

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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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

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 parental-like (+) 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 *oriR*s 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 (Rietveld *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 element(s) 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 (3NTR) of enterovirus genomic RNAs (Pilipenko *et al.*, 1992b). We proposed that these structures may serve as *oriR*. An alternative folding for the poliovirus 3NTR (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 interaction (Figure 1). We (Pilipenko *et al.*, 1992b) proposed base pairing between loops of two hairpins of a novel, 'kissing', type (Chang and Tinoco, 1994), whereas a classical pseudoknot (Pleij, 1994) 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 3NTR (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

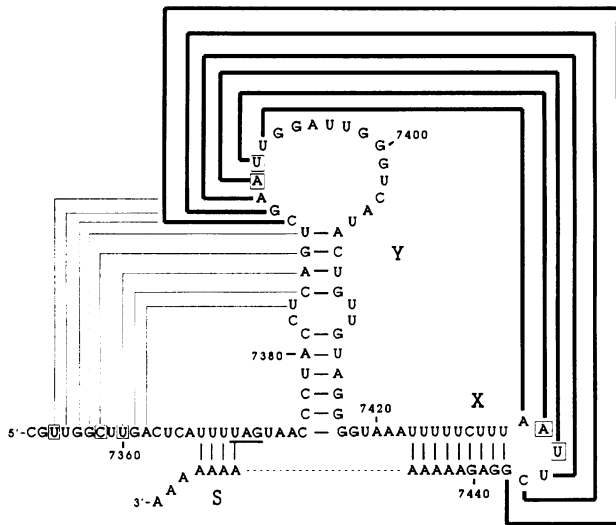


Fig. 1. Models for the poliovirus 3NTR secondary and tertiary structures. The secondary structure, with its domains S, X and Y, is given according to Pilipenko *et al.* (1992b). Proposed tertiary interactions between the loops of domains X and Y (Pilipenko *et al.*, 1992b) and between the loop of domain Y and the preceding non-structured segment (Jacobson *et al.*, 1993) are shown by thick and thin lines, respectively. The termination codon of the polyprotein reading frame is underlined. Nucleotides changed in the mutants engineered in the present study are boxed.

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_0 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, C₇₃₅₈→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_0 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

