown in Fig. 1D. The construct depicted in Fig. 1F, carrying a 3'-UTR 53 nts in length (delta 12) was still unable to generate infectious virus or to direct viral RNA synthesis in transfected cells.

Computer assisted analysis of poliovirus 3'-UTR [8] had predicted the formation of a stable stem-and-loop structure extending between nucleotides 7376 and 7418 (domain 'Y' according to Pilipenko et al [9]), which was supposed to play a significant role in RNA replication [10]. The 12-nt deletion (delta 12) introduced removed entirely the 5'-distal arm of the stem (Fig. 2C) and this raised the question as to whether the observed inhibition of RNA synthesis was a consequence of the shortening of the 3'-UTR, or a result of the loss of the stem-and-loop structure.

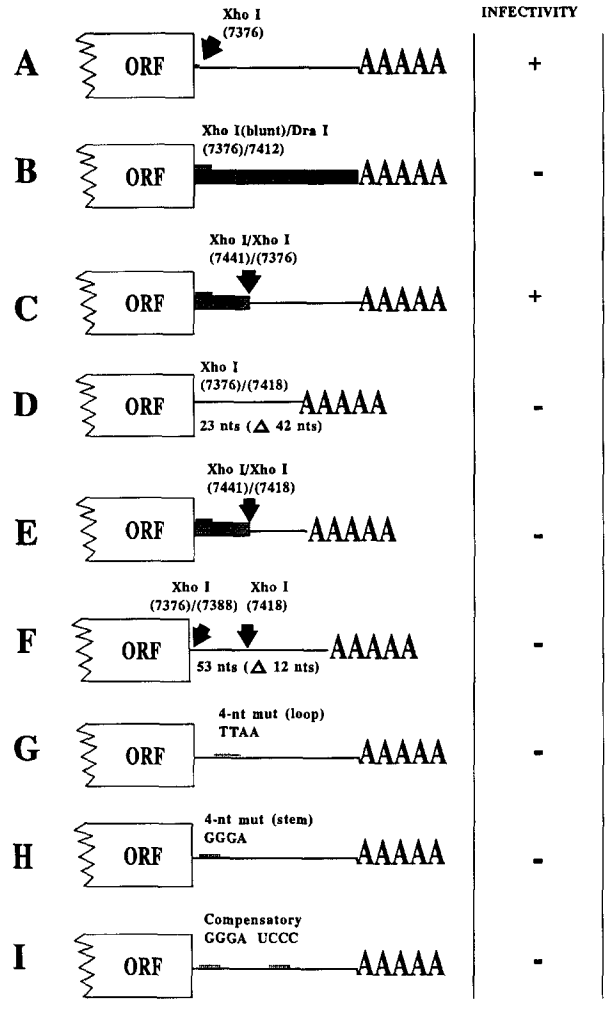

Fig. 1. Schematic representation (not to scale) of 3'-UTR's of poliovirus (thin line) and hepatitis A (grid area) RNAs. Grey boxes on top of the 3'-UTRs indicate the mutations introduced, and vertical arrows show the junctions at the *Xho*I sites. The infectivity of these constructions was assayed as in section 2.

3.3. Role of secondary folding in RNA replication

We engineered a 4-base mutation in the 5'-distal portion of the predicted loop (Figs. 1G and 2D) to prevent possible long-distance interaction(s) and/or knot formation, suggested by Jacobson et al [10]. The cDNA clone so generated was not infectious nor was it possible to detect by dot-blot analysis any viral RNA synthesis following transfection in COS-1 cells (Fig. 3).

A second 4-nt sunstitution was then introduced, aimed at destabilizing the stem of the stem-and-loop structure (Fig. 2E), and this mutation was also unable to support either plus or minus strand RNA synthesis (Fig. 3).

The original secondary folding was then restored by means of a compensatory mutation introduced with a synthetic double-stranded oligonucleotide as described:

TCGAGGGGAACCTCAGTCGAATTGGATTGGGTCATACTGTTGTTCCCC
CCCCTTGGAGTCAGCTTAACCTAACCCAGTATGACAACAAGGGGAGCT

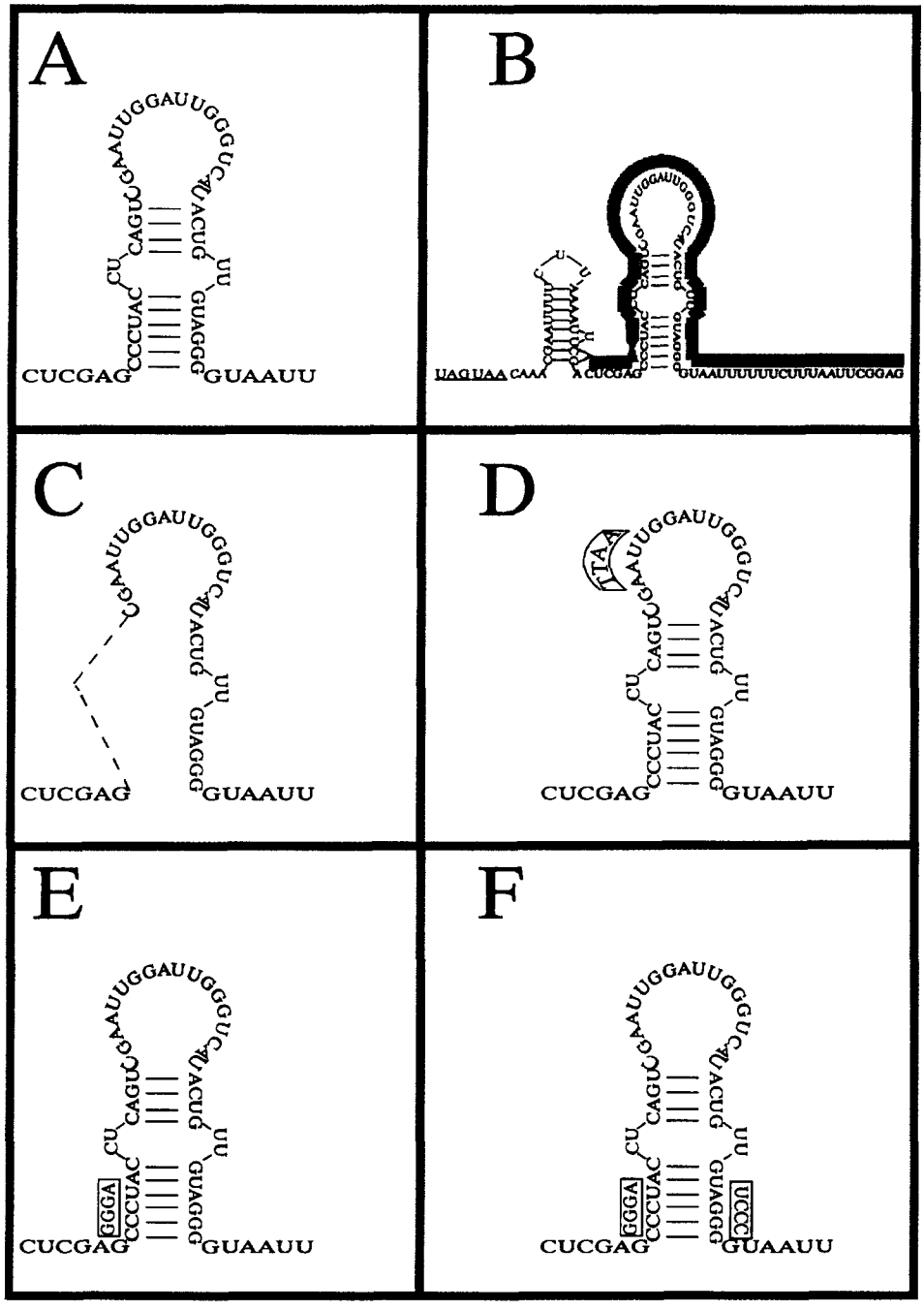

Fig. 2. (A) Predicted secondary structure of poliovirus 3′-UTR carrying a *Xho*I site (CUCGAG) as in Fig. 1A. (B) Predicted secondary structure of the 3′-UTR of the infectious chimera polio/hepatitis A (Fig. 1C). Note the poliovirus stop codon (underlined), the poliovirus 3′-UTR sequences (shaded bars), and the second stem-and-loop introduced with the hepatitis A sequences. (C) 12-nt deletion (Fig. 1F). (D) 4-nt mutation in the loop (Fig. 1G). (E) 4-nt mutation in the stem (Fig. 1H). (F) Compensatory mutation in the stem (Fig. 1I).

where the bases underlined indicate the positions substituted (Fig. 2F).

Following transfection of COS-1 cells, the synthesis of poliovirus plus and minus strand RNA was monitored and quantitated by dot-blot hybridization, using strand-specific riboprobes transcribed in vitro (Fig. 3). While the above mutation reconstructed the native stem-and-loop structure of domain 'Y', no viral RNA synthesis of either minus and plus strand could be detected by dot-blot hybridization (Fig. 3). Moreover, we could not recover any infectious virus, even after 3 cycles of attempted amplification in Vero cells.

