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Structural requirements of the higher order RNA kissing element in the enteroviral 3′UTR

Jinhua Wang, Judith M. J. E. Bakkers, Joep M. D. Galama, Hilbert J. Bruins Slot[1], Evgeny V. Pilipenko[2,3], Vadim I. Agol[2,3] and Willem J. G. Melchers*

University of Nijmegen, Department of Medical Microbiology, PO Box 9101, 6500 HB Nijmegen, The Netherlands, [1]University of Nijmegen, CAOS/CAMM Center, PO Box 9010, 6500 GL Nijmegen, The Netherlands, [2]M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Moscow Region 142782, Russia and [3]Moscow State University, Moscow 119899, Russia

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ABSTRACT

The origin of replication (*oriR*) involved in the initiation of (–) strand enterovirus RNA synthesis is a quasi-globular multi-domain RNA structure which is maintained by a tertiary kissing interaction. The kissing interaction is formed by base pairing of complementary sequences within the predominant hairpin-loop structures of the enteroviral 3′ untranslated region. In this report, we have fully characterised the kissing interaction. Site-directed mutations which affected the different base pairs involved in the kissing interaction were generated in an infectious coxsackie B3 virus cDNA clone. The kissing interaction appeared to consist of 6 bp. Distortion of the interaction by mispairing of each of the base pairs involved in this higher order RNA structure resulted in either temperature sensitive or lethal phenotypes. The nucleotide constitution of the base which gaps the major groove of the kissing domain was not relevant for virus growth. The reciprocal exchange of the complete sequence involved in the kissing resulted in a mutant virus with wild type virus growth characteristics arguing that the base pair constitution is of less importance for the initiation of (–) strand RNA synthesis than the existence of the tertiary structure itself.

INTRODUCTION

Structural analysis of the enteroviral 3′ untranslated region (3′UTR) showed that this region contains two (poliovirus-like subgroup) or three (coxsackievirus B-like subgroup) hairpin structures, designated as domains ‘X’, ‘Y’ and ‘Z’ (Fig. 1A). The overall secondary structure can be closed by the interaction of a small poly(U)-stretch with a genetically encoded poly(A)-tail (1–3). We and others have recently shown that sequences within the loop-structure of the domain ‘X’ base pair with complementary sequences in the loop-structure of domain ‘Y’ to form an intramolecular tertiary RNA structure designated as a ‘kissing’ interaction (2–4). The kissing domain ‘K’ can be stacked to the helix of domain ‘X’ to form one coaxial helical domain which, connected to the ‘Y’-domain in poliovirus-like enteroviruses or the stacked coaxial helical ‘Y–Z’ domain (coxsackievirus B-like enteroviruses) forms the overall structure of the origin of replication (*oriR*) for the initiation of (–) strand RNA synthesis (2,3). We have recently argued that the kissing interaction most probably contains 6 bp because this appears to be (i) phylogenetically conserved and (ii) the major groove of this 6 bp domain can be folded back from the kissing domain to the extended region of domain ‘X’ by a single nucleotide (Fig. 1B). In this report we have used a genetic approach to study the functional requirements of the overall kissing interaction. Mutations were introduced in an infectious coxsackie B3 virus cDNA clone in the nucleotides possibly involved in the kissing interaction such that the interaction was either disturbed or restored. The mutated cRNAs were subsequently transfected to susceptible cells and the growth characteristics of the mutants were analysed.

MATERIALS AND METHODS

Cells and viruses

Virus propagation and viral RNA transfections were performed with Vero cells. The cells were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. After infection, cells were fed with MEM containing 3% serum and after transfection, MEM containing 10% serum was added to the cells. Virus titers were determined in eight replicate wells by titrating decimal dilutions in 96-well microtiter plates (5). $TCID_{50}$ values were calculated according to Reed and Muench (6).