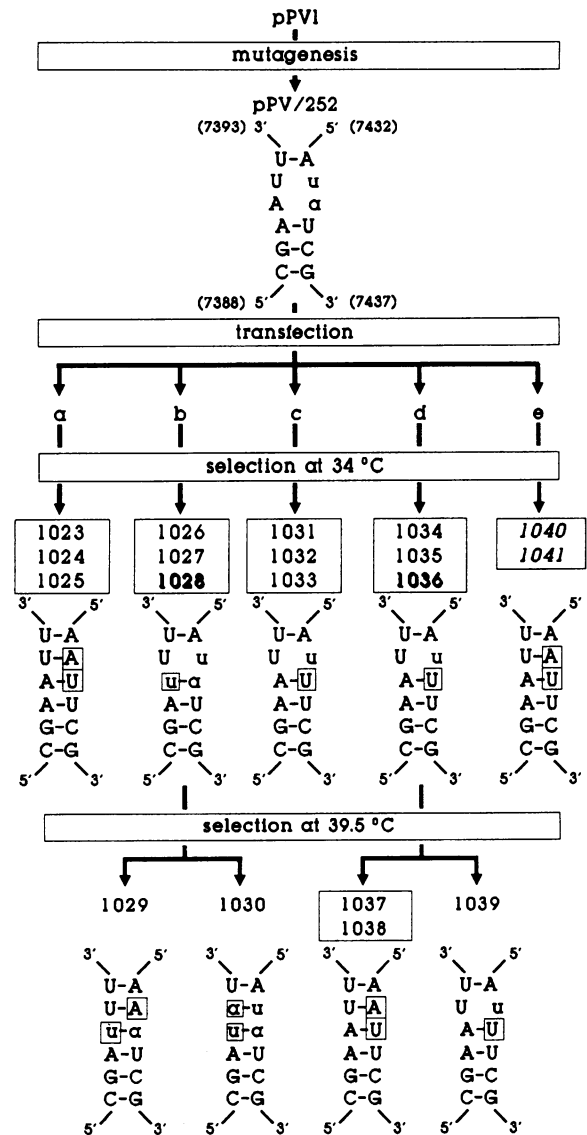


Fig. 2. The destabilized putative kissing interaction in the *oriR* of the engineered mutant pPV/252 and the structure of the relevant regions in the genomes of revertants selected at 34 and 39.5°C. The U₇₃₉₃–A₇₄₃₂ pair in pPV/252 (which probably does not exist) is shown only to demonstrate the extent of tertiary base pairing in the wild-type RNA. The nucleotides differing from the wild-type are in lower case; the nucleotides changed upon viral passages are boxed. The nucleotide sequence of the *oriR* in all the secondary clones within a given box are identical. The clones whose numbers are printed in bold were subjected to further selection at 39.5°C. The clones whose numbers are printed in italics contain an insertion of a U residue within the pentauridylic stretch between positions 7423 and 7427 (see text).

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

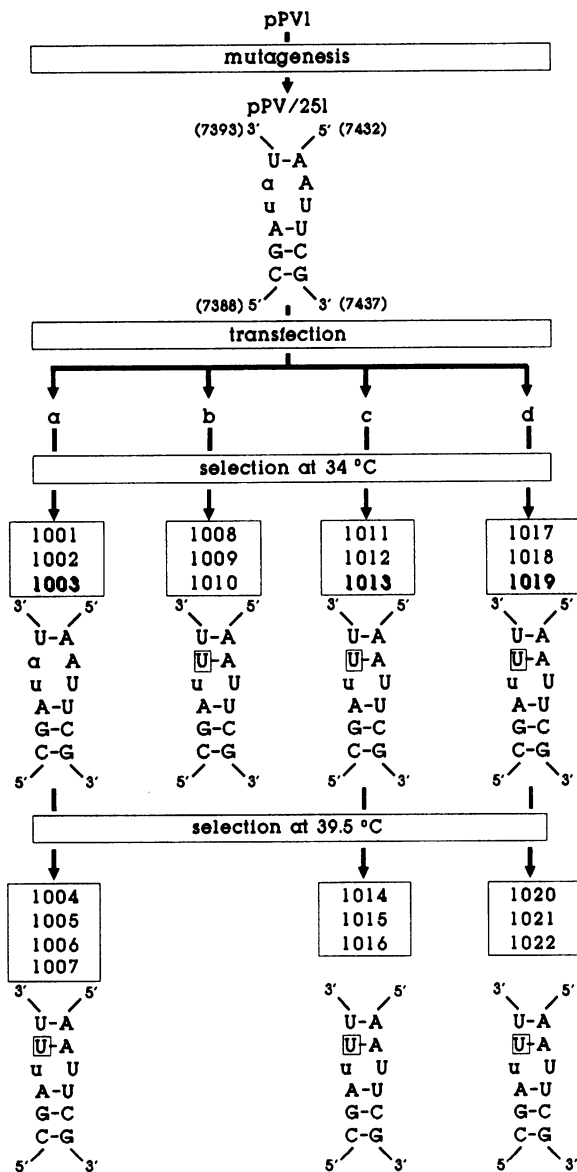


Fig. 3. The destabilized putative kissing interaction in the *oriR* of the engineered mutant pPV/251 and the structure of the relevant regions in the genomes of its revertants. For other details, see legend to Figure 2.

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/253 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

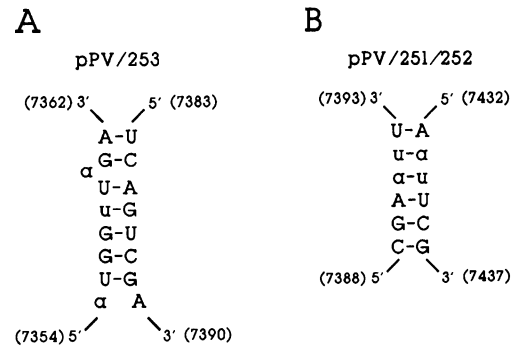


Fig. 4. The putative tertiary interactions in the genomes of engineered mutants pPV/253 (A) and pPV/251/252 (B). The nucleotides differing from those in the wild-type genome are shown in lower case. Note that the remaining (hypothetical) pseudoknot in the *oriR* of pPV/253 contains one terminal and two adjacent internal G-U pairs.

