Cis-element, oriR, involved in the initiation of (−) strand poliovirus RNA: a quasi-globular multi-domain RNA structure maintained by tertiary ('kissing') interactions

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Introduction

Replication of viral single-stranded RNA genomes of positive (mRNA-like) polarity is accomplished by virus-encoded RNA-dependent RNA polymerases, usually assisted by a multi-component replication machinery composed of both viral and host proteins. The general scheme of the replicative process consists of the synthesis of a complementary, or (−) strand, by using the parental strand as a template, followed by the synthesis of parent-strand (+) strands on the (−) template. Initiation of a (−) strand demands positioning of the polymerase in close proximity to the 3′ end of a (+) strand, whereas the enzyme should be placed at the 3′ end of a (−) strand to initiate a daughter (+) strand. Since the 3′ ends of the viral complementary strands are dissimilar, the replication machinery should be able to recognize two different types of cis-acting elements to initiate (−) and (+) strands. These elements will be referred to as oriR and oril, respectively. In spite of the importance of the ori signals, they are defined vaguely in the replication system of viruses with positive RNA genomes. Relatively detailed studies were carried out with oris of some plant viruses, where the pivotal role of tertiary interactions for maintaining a tRNA-like terminal structure and upstream subterminal folding as well as for replicative functions was documented (Raetsky et al., 1984; Mans et al., 1991; Lahser et al., 1993).

Here, we wish to characterize the oriR of the poliovirus genome. This viral RNA is a 7.5 kb single-stranded molecule containing a poly(A) tail at its 3′ terminus (Wimmer et al., 1993). Obviously, this ubiquitous homopolymeric stretch cannot serve as an ori, since the viral replication machinery is known to exhibit a remarkable specificity and replicates, in the infected cell, exclusively virus-specific RNAs. It is natural to assume that an ori structure should contain heteropolymeric elements conserved among related viruses. An analysis of published nucleotide sequences as well as chemical and enzymatic probing allowed us to define consensus secondary and tertiary structures in the 3′-non-translated region (NTR) of enterovirus genomic RNAs (Pilipenko et al., 1992b).

We proposed that these structures may serve as oriR. An alternative folding for the poliovirus NTR (not conserved in other enteroviruses) has also been published (Jacobson et al., 1993). The two models differ from one another by the character of the postulated tertiary interactions (Figure 1). We (Pilipenko et al., 1992b) proposed base pairing between loops of two hairpins of a novel, ‘kissing’, type (Chang and Tinoco, 1984), whereas a classical pseudoknot (Pleij, 1984) involving a hairpin loop and a neighboring (non-looped) single-stranded element was suggested by Jacobson et al. (1993). To evaluate the validity of the models, site-directed mutagenesis was used here to modify partners of the potential tertiary interactions in the oriR.

The capacity of the mutant RNAs to replicate in the transfected cells clearly depended on the preservation of the potential for the kissing interaction; the mutant transcripts with a lowered potential exhibited severely impaired replicative activities. Nevertheless, the mutant transcripts proved to be infectious, yielding some progeny with true or second-site reversions. A comparison of the phenotypic properties of these revertants with the structure of their oriR not only supported the original structural model of the poliovirus NTR (Pilipenko et al., 1992b), but demonstrated unambiguously the functional significance of the kissing interaction for the poliovirus oriR function.

Based on these and previous (Pilipenko et al., 1992b) experimental data, a three-dimensional structure of the RNA tertiary structure/viral RNA replication

The key steps in the replication of the poliovirus genome, initiation of (−) and (+) strands, require two different cis-acting elements, oriR and oril, respectively. It has been proposed that the spatial organization of these elements is maintained by tertiary ('kissing') interactions between the loops of two constituent hairpins. Here, the putative partners of the kissing interaction within the oriR of the full-length poliovirus RNA were modified by site-directed mutagenesis. The destabilization of this interaction resulted in a severe suppression of the viral RNA synthesis, but the mutant transcripts proved to be infectious. With a single exception, the potential for the kissing interaction within the oriR of the recovered viruses was partially or completely restored due to either true reversions or second-site compensatory mutations. There was a good correlation between the restoration of this potential and the phenotypic properties of the viruses. It was concluded that the kissing interaction in the poliovirus oriR is functionally important. Using the above experimental data, a three-dimensional structure was derived by molecular modeling techniques, which demonstrated the overall feasibility of the proposed interactions and displayed the poliovirus oriR as a quasi-globular multi-domain structure.