To discriminate between RNA replication and RNA merely transcribed from the transfecting cDNA plasmid, Actinomycin D was added to the cultures 1 h after the end of transfection. Since RNase treatment abrogated the virus-specific signal detected at T_0, it is clear that this cannot be due to some residual plasmid DNA left. In our view, the minus strand RNA recovered 1 h after transfection and before the addition of any Acti-

nomycin D, represents primary transcription of the opposite strand of plasmid DNA by the cellular polymerases. This is substantiated by the observation (Degener et al., unpublished) that pSV-L constructs carrying full length poliovirus cDNA in the reverse orientation (i.e. transcription of a plus strand poliovirus RNA should start opposite to the SV-40 promoter) are also infectious, implying that the transcription machinery of the host cell can (randomly?) start rounds of RNA synthesis in the absence of an efficient eukaryotic promoter. The signal falls to background levels 24 h post transfection (T_1), and reach significant levels (wt) the next day (T_2) (Fig. 3).

4. Discussion

The nature of the signal(s) recognized by the picornavirus replication complex at initiation of minus strand RNA synthesis has so far remained elusive. Conceivably, the specific recognition of the plus strand RNA template may be mediated by primary sequence and/or secondary/tertiary folding of the 3′-terminal sequences adjacent to the poly(A) tract. Moreover, it was not clear whether these signal(s) (whatever their nature) were sufficiently conserved throughout the *Picornaviridae* to allow cross RNA transcription, i.e. the specific recognition of the genomic RNA of one picornavirus by the polymerase complex of another one. In an attempt to locate the sequences of poliovirus RNA specifically required to initiate RNA transcription in vivo we have performed a mutational analysis of different domains of the poliovirus 3′-UTR. To that end, we transfected COS-1 cells with cDNA constructs inserted in the eukaryotic expression vector pSV-L under the transcriptional control of the late promoter of SV-40. This approach has several advantages over the transfection with RNA transcripts: the double-stranded DNA is far more stable and less prone to nuclease degradation than RNA; moreover, the entire process, i.e. transcription of the first plus strands RNA by the cellular RNA Polymerase II, the transportation of the transcript to the cytoplasm perhaps in association with cellular proteins, and its presentation to the ribosomes resembles more closely the physiological conditions as compared to transfection with phenol-extracted RNA obtained by in vitro transcription directed by the T7 phage RNA polymerase.

In the course of these studies we used dot-blot hybridization to check de novo RNA synthesis and the amount of labeled probe retained on the membrane was measured by quantitative image analysis. Although the method does not provide absolute values because of the different specific activity of each probe [17], it nevertheless allows comparison of the amounts of RNA present in the dots of individual membranes. We could, therefore, correlate the loss of infectivity of the mutated constructs with the changes in the amount of RNA synthesized.

In a first approach, we tried to investigate whether the sequences recognized by the replication complex are entirely contained in the 3′-UTR. Since the insertion of extended HAV sequences between the ORF and the 3′-UTR (Figs. 1C and 2B) was compatible with poliovirus replication, we conclude that specific template recognition does not require a 3′-UTR necessarily contiguous with the coding sequences. The HAV sequences of the 'chimera' would fold to constitute an extra domain, similar to the stem-and-loop 'Z' of Coxsackie B virus RNA described by Pilipenko et al. [9], between the ORF and domain 'Y'.