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 accumulation of the viral RNA upon transfections (Figure 5A and B), suggested that the primary plaques formed upon transfections with pPV/251 and pPV/252 RNAs might be initiated by revertants rather than by the mutants themselves. In other words, these two mutant RNAs could be classified tentatively as quasi-infectious (cf. Gmyl *et al.*, 1993). By contrast, when the mutations that had been introduced individually into pPV/251 and pPV/252 were combined in a single construct (pPV/251/252), thereby providing for a tertiary interaction of wild-type strength, the wild-type-like phenotype was retained (not shown). Likewise, transcripts of pPV/253 (with a destabilized pseudoknot) generated early appearing plaques of wild-type size (not shown). The viruses retained their phenotype upon *in vitro* cultivation (Table I).

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 (A₇₄₃₄→U in clones 1031–1036) or a second-site mutation in the loop of domain Y (A₇₃₉₁→U in clones 1026–1028) (Figure 2). All the revertants with a partially destabilized kissing interaction exhibited thermosensitivity of growth, although

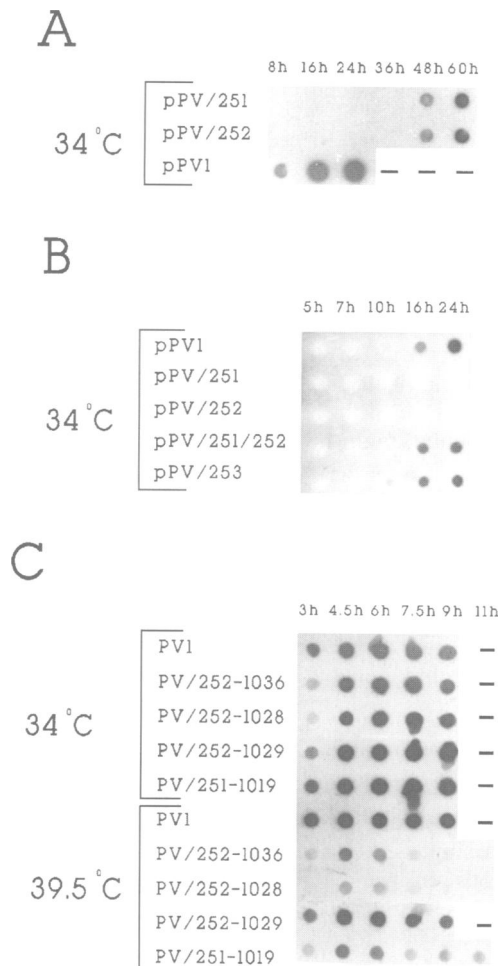


Fig. 5. Dot hybridization assay for the viral RNA synthesis triggered by the mutant and revertant genomes. The cells were either transfected with the wild-type and mutant transcripts (**A** and **B**) or infected with appropriate viruses (**B** and **C**) at the indicated temperatures. Total RNA was isolated at the indicated time intervals and assayed for the presence of virus-specific species as described in Materials and methods. The RNA from cells undergoing the productive infection could not be analyzed at late stages (dashes) due to the cytopathic effect.

to somewhat different extents. The ratio between efficiencies of plaquing at 34 and 39.5°C (EOP_{pr}) varied between 7×10^2 and 2.6×10^1 , as compared with the wild-type value of 1.3; likewise, the average plaque size (at 34°C) of these revertants varied between 3.5 and 5 mm (compared with the wild-type value of 6.8 mm) (Table I), with the difference between the mutant and wild-type plaque size being much greater at 39.5°C (not shown). The five remaining pPV/252-derived clones (1023–1025, 1040 and 1041; from two primary plaques) all exhibited the complete restoration of the potential for the kissing interaction due to the back reversions of the two mutated bases, $UA_{7434} \rightarrow AU$ (Figure 2). Clones 1040 and 1041 also harbored an additional mutation: a sixth residue U was inserted into the pentauridylic stretch between positions 7423 and 7427, with an expected lengthening of the stem of domain X by a U–A pair. All of the full revertants exhibited a wild-type phenotype with regard to both the plaque size and EOP_{pr} (as an example, see PV/252e-1041 in Table I).