Keywords: RNA tertiary structure/viral RNA replication
poliovirus oriR was derived by computer-assisted molecular modeling. According to the model, this RNA control element is represented by a quasi-globular multi-domain structure.

Results

**Engineered mutant genomes and their biological properties**

To check the validity of the proposed models for the poliovirus oriR folding, mutations were introduced into the domains involved in the two putative tertiary interactions. The potential for the kissing interaction between the loops of domains X and Y (Pilipenko et al., 1992b) (Figure 1) was decreased by disturbing two neighboring base pairs through altering either nucleotides 7433–7434 (AU→UA) in loop X (pPV/252; Figure 2) or nucleotides 7391–7392 (AU→UA) in loop Y (pPV/251; Figure 3). Using published thermodynamic parameters (Freier et al., 1986), the ΔG° values for the kissing interaction were calculated to decrease by 2.9 kcal/mol in both cases. To destabilize the pseudoknot proposed by Jacobson et al. (1993) without impairing the kissing interactions, the coding sequence of the poliovirus RNA should have been changed. Three positions were altered to generate plasmid pPV/253 (Figure 4A): U7354→A, C7355→U and U7360→A (cf. also Figure 1). This resulted in modifications of two codons (without altering the encoded amino acids), CGU→CGA and CUU→UUA, as well as in a change in the calculated ΔG° value for the putative pseudoknot interaction from −7.7 to −4.6 kcal/mol.

Viral RNA replication in cells transfected with transcripts of the engineered plasmids was investigated by dot hybridization of the total RNA from transfected cells with the labeled riboprobe. Although the riboprobe used should detect the accumulation of viral RNA (+) strands, this could be taken as a reliable measure of the ability to initiate (−) strands in mutants with a defective oriR. The destabilization of the kissing interaction caused by mutations in the loops of domains X (pPV/252) or Y (pPV/251) resulted in a severe suppression of the genome replication. In the experiment shown in Figure 5A, significant amounts of virus-specific RNA species accumulated in cells transfected with either of these transcripts only by 48 h incubation at 34°C, whereas a clear signal could be observed already at 8 h after transfections with the wild-type genome. Moreover, further replication of the viral genome proceeded much faster in the latter case; by
affect the translational template activity of the viral transcription-translation system. The mutations did not logically important.

The kissing interaction within this control element is physio­

did not impair the replication appreciably (Figure 5B). No

24 h the cell monolayers transfected with the wild-type transcripts were destroyed completely. On the other hand, the same alterations in domains X and Y, but combined in a single construct pPV/251/252 so that the potential for the kissing interaction has been restored fully (Figure 4B), did not impair the replication appreciably (Figure 5B). No changes in the ability to synthesize viral RNA were detected upon the destabilization of the pseudoknot proposed by Jacobson et al. (1993), as evidenced by the hybridization results obtained with the pPV/251 RNA-transfected cells (Figure 5B).

These data strongly supported our model for the oriR folding and, in addition, demonstrated that the tertiary kissing interaction within this control element is physiologically important.

The engineered constructs were also tested in a coupled transcription-translation system. The mutations did not affect the translational template activity of the viral genomes (data not shown). We concluded that the mutations engineered into the poliovirus 3NTR primarily affected replication rather than translation of the viral genome.