Site-directed mutagenesis

A full-length copy DNA of coxsackie B3 virus (pCB3/T7) which was cloned behind a T7 RNA polymerase promoter was used in the experiments (7). For oligonucleotide-directed site-specific mutagenesis the 3′UTR was cloned into phagemid pALTER™-1 (2) and mutations were introduced using the Altered Sites™ *in vitro* mutagenesis system (Promega) according to the recommendations

*To whom correspondence should be addressed. Tel: +31 24 3617574; Fax: +31 24 3540216; Email: w.melchers@mmb.azn.nl

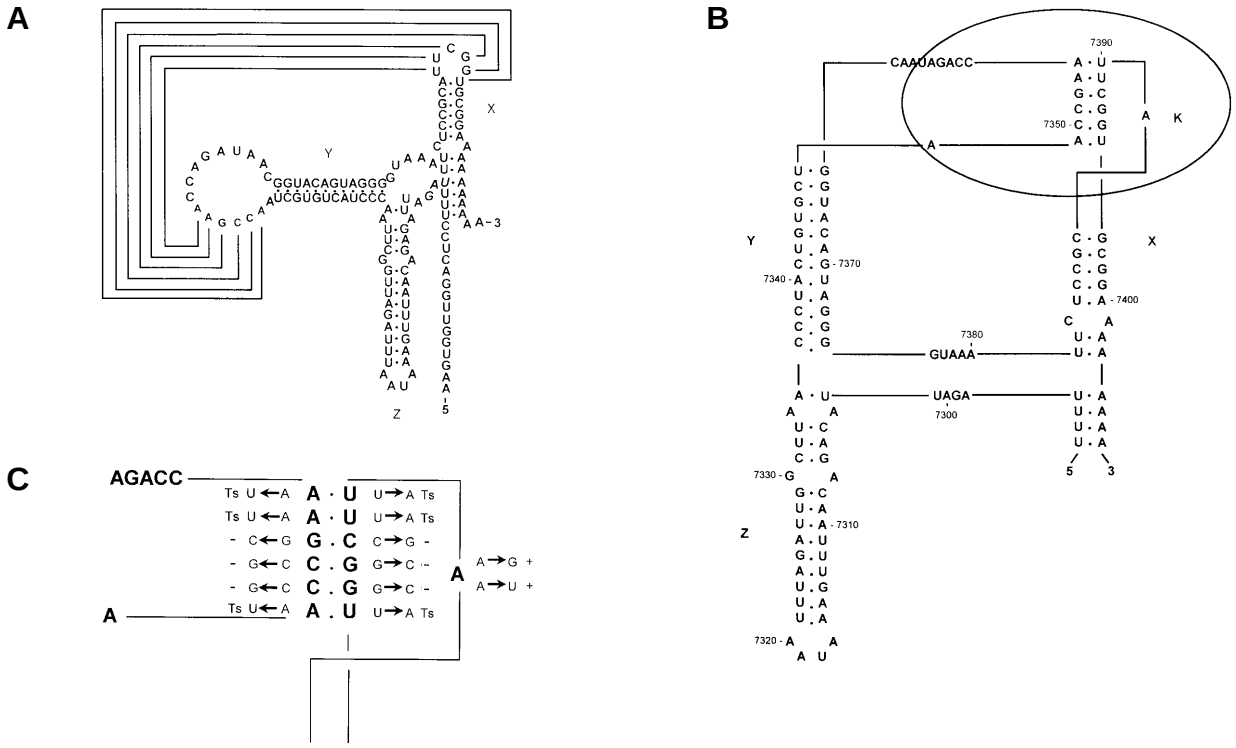

Figure 1. Structure models of the enteroviral 3′UTR. (**A**) The secondary and (**B**) the tertiary structure of the coxsackie B3 virus 3′UTR as a representative for the coxsackievirus B-like subgroup. The 3′UTR consists of three hairpin structures designated as domains 'X', 'Y' and 'Z'. The structure can be closed by an interaction between the poly(A) with a 4 nt U-stretch overlapping the 3′UTR and the 3D-coding region. The 'X' domain can be stacked to the tertiary 'kissing' interaction to form one coaxial helical element which is connected by a single-stranded nucleotide stretch ($GUAAA_{7376–7380}$ and $AGAU_{7298–7301}$) to a second coaxial helical domain 'Z–Y'. (**C**) Genotypes of the engineered mutations in the coxsackie B3 virus 3′UTR and the outcome of the mutations on viral growth. –, lethal phenotype; ts, temperature sensitive phenotype; +, wild type phenotype. Specific mutations were introduced in an infectious coxsackie B3 virus cDNA clone using the Altered Sites™ *in vitro* mutagenesis system. The mutated fragments were analysed by sequence analysis to verify the mutations. Mutations pCB3–3′UTR:G_{7352} → C and pCB3–3′UTR:C_{7392} → G have previously been described (2), all specific mutations are indicated in the figure.