The fact that all the infectious descendants of the pPV/

Table I. Biological properties of the PV/251 and PV/252 revertants and the mutant viruses

Virus	Plaque size (mm)	EOP_{pr}^a
PV1	6.8	1.3
PV/251a-1001	2.3	8.0×10^2
PV/251a-1003	4.8	3.0×10^2
PV/251a(1003)-1004	6.1	1.0×10^1
PV/251a(1003)-1005	5.8	3.0×10^1
PV/251a(1003)-1007	4.9	6.0×10^1
PV/251c-1013	4.7	1.3×10^1
PV/251c(1013)-1016	5.7	1.7
PV/251d-1019	6.7	2.5
PV/251d(1019)-1022	6.3	4.2
PV/252b-1027	4.1	7.0×10^2
PV/252b-1028	3.5	4.0×10^2
PV/252b(1028)-1029	7.4	3.9
PV/252b(1028)-1030	6.3	1.0
PV/252d-1035	5.0	2.6×10^1
PV/252d-1036	3.5	7.0×10^1
PV/252d(1036)-1039	5.7	3.2
PV/252e-1041	7.6	1.9
PV/251/252-1042	7.1	2.0
PV/253-1114	7.3	2.6

^a EOP_{pr} 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 ($U_{7433} \rightarrow A$ in clones 1029, 1037 and 1038) or by a second-site mutation in domain Y ($U_{7392} \rightarrow A$ 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 I).

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, $A_{7392} \rightarrow U$;

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 V1) 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 nuclease 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 U₇₄₃₁ 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 ~35° 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 G₇₄₁₈–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.