Revertant viruses and their properties

Despite the failure to trigger replication of the viral genome detectable in the dot hybridization assay, the transcripts of pPV/251 and pPV/252 proved to be infectious when transfected into primary monolayers of African green monkey kidney (AGMK) cells at 34°C. The plaques generated were heterogeneous with respect to the size and the time of their appearance, most of them becoming visible on days 3–5 rather than day 2, as is typical for the wild-type. Such behavior, together with the late accumu­

The plaques produced by these RNAs were picked up and used to infect RD cells at 34°C. Five plaques (a–e) generated by the pPV/252 RNA were used separately to produce secondary plaques and, eventually, viral pools. The sequences downstream of position 7345 of the 14 viral RNA species thus generated were determined in no case was the parental mutant genome conserved. The potential to form one (out of the two lost) tertiary base pair was restored in nine RNAs, descending from three primary plaques. This was achieved by either a back mutation in the loop of domain X (A1031U) or a second-site mutation in the loop of domain Y (A1026U) in clones 1031–1036 or 1026–10281 (Figure 2). All the revertants with a partially destabilized kissing interaction exhibited thermosensitivity of growth, although
Table 1. Biological properties of the PV/251 and PV/252 revertants and the mutant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque size (mm) EOP pv&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV1</td>
<td>6.8</td>
</tr>
<tr>
<td>PV/251a-1001</td>
<td>2.3</td>
</tr>
<tr>
<td>PV/251a-1003</td>
<td>4.8</td>
</tr>
<tr>
<td>PV/251a-1005</td>
<td>6.1</td>
</tr>
<tr>
<td>PV/251a-1007</td>
<td>7.8</td>
</tr>
<tr>
<td>PV/251c-1013</td>
<td>4.7</td>
</tr>
<tr>
<td>PV/251c-1016</td>
<td>5.7</td>
</tr>
<tr>
<td>PV/251d-1019</td>
<td>6.7</td>
</tr>
<tr>
<td>PV/251d-1022</td>
<td>6.3</td>
</tr>
<tr>
<td>PV/252b-1027</td>
<td>4.1</td>
</tr>
<tr>
<td>PV/252b-1035</td>
<td>5.0</td>
</tr>
<tr>
<td>PV/252d-1035</td>
<td>5.0</td>
</tr>
<tr>
<td>PV/252d-1036</td>
<td>5.7</td>
</tr>
<tr>
<td>PV/252d-1036b-1039</td>
<td>7.6</td>
</tr>
<tr>
<td>PV/252e-1041</td>
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</tr>
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<td>7.4</td>
</tr>
<tr>
<td>PV/251-1114</td>
<td>7.3</td>
</tr>
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<sup>a</sup>EOP<sub>pv</sub> is the ratio between efficiencies of plaquing at 34 and 39.5°C.

252 RNA demonstrated partial or complete restoration of the potential for the kissing interaction strongly suggested that the latter played an important part in the viral reproduction. This notion was supported further by the results of additional selection at 39.5°C of the revertants with partial reversions (Figure 2). The potential for the kissing interaction was fully restored in four out of the five investigated descendants of clones 1028 and 1036. This was achieved either by a back mutation in domain X (U<sub>733</sub>→A in clones 1029, 1037 and 1038) or by a second-site mutation in domain Y (U<sub>790</sub>→A in clone 1030). Only one clone (1039) selected at 39.5°C did not undergo any changes within the segment sequenced (this exception will be discussed below). All the clones selected at this temperature exhibited a wild-type-like phenotype (Table 1).

The effect of temperature on the ability of revertants to trigger RNA synthesis in the infected cells was studied by using the dot hybridization assay (Figure 5C). Upon wild-type infections (PV1) at either the permissive (34°C) or the restrictive (39.5°C) temperature, a prominent signal could be detected by as early as by 3 h post-infection (the earliest point investigated), showing much faster viral RNA accumulation than in the transfection experiments with transcripts (cf. Figure 5A and B) perhaps because of a much greater multiplicity of the infection. A partial reversion of the destabilized kissing interaction, due to either a back mutation (clone 1036) or a pseudo-reversion (clone 1028), was accompanied by nearly normal RNA synthesis at 34°C but not at 39.5°C (Figure 5C). On the other hand, the full restoration of the potential for the tertiary interaction in the oriR (clone 1029) led to a nearly wild-type level of viral RNA accumulation at both temperatures (Figure 5C), even though the primary structure of this region was changed (Figure 2).