of the manufacturer. Synthetic oligonucleotides (Isogen Bioscience, The Netherlands) were used to introduce site-specific mutations. The mutated fragments were cloned into pCB3/T7 and the nucleotide sequence of the mutant cDNAs was verified by sequence analysis as described previously (2).

Transfection of cells with copy RNA transcripts

pCB3/T7 plasmids were linearised by digestion with *Sal*I and transcribed *in vitro* by T7 RNA polymerase as described previously (2). Vero cells were transfected in duplo with 4 µg of copy RNA using the DEAE–dextran method (2). The cells were grown at 33 and 36°C until a cytopathic effect (CPE) was observed. When no CPE was observed 5 days after transfection, the cell cultures were subjected to three cycles of freezing and thawing and 250 µl were subsequently passaged to fresh Vero cell monolayers. When the CPE was complete the cultures were subjected to three cycles of freezing and thawing and the viruses were stored in 1 ml aliquots at –80°C. When no CPE was observed 5 days after passage, the mutations were considered to be lethal.

Single-cycle growth analysis

For determining the virus yield in a single replicative cycle, 80% confluent Vero cell monolayers were infected with virus at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell and grown at 33, 36 and 39°C for 4, 6 and 8 h (2). Viruses were released by three successive cycles of freezing and thawing and virus titers were determined by titration (2).

Sequence analysis of mutant viruses

Total RNA was isolated from 100 µl of cellular lysates obtained from the 8 h time point of the growth curve using a single extraction procedure with guanidinium thiocyanate–phenol–chloroform (2). Mutated RNA was PCR amplified using a poly(T) primer and a primer located in the 3D-coding region (5′-GTTGTTT-GACCCTCCCCGCG-3′ nt 7241–7260) as described previously (2). The resulting 179 bp PCR products were purified from low-melting agarose and the nucleotide sequence of the 3′UTR was determined using the Ampli Cycle™ sequencing kit according to the instructions of the manufacturer (Perkin Elmer).

Molecular dynamics

The starting model of the overall 3′UTR three-dimensional structure was re-optimised with MacroModel/Batchmin (8) using the AMBER* force field (9,10) applying the GB/SA implicit water model (11). The simulation was performed at 300 K and a timestep of 1 fs. All bond lengths were constrained using SHAKE (12) and the non-bonded interaction assay was recalculated every ps to correct for large movements in (part of) the model. The final geometry was obtained by gradually removing the kinetic energy

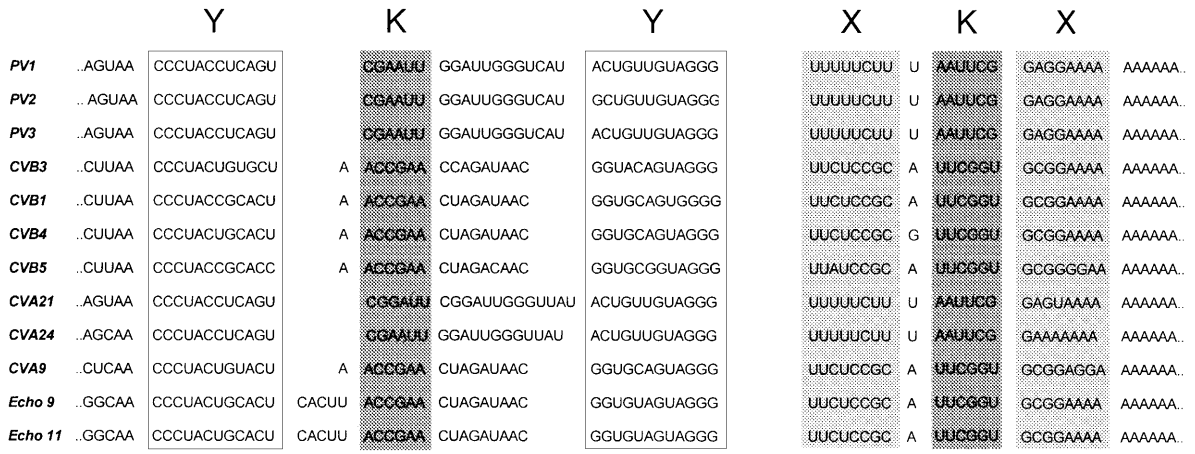

Figure 2. Sequence alignment of the enteroviral 3′UTRs. A comparative alignment was performed on all enteroviral 3′UTR sequences available. The specific domains are indicated in the figure.