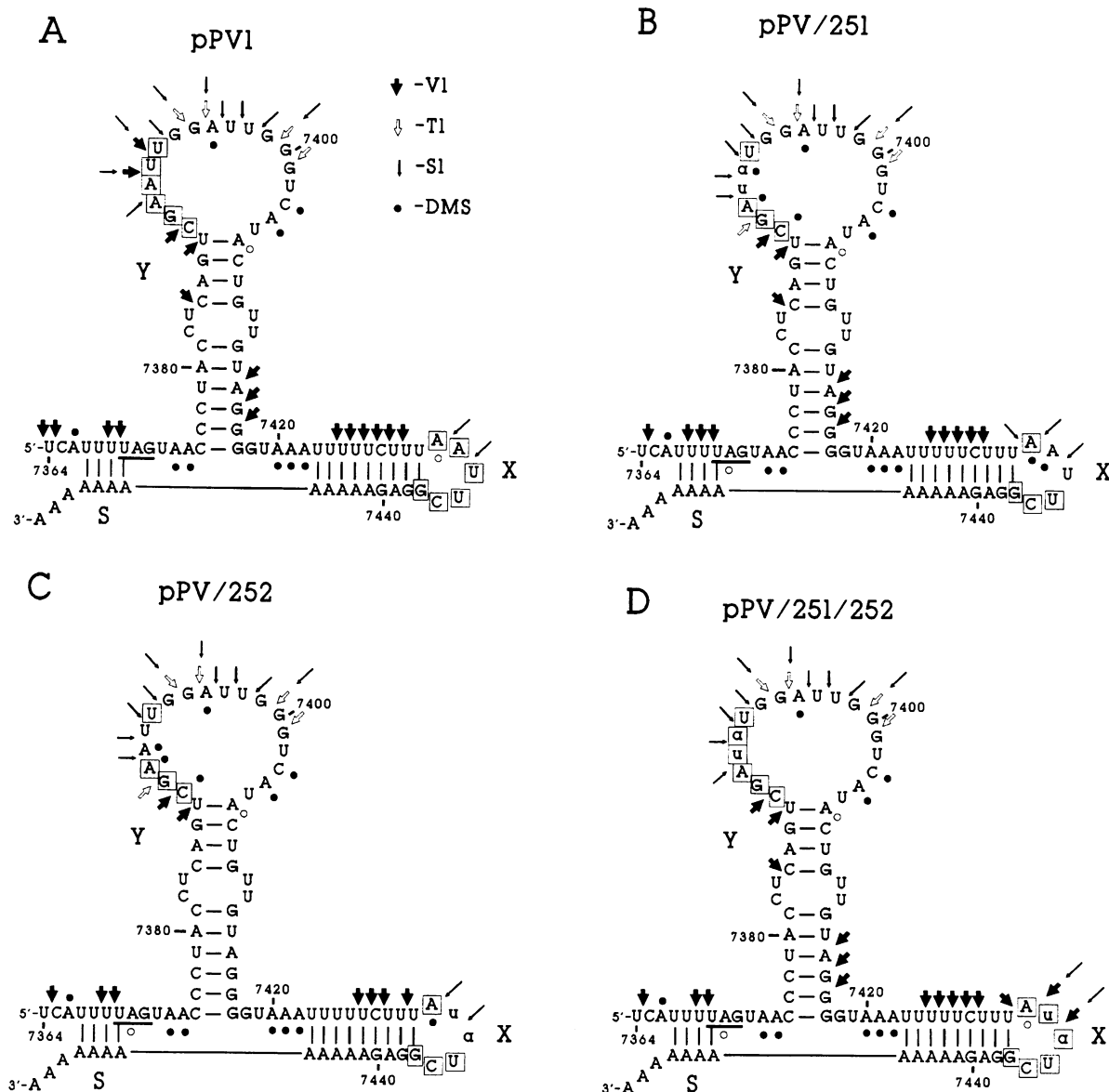


Fig. 6. Probing of the structure of *oriR* of wild-type (A), pPV/251 (B), pPV/252 (C) and pPV/251/252 (D) poliovirus RNA species. The transcripts were treated with nucleases V1, T1 and S1 as well as with DMS as described in Materials and methods. The appropriate cleavage/modification sites are denoted 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.

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 I), not accompanied by any changes in the *oriR* primary structure, also suggests the