A similar selection protocol was used to obtain revertants of pPV/251 (Figure 3). A total of 12 virus pools formed at 34°C were prepared. Nine clones (1008–1013 and 1017–1019) had an identical true reversion, A<sub>733</sub>→U;
as a result, a single internal U–U mismatch should be present within the kissing interaction. The viruses exhibited either a slight ts (e.g. 1013) or nearly wild-type (e.g. 1019) plaque phenotype (Table I), although even in the latter case the level of RNA accumulation at 39.5°C was still markedly lower than at 34°C (Figure 5C). All the three descendants (1001–1003) of the primary plaque PV/251a were different from the remaining set of the revertants, having no reversions within the mutated oriR (Figure 3); the significance of this finding will be discussed below. These viruses exhibited a ts phenotype (Table I). Further selection of clone 1003 at 39.5°C resulted in a partial restoration of the strength of the kissing interaction due to A7392→U transversion (Figure 3), accompanied by some phenotypic improvement (Table I). Cultivation at 39.5°C of partial revertants 1013 and 1019 resulted in no changes in the oriR structure (Figure 3) and minimal, if any, phenotypic alterations (Table I).

Thus, (i) nearly all viable descendants of pPV/251 and pPV/252 exhibited at least partially restored potential for the kissing interaction within the oriR, and (ii) there was a reasonable correlation between the restoration of the tertiary structure and the reversion of the replicative capacity.

Probing of the structure of mutant RNAs

Full-length transcripts of the wild-type and mutant plasmids were subjected to treatments with reagents specific for base-paired (nuclease VI) or unpaired [nucleases T1, and S1 as well as dimethyl sulfate (DMS)] regions (Figure 6). The results of such probing of the wild-type poliovirus RNA were in good agreement with those reported previously (Pilipenko et al., 1992b). For the present work, it was particularly relevant that the PV1 RNA region assumed to be involved in the kissing interaction (boxed positions in Figure 6A) was susceptible to the double strand-specific nuclease V1. This was also true of the pPV/251/252 RNA, where the V1 susceptibility concerned not only the loop of domain Y but also the loop of domain X (Figure 6D). This region also showed some susceptibility to the single strand-specific nucleases S1, which might suggest that paired and unpaired conformations were in a dynamic equilibrium or that the RNA population was composed of different conformers.

The mutations in the loops of domains Y (pPV/251) or X (pPV/252), aimed at destabilization of the kissing interaction, were accompanied by the disappearance of the nuclease V1 susceptibility of the loop of domain Y around positions 7392–7393 as well as an increase in the sensitivity to DMS within the relevant portions of domains X and Y (Figure 6B and C). These data constituted independent support for our structural predictions.

The structural model for the poliovirus oriR

A comparative analysis of the nucleotide sequences of the enterovirus 3NTRs allowed us to propose a fully conserved model for their tertiary structure which represents a slight modification of that described previously (Pilipenko et al., 1992b). According to this model, the helix K generated by the tertiary (kissing) interaction between the loops of domains X and Y can be stacked to the stem of domain X in the way shown in Figure 7A. Based on these data, a three-dimensional structure for the poliovirus 3NTR was predicted using computer-aided molecular modeling (Figure 7B–D). The model demonstrates the feasibility of folding back of residue U7391 along the major groove of the six base pair long ‘kissing helix’ (involving positions 7388–7393 and 7432–7437) to return to the stacked domain X (cf. Gregorian and Crothers, 1995). The two helical elements, X and Y, being connected by the tertiary interaction, form an angle of ~36° and thus do not acquire a typical L-shaped tRNA-like conformation as assumed previously (Pilipenko et al., 1992b). The bridging strands A7371→A7375 and G7412→A7422 connecting the X and Y domains opposite the kissing interaction simply do not allow geometrically for such an overall L-shaped conformation. Due to the kissing interaction, the oriR has a quasi-globular, multi-domain structure.