in an 18 ps MD run, after which the structure was energy minimised until the RMS gradient was <0.05 kJ/Å. A full analysis of the molecular dynamics simulation will be reported elsewhere (H.J.Bruins Slot, E.V.Pilipenko, V.I.Agol and W.J.G.Melchers, in preparation).

RESULTS

Tertiary structure of the enteroviral 3′UTR

Figure 1A shows the secondary and Figure 1B the tertiary model of the coxsackie B3 virus 3′UTR as an example of the coxsackie B-like enteroviruses. A tertiary kissing interaction ('K') between the loops of domains 'X' and 'Y' can be stacked on domain 'X' to form one coaxial helical domain interconnected to a super helical domain formed by stacking of domain 'Y' to domain 'Z'. An identical tertiary structure can be built up for every human enterovirus sequenced today. Comparative alignments of all enteroviral 3′UTRs available (Fig. 2) showed that the kissing interaction always contains 6 bp, and a single nucleotide is used to gap the major groove.

Construction of mutants

The structural requirements of the kissing interaction for maintenance of the overall structure of the enteroviral 3′UTR was examined by a genetic analysis. A series of constructs, containing mutations either to disrupt or to retrieve the higher order RNA structures were generated by site-directed mutagenesis. The mutations were verified by sequence analysis and introduced into the infectious coxsackie B3 virus cDNA clone pCB3/T7. The genotype of the different constructs is shown in Figure 1C.

Effect of the mutations on viral growth

To study the effect of the mutations on virus viability, Vero cells were transfected with copy RNA transcripts of the different constructs. All mutations disturbing either a G•C or C•G base pair (Fig. 1C) displayed a lethal phenotype. A CPE was observed upon transfection of all mutated RNAs in which either one of the A•U base pair was modified into a mispair (either A•A or U•U; Fig. 1C). All six individual mutated RNAs in which the specific base pairs were mirrored (for example A•U into U•A and C•G into G•C) grew viruses upon transfection. The growth characteristics of the viruses obtained were further analysed by single-cycle growth analysis at 33, 36 and 39°C (e.g. Fig. 3). The mutant viruses in which an A•U base pair was changed into either an A•A or U•U mispair (vCB3–3′UTR/A_{7354} → U; vCB3–3′UTR/A_{7353} → U; vCB3–3′UTR/A_{7349} → U; vCB3–3′UTR/U_{7390} → A; vCB3–3′UTR/U_{7391} → A; and vCB3–3′UTR/U_{7395} → A) exhibited a temperature sensitive phenotype with a virus yield of ~10% of that of wild type at 39°C. Restoration of the kissing interaction by mirroring the specific individual base pairs, however, resulted in mutant viruses with wild type growth characteristics at all temperatures. Transfection of constructs pCB3–3′UTR/$ACCGAA_{7349–7354}$ → UGGCUU and pCB3–3′UTR/$UUCGGU_{7390–7395}$ → AAGCCA, in which both strands involved in the kissing were individually mutated to their complementary sequences, resulted in lethal phenotypes, while the double mutant in which a mirror image of the complete kissing interaction was created, yielded a virus exhibiting the growth characteristics of the wild type-like phenotype.

The adenine at position 7389 which gaps the major groove of the kissing domain and folds back from the stacked coaxial X domain (Fig. 1B), is not conserved on the nucleotide level within the different enteroviruses sequenced today (Fig. 2). Both a guanine and a uracil may replace the adenine, and indeed alteration of the A_{7389}-residue into a uracil in mutant pCB3–3′UTR/A_{7389} → U or a guanine pCB3–3′UTR/A_{7389} → G (Fig. 1C), yielded a virus which exhibited the growth characteristics of the wild type virus, as examined by single-cycle growth analysis at 33, 36 and 39°C (Fig. 3).