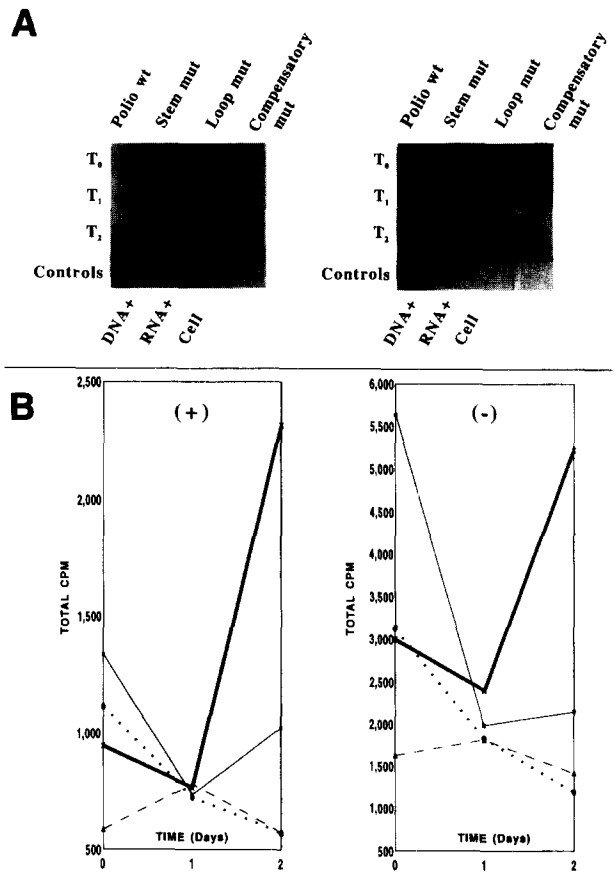

Fig. 3. (A) Dot blot analysis of plus and minus strand poliovirus RNA synthesized in COS-1 cells transfected with constructs carrying a full cDNA representation of poliovirus genome (wt) or mutated in the stem (Fig. 1H), in the loop (Fig. 1G), or carrying the compensatory mutations (Fig. 1I), at the end of transfection (T_0) or 1 (T_1) and 2 (T_2) days afterwards. (B) The relative amounts of RNA in the above dot blot were determined by image analysis as described in section 2. Thick line: Polio wt; dotted line: Stem Mut; dashed line: Loop Mut; thin line: Compensatory Mut.

These sequences are quite specific, and cannot be replaced with the topologically equivalent ones of HAV RNA (Fig. 1B), suggesting that the elements present in poliovirus 3′-UTR differ sufficiently from those of HAV RNA to prevent the recognition of a template containing them. Rohll et al. [18] have recently succeeded in the construction of recombinant 'replicons' in which sequences of poliovirus 3′-UTR were substituted with those of coxsackie B4 rhinovirus 14 (HRV14), suggesting that enterovirus and rhinovirus groups are more closely related between them than to HAV.

We tried to determine the minimum required length of the 3′-UTR by introducing nested deletions that removed short stretches of the region: the 5′-most two-thirds of the 3′-UTR seem to be strictly indispensable since deletion of even short tracts of it (Figs. 1D and 1F) abolished both RNA synthesis and viral replication. This is in line with recent findings of Todd et al. [21], who identified a short sequence involved in replication of rhinovirus RNA: removal of this segment delayed and impaired RNA replication. This highly conserved domain, 7-nt downstream from the end of the ORF in HRV14, constitutes the base of the predicted stem of domain 'Y' [9].

While there seems to be little nucleotide sequence homology among different members of the picornavirus family, previous studies [9] suggested the predicted secondary structure may constitute a much more conserved element, serving perhaps as a functional signal at initiation of RNA replication [10]. To test this view we mutated either the loop (Fig. 1G) or the stem of domain 'Y' and looked at both infectivity and viral RNA synthesis (Fig. 3).

Infectivity was lost when mutations were introduced in the free loop reportedly engaged in long rate intra-molecular interaction [9,10] with sequences that tertiary folding would bring into vicinity (Fig. 2D).

In the mutant depicted in Fig. 1H, the four nucleotides mutated (CCCU) map at the base of the stem (Fig. 3E), and are likely to be indispensable to stabilize the entire structure. Disruption of this stem, therefore, seems to be incompatible with infectivity. Interestingly, the mutated motif CCCUAC is highly conserved among enteroviruses where it contributes to form the stem of domain 'Y'. This is reminiscent of the organization of rhinoviruses, where the conserved motif is ATATAGA [19]. RNA synthesis, however, was not restored by a compensatory mutation (GGGA-TCCC) which should reconstruct the native stem-and-loop (Fig. 3F).

Two main principles emerge from these findings: the integrity of the whole 3′-UTR, on the one hand is a stringent requirement for initiation of minus strand RNA synthesis, and no deletions are tolerated. Insertion of foreign sequences instead seems to be compatible with RNA replication, provided they are not adjacent to the 3′-terminal poly(A) stretch, and they do not interfere with the folding of the major domain 'Y' [9]. On the other hand, the secondary structure alone is not per se sufficient to determine template recognition at initiation of minus strand synthesis, and primary sequence plays a significant role in this process. This signal would be shared by enteroviruses and possibly by rhinoviruses, but may not necessarily be the same in the slow-replicating HAV. The question is still open as to whether the peculiar structure of the 3′-UTR of HAV correlates with its unique growth characteristics.

Acknowledgements: This work was partly supported with funds of the Canadian Medical Research Council, NATO Collaborative Research Grant 890157, Italian National Research (CNR) (Progetti Finalizzati 'Ingegneria Genetica' and 'Biotecnologie e Biostrumentazione'), and Istituto Pasteur/Fondazione Cenci-Bolognetti of Rome.

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