Discussion

The results presented above strongly suggest that the tertiary kissing interaction between the loops of domains X and Y is of crucial importance for the recognition of the poliovirus oriR by the replication machinery. This notion is supported by the following facts: (i) the viral RNA synthesis, upon transfection of susceptible cells with engineered mutant genomes having a destabilized tertiary structure of the oriR, was dramatically delayed, as compared with transfections with the wild-type RNA; (ii) the viral RNAs found in the late appearing plaques always (with a single exception discussed below) demonstrated reversions ensuring at least partial stabilization of the kissing interaction; and (iii) the phenotypic improvement (an increase in plaque size and the ts→ts’ conversion) of the mutants with partially stabilized tertiary structures was usually accompanied by a further increase in the strength of the kissing interaction. Moreover, an alteration of four contiguous nucleotides (7390–7393) in the loop of domain Y, which is expected to result in a complete destruction of the kissing interaction, generated a dead virus (Pierangeli et al., 1995).

The engineered mismatches aimed at disturbing the kissing interaction (in clones 251 and 252) were represented by both A→A and U→U combinations. Characteristically, the partial reversions caused by either true or second-site mutations were invariably achieved by A→U rather than U→A transversions (Figures 2 and 3). The reason for such a bias was probably due to a much stronger selective pressure against A→A as compared with U→U mismatches, because the former should exert a higher destabilizing effect. In the frame of this reasoning, some structural and functional differences between the partial revertants originating from pPV/251 and pPV/252, respectively, could be explained readily. Indeed, the kissing interaction in the wild-type RNA can potentially involve six base pairs (Figures 1 and 7A), and maximally three of them could survive the mutagenesis, if any kissing was now possible at all (Figures 2 and 3; the ‘upper’ lone U–A pair, shown only to indicate that the respective bases were not altered, should not exist in reality). The number of base pairs involved is five and four in the partial revertants of pPV/251 and pPV/252, respectively. This implies that the former should be more stable, especially because an internal U–U pair in descendants of pPV/251 is hardly expected to exert a strong destabilizing effect.
As a consequence, no further stabilization of the kissing interaction of these viruses was observed upon the secondary selection at 39.5°C (Figure 3), whereas such a stabilization was documented to occur in the pPV/252 progeny (Figure 2).

It may also be noted that the transversions occurred remarkably easily. Indeed, the number of plaques per μg of RNA generated by the mutant transcripts pPV/251 and pPV/252 was only a few times lower (or sometimes not even lower at all) than in the case of the wild-type pPV1 transcript (not shown). Since nearly all of the recovered viruses had an A → U transversion, this observation means that a specific position in the viral genome could be changed by a transversion in at least one of a few primarily infected cells.

Although there is good correlation between the expected stability of the kissing interaction in the oriR, on the one hand, and the phenotype of the mutants and revertants, on the other, a few exceptions are enlightening and deserve special comment. The descendants of plaque PV/251a, while retaining the engineered destabilizing mutations, were viable and relatively genetically stable upon cultivation at 34°C, whereas all the other 23 clonal descendants of pPV/251 and pPV/252 acquired at least one stabilizing reversion or pseudoreversion (Figures 2 and 3). Moreover, the phenotypic properties of clones 1001—1003 (with two base pairs of the kissing site disturbed) were not significantly different from those of 1027 and 1028 (with only one pair disturbed). The most likely explanation for the non-concordant properties of clones 1001—1003 is that they acquire an additional mutation(s), outside oriR, able to partially suppress the phenotypic effects of the destabilized kissing interaction. Some phenotypic improvement of descendants of PV/251c-1013 and PV/252d-1036 upon selection at 39.5°C (Table 1), not accompanied by any changes in the oriR primary structure, also suggests the