Sequence analysis of the 3′UTR of all mutants analysed showed that the mutations introduced by site-directed mutagenesis were retained in the mutant viral RNAs and that no other mutations had occurred.

DISCUSSION

Replication of enterovirus is initiated in the cytoplasm of the host cell by the synthesis of a complementary RNA strand of negative polarity. We and others have recently described that a *cis*-acting

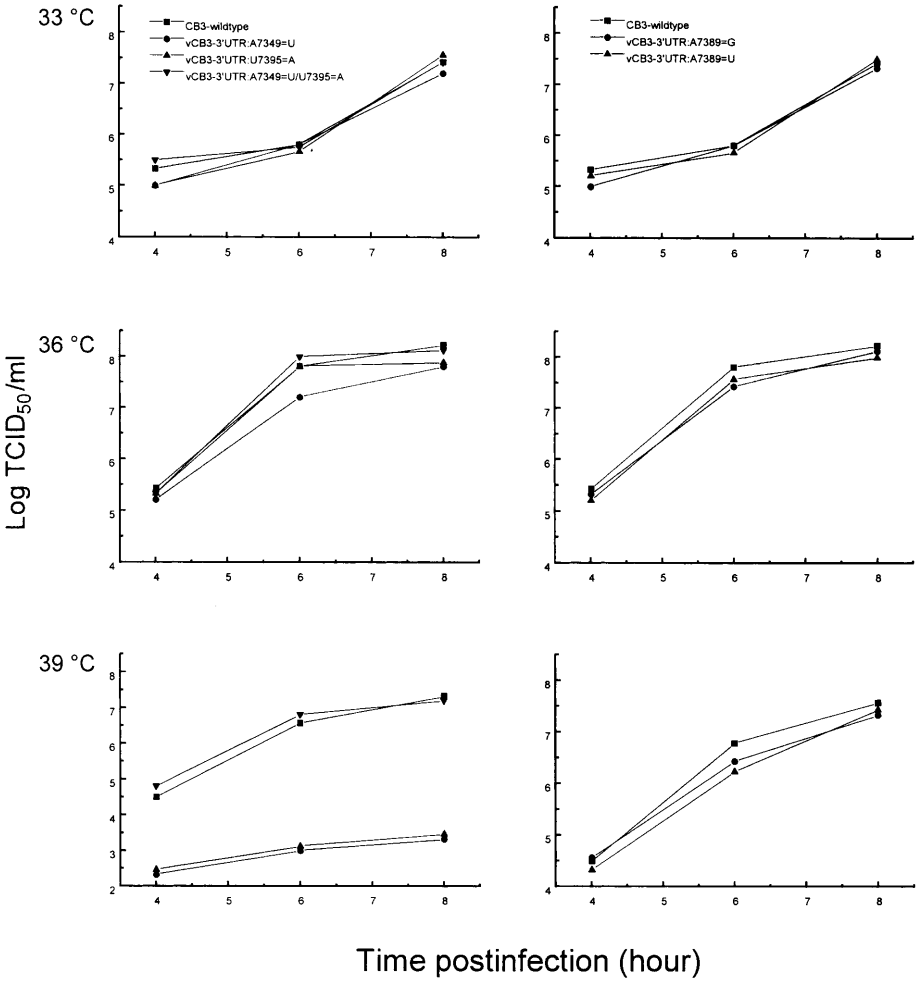

Figure 3. Single-cycle growth curves of structural mutants. Vero cells were infected with wild-type and mutant viruses at an MOI of 1 $TCID_{50}$ per cell. The cells were grown at 33, 36 and 39°C for 4, 6 and 8 h. Virus titers were determined as described in detail previously (5). The specific mutants are indicated in the figure.

element, the *oriR*, is involved in the initiation of (–) strand RNA synthesis. This RNA structure is maintained by an intramolecular tertiary kissing interaction formed between the loop-structures of the two predominant hairpin-structures within the different enteroviral 3′UTRs (2–4). The results presented here show that the kissing interaction between the loops of domains X and Y is of crucial importance for the recognition of the coxsackie B3 *oriR* by the replication machinery.