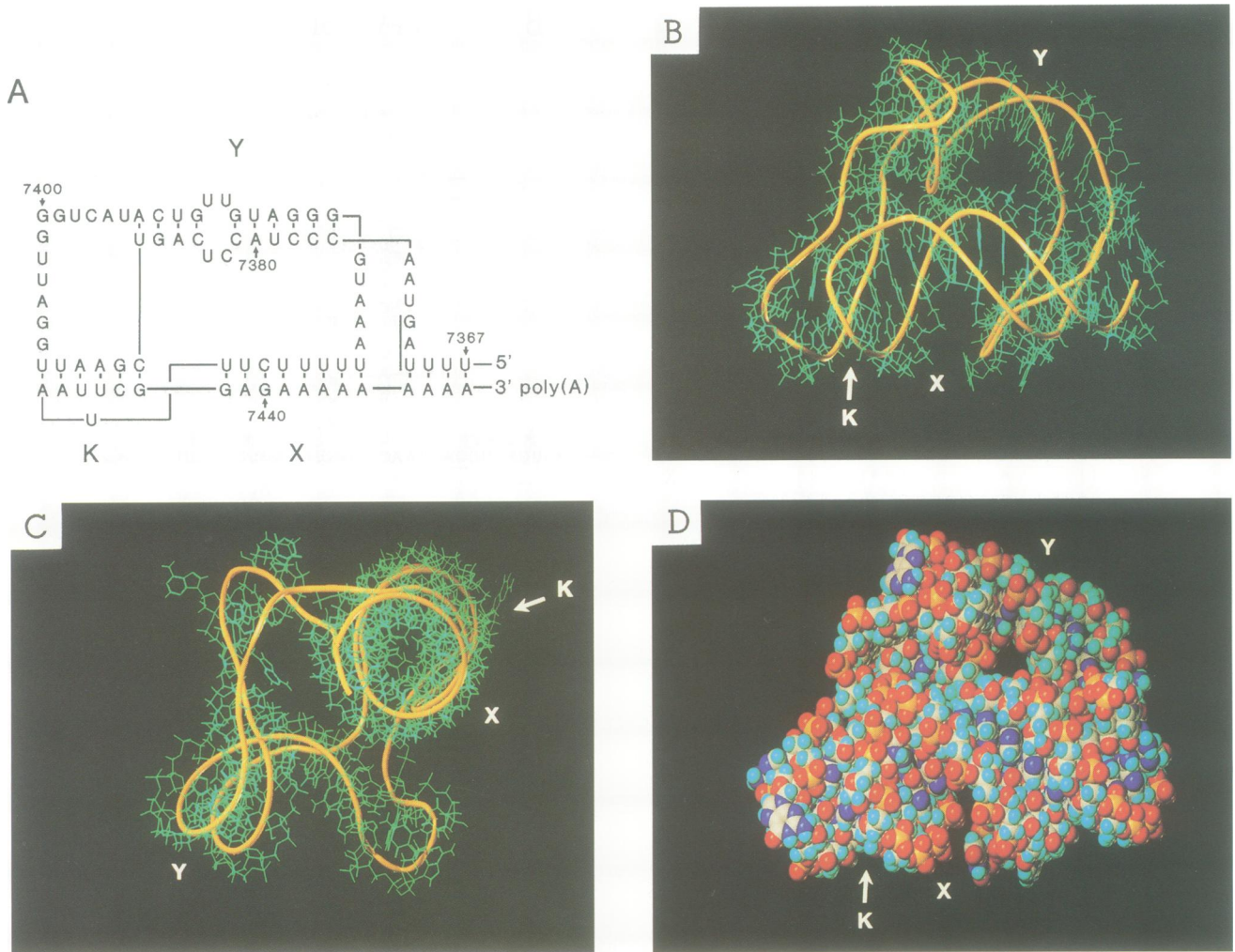


Fig. 7. Three-dimensional model of poliovirus type 1 *oriR*. (A) Schematic representation of the model. (B) and (C) Stick display style views (green) with a tube (orange) illustrating the overall folding in two projections. (D) Space-filled view of the projection shown in (B). Color coding is by atom type: P, orange; O, red; N, blue; C, white; H, cyan. X and Y correspond to the respective domains, K stands for the kissing interaction.

existence of external suppressor mutations. Characterization (which is now in progress) of such suppressors may lead to the identification of viral proteins and their domains involved in the interaction with *oriR*. A similar approach proved to be quite successful in the discovery of a specific interaction between the poliovirus *oriL* and a domain of the viral 3C protease (Andino *et al.*, 1990, 1993).

The molecular model that takes into account the previous (Pilipenko *et al.*, 1992b) and present results displays the poliovirus *oriR* as a relatively compact, quasi-globular multi-domain entity with an elaborate surface. This surface bears recognition signals for the viral RNA-dependent RNA polymerase and other replicative proteins of viral (Harris *et al.*, 1994) and, possibly, cellular (Todd *et al.*, 1995) origin. The poliovirus *oriR* appears to function as a structurally separated domain, and insertion of an extended spacer between the coding sequence and the 3NTR was not accompanied by a significant alteration of the replicative functions (Pierangeli *et al.*, 1995).

There are common and distinct features of the *oriR*s of poliovirus and other picornaviruses. The poliovirus RNA 3NTR is somewhat shorter [68 nucleotides, not taking into account the poly(A) tail] than that in the genomes of coxsackie B viruses and related enteroviruses (100

nucleotides), which contain an additional hairpin. Notwithstanding this, the 3NTRs of all enterovirus RNAs were proposed to form very similar tertiary structures stabilized by homologous kissing interactions (Pilipenko *et al.*, 1992b). Recent data demonstrate that the kissing interaction in the coxsackie B3 virus RNA 3NTR is also essential for the *oriR* function (W.J.G.Melchers, J.G.J. Hoenderop, H.J.Bruins Slot, C.W.A.Pleij, E.V.Pilipenko, V.I.Agol and J.M.D.Galama, submitted). In view of a high structural similarity, it is not surprising that the *oriR*s of polio and coxsackie B viruses are interchangeable (Rohll *et al.*, 1995). Less expected was the functional exchangeability of the polio *oriR* with the structurally dissimilar (Pilipenko *et al.*, 1992b) *oriR* of human rhinovirus 14 (Rohll *et al.*, 1995). This fact may indicate that the picornavirus *oriR*'s recognition signals are to some extent degenerate. On the other hand, the *oriR*s of bovine enterovirus and hepatitis A virus were only marginally, if at all, active as substitutes for the poliovirus *oriR* (Pierangeli *et al.*, 1995; Rohll *et al.*, 1995). The *oriR* of encephalomyocarditis virus, a representative of another genus of picornaviruses, also has an elaborate but unrelated structure, again involving a functionally important tertiary interaction (Cui and Porter, 1995).

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 *oriLs*, 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 *oriRs* (and probably *oriLs*) 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-globular conformation. *oriRs* 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 (replicational 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, pBSM13⁻ (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 *PvuII*–*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*–*PvuII* (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 nucleases 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)₄CTCCG (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 pPV/251 or pPV/252 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 plated 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 at 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 plating at 34 and 39.5°C (EOP_{p/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 (Gmyl *et al.*, 1993) using sense and antisense oligodeoxyribonucleotide primers corresponding to positions 7040–7060 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 T7 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 cytoplasmic RNA was prepared as described (Marc *et al.*, 1989). The cytoplasmic 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, ε₀ = 40, 8 Å non-bonded 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.

Acknowledgements

This research was supported by grants from the International Science Foundation (RA1000), Human Frontiers Science Program Organization (RG 470/93) and Russian Foundation for Basic Research (93-04-07594) to V.I.A. V.I.A. is a Soros Professor.

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Received on March 15, 1996; revised on June 12, 1996