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Fig. 6. Probing of the structure of oriR of wild-type (A), pPV/251 (B), pPV/252 (C) and pPV/251/252 (D) poliovirus RNA species. The transcripts were treated with nucleases V1, T1 and S1 as well as with DMS as described in Materials and methods. The appropriate cleavage/modification sites are denoted as shown in (A). The mutated nucleotides are shown by lower case letters. The nucleotides able to participate in the kissing interaction are boxed. The termination codon is underlined.
Our experiments did not reveal any significant physiological role for the pseudoknot proposed by Jacobson et al. (1993). In this regard, it may be noted that the eight nucleotide linker between positions 7387 and 7388 in their poliovirus mutant 3NC202 did not diminish markedly the potential for the generation of either the putative pseudoknot or the kissing interaction (not shown). Moreover, the suggested pseudoknot, in this mutant, did not change its location, whereas the nucleotides in the loop of domain Y involved in the kissing interaction were separated from the stem of this domain by eight nucleotides. The wild-type-like arrangement of the kissing interactions should be restored in the revertants of 3NC202 described by Jacobson et al. (1993). Therefore, we are inclined to explain the ts phenotype of 3NC202 by the overall destabilization of the compact tertiary structure of the oriR.

It was suggested that the enterovirus and rhinovirus oriRIs, the cis-elements controlling initiation of the (+) RNA strand synthesis, being structurally related to the oriRs of enteroviruses, are also stabilized by a similar kissing interaction (Pilipenko et al., 1992b); however, its functional importance has yet to be tested.

Thus, the picornavirus orIr's (and probably oriRIs) belong to an emerging class of complex RNA cis-elements composed of extended (up to several hundred nucleotides long) segments apparently acquiring a multi-domain, quasi-global conformation. oriRIs of many plant viruses are represented by complex tRNA-like structures which include, and adjoin, functionally important pseudoknots (Rietveld et al., 1984; Mans et al., 1991; Lahser et al., 1993; Gordon et al., 1995). The internal ribosome entry sites (IRES) of the genomes of picornaviruses (reviewed in Agol, 1991; Wimmer et al., 1993), hepatitis C virus (Tsukiyama-Kohara et al., 1992; Wang et al., 1994, 1995; Reynolds et al., 1995) and pestiviruses (Le et al., 1995; Poole et al., 1995) as well as certain translational control elements of Escherichia coli mRNAs (Philippe et al., 1993; Spedding and Draper, 1993; Spedding et al., 1993) are other remarkable examples of such complex RNA structures, with elaborate surfaces, potentially capable of very specific multi-point interactions with diverse components of synthetic (replication or translational) machinery.

Materials and methods

Construction of plasmids
An intermediate construct, pMHE, containing a HindIII–EcoRI poliovirus 1 CDNA segment (nt 6517–7525 including 84 3′-terminal A residues) in an M13-based vector, pBAM13 (Vector Cloning Systems, San Diego, CA), was made. All mutations were engineered by oligonucleotide-directed mutagenesis using this intermediate construct. The full-length mutant polioviral genomes were assembled by the three-segment ligation involving fragments PolV–EcoRI (nt 7056–7525, containing engineered mutations) from pMHE, EcoRI–BglII (nt 7526–5601, containing appropriate regulatory elements and a T7 RNA polymerase promoter) and BglII–PolVI (5602–7055) from the wild-type pPV1 (Pilipenko et al., 1992a).

Generation of transcripts
For the generation of full-length RNAs, the plasmids were linearized by digestion with EcoRI and transcription was carried out as described (Pilipenko et al., 1992a). The transcripts aimed at secondary structure probing were partially purified by 2 M LiCl precipitation (Pilipenko et al., 1994).