In this report we show that all 6 bp potentially involved in the kissing interaction are required for the optimal functioning of the structure in viral replication. Any mutation affecting either one of the base pairs within the kissing interaction resulted in an altered phenotype. The effect of the mutations on viral growth was dependent on the base pair modified; alteration of any A•U base pair into a mispair (mutants A_{7349}–A_{7395}, A_{7353}–A_{7391}, A_{7354}–A_{7390}, U_{7349}–U_{7395}, U_{7353}–U_{7391}, U_{7354}–U_{7390}; Fig. 1) resulted in viruses which exhibited a temperature sensitive phenotype while mispairing of either one of the three G•C base pairs (mutants C_{7350}–C_{7394}, C_{7351}–C_{7393}, C_{7352}–C_{7393}, G_{7350}–G_{7394}, G_{7351}–G_{7393}, G_{7352}–G_{7393}; Fig. 1) resulted in lethal phenotypes. The results obtained by mispairing C_{7350}•G_{7394} and C_{7351}•G_{7393} confirmed the importance of maintaining the C•G base pair as previously described for the coxsackie B3 virus C_{7352}•G_{7393} base pair (2). Coxsackie A9 virus is closely related to coxsackie B3 virus and contains an identical kissing sequence (4). Interestingly the equivalents of base pairs C_{7350}•G_{7394} and C_{7351}•G_{7393} in coxsackie A9 virus were shown likewise to be important (4). The importance of the kissing A•U base pair extremities (A_{7349}•U_{7395} and A_{7354}•U_{7390}) indeed confirmed that the kissing interaction consists of 6 bp. Both the mutants in which each individual base pair was restored and the mirror kissing mutant yielded viruses with wild type growth characteristics, indicating that the base pair constitution of the kissing domain is of less importance for the initiation of (–) strand RNA synthesis than the formation of the tertiary structure itself. This is in agreement with differences in base pair constitution of the kissing interaction observed in various sequenced enteroviruses (Fig. 2) and the finding that base pair A_{7353}•U_{7391} can be exchanged into an alternative base pair without effecting viral growth (2).

The differences in the number of G•C base pairs, either two for the poliovirus-like or three for the coxsackie B virus-like enteroviruses, may reflect a virus-type specific acquisition. Alteration of the G_{7352}•C_{7392} base pair in coxsackie B virus into