Secondary structure probing
Treatments of the transcripts with DMS and nuclease V1, S1 and T1 were performed as described (Pilipenko et al., 1994). To identify the sites of modifications or cleavages, a 5′ end-32P-labeled oligodeoxyribonucleotide (T)5-CTCCG (complementary to nt 7436–7454 of poliovirus RNA) was used as a primer for cDNA synthesis as described (Pilipenko et al., 1989).

Transfection, isolation of viral clones and temperature sensitivity assay
Plaque assays were performed on primary cultures of AGMK cells, whereas a continuous monkey rhabdomyosarcoma (RD) cell line was used for the virus-accumulating passages. Infectivity of the viral transcripts was investigated by the plaque assay at 34°C using the DEAE-dextran transfection method (Pilipenko et al., 1992a). The appearance of plaques was inspected over the period of 1 week.

A few plaques were picked up on days 4–6 from different flasks transfected with either the pPV251 or pPV252 RNAs. The viral stocks of each individual clone were prepared by an accumulating passage at 34°C. The stocks intended for the selection of revertants were plaque-puriﬁed at the same temperature, and several secondary plaques were collected on day 3. The progeny of individual secondary plaques were subjected to 1–3 passages at 34°C. Some viral clones were subjected to further selection at 39.5°C. In this case, a passage was carried out at this temperature, followed by a plaque assay at 39.5°C. A few plaques were then picked up on random on days 3 or 4, and the viral stocks were prepared at 37°C.

The sizes of plaques produced by either viruses or transcripts on AGMK monolayers at 34°C were measured on day 3 and averaged. The ratio of efficiencies of plaquing at 34 and 39.5°C (EOPp/r) was determined on day 3.

Viral genome sequencing
RNAs were extracted from the virus-infected RD cells as described (Pilipenko et al., 1995). cDNA was prepared by RT–PCR (Gyml et al., 1993) using sense and antisense oligodeoxyribonucleotide primers corresponding to positions 7340–7360 and 7436–7454 of the poliovirus type 1 RNA, respectively. The amplified DNA products were puriﬁed by agarose gel electrophoresis and sequenced as described (Pilipenko et al., 1995) using a sense primer corresponding to positions 7324–7341.

Translation
The template activity of the mutant constructs was assayed in the TNT coupled transcription/translation reticulocyte lysate system kit (Promega) supplemented with HeLa S10 extract (final concentration 4% v/v), essentially using the manufacturer’s protocol. The probes contained 20 μg/ml of the respective full-length plasmid linearized by the digestion with EcoRI. Incubations were carried out at 30°C for 120 min. The template activity was quantiﬁed by the measurement of the [35S]methionine incorporation into the acid-insoluble material. The labeled products were subjected to SDS–PAGE (Pilipenko et al., 1994), followed by ﬂuorography.

Dot hybridization of viral RNA
RD cell monolayers were transfected with 2 μg of the full-length viral transcripts or infected with the viruses at an input multiplicity of 20 p.f.u./cell. The cells were incubated at 34 or 39.5°C for different time intervals as speciﬁed in the Results. The cytoplasmasic RNA was prepared as described (Marc et al., 1989). The cytoplasmasic RNA (2 μg) was denatured, immobilized onto Zeta-Probe Blotting Membrane (Bio-Rad) and hybridized to a 32P-labeled riboprobe complementary to nucleotides 3417–4830 of the poliovirus RNA followed by ﬂuorography, using a previously described protocol (Marc et al., 1989).

Molecular modeling
The model was constructed on the basis of phylogenetic predictions, secondary structure probing and genetic evidence (Pilipenko et al., 1992b; this work) using the SYBYL software, Version 6.1A (Tripos, Inc., St Louis, MO). The initial model was optimized using energy minimizations [AMBER 3.0A all-atom force field (Weiner et al., 1986) and charges, distance-dependent dielectric function, ε∞ = 40, 8 A non-boundary cut-off, Powell minimizer with line searching] until the root mean square gradient converged to <0.05 kcal/Å2. Counterions or solvent molecules were not added.
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