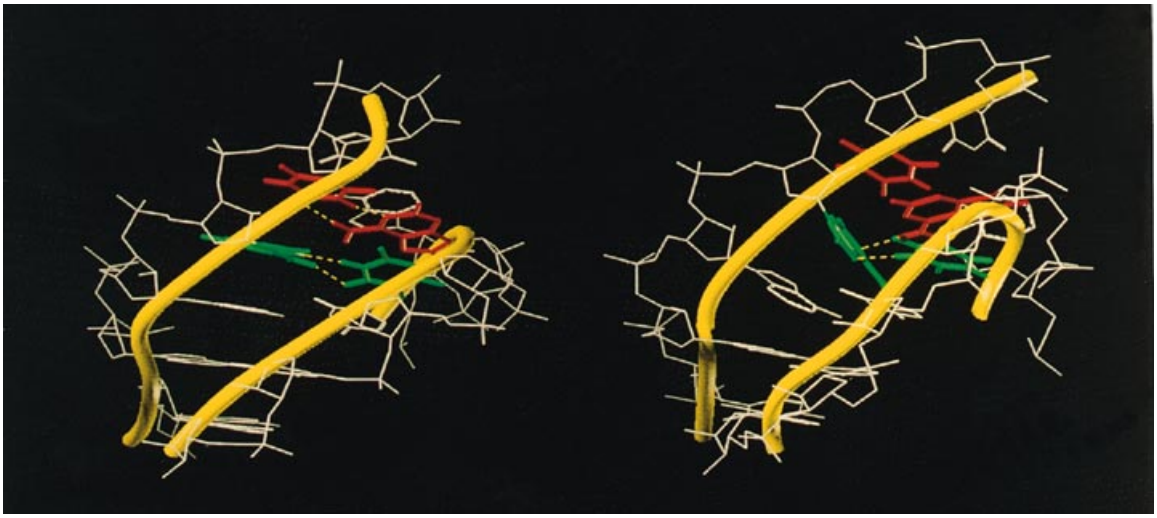

Figure 4. Three dimensional model of the kissing structure. Three dimensional model of the poliovirus kissing structure at the start of the molecular dynamics simulation (**left**) and after 1 ns simulation (**right**). A Hoogsteen base pair between A_{7391}•U_{7434} (green capped sticks) and a Watson–Crick base pair (red capped sticks) between U_{7392}•A_{7433} (left) convert during the simulation to two unclassified base pairs (right). To simplify the figure, only the kissing interaction extracted from the overall 3′UTR three-dimensional structure is shown as white lines. Hydrogen bonds are shown in yellow dashed lines. The overall kissing structure is presented in a tube-display style.

a G•U wobble-pair, indeed resulted in a virus which exhibited a temperature sensitive phenotype which could not be restored by an A•U base pair, arguing for the importance of the G•C base pairs (2).

The constitution of the nucleotide that gaps the major groove of the kissing domain to fold back the kissing domain to domain X, A_{7389}, is of minor importance as shown by mutants vCB3–3′UTR/A_{7389} → U and vCB3–3′UTR/A_{7389} → G which yielded viruses with wild type growth characteristics. This appears phylogenetically correct since both a guanine and a uracil can be found at this position in the different enteroviruses (Fig. 2) and indicates that this nucleotide is only required to gap the major groove formed by the 6 bp of the kissing interaction.

The essential part of the dimer linkage structure of the RNA of human immunodeficiency virus (HIV) type 1 is also formed by an (inter)molecular kissing interaction. Modification of this HIV kissing interaction results in a reduced viral infectivity and implicates an important biological function for this tertiary structure as well (13,14). Moreover, although considerable sequence divergence exists between different HIV 1 strains, the kissing interaction always contains a 6 nt self-complementary sequence (15,16). It is not known yet why 6 nt seem to be important for the formation of the structure. However, using computer simulation of the three-dimensional model of the poliovirus 3′UTR by molecular dynamics it was shown that the kissing interaction was the most stable structure within the overall *oriR* (H.J.Bruins Slot, E.V.Pilipenko, V.I.Agol and W.J.G.Melchers, in preparation). The stability seems to be due to the structural fitness of the complex *oriR* structure which is mainly dependent on the right orientation of all strands nearby the kissing rather than on the thermodynamic stability of the kissing interaction itself. Interestingly the canonical A_{7391}•U_{7434} and U_{7392}•A_{7433} base pairs converted during the simulation from an Hoogsteen and a Watson–Crick base pair, respectively, to two unclassified base pairs (Fig. 4) (H.J.Bruins Slot, E.V.Pilipenko, V.I.Agol and W.J.G.Melchers, in preparation; 17). This conversion results in alternating helical twists between adjacent base pairs and distortions in the kissing helix possibly similar to those found in the HIV kissing interaction (18). It has been suggested that RNA helix distortions are important to create active sites for RNA and protein recognition (18,19). In the case of enteroviruses, helical distortions in the kissing interaction may facilitate the formation of an RNP complex at the *oriR*. Indeed, both viral (20) and cellular (21,22) proteins specifically recognise the enteroviral 3′UTR to initiate (–) strand RNA synthesis, and recently a cellular protein was identified which was dependent on the existence of the kissing element for binding (23). However, the viral proteins involved in the recognition of the *oriR*, 3AB-CD (20) also recognize the *cis*-acting element (*oriL*) involved in the initiation of (+) strand RNA synthesis (24). The *cis*-acting element in the enteroviral 5′UTR is formed by a cloverleaf-like structure contained by the first 90 nt of the 5′UTR. This structure interacts with the viral 3CD precursor and cellular factors (25–27). It is quite possible that the viral replication machinery is able to identify two such dissimilar types of *cis*-acting elements to initiate both (–) and (+) strand RNA synthesis (17,28). On the other hand, Pilipenko *et al*. (1) have suggested that the 5′UTR cloverleaf-like structure is also folded in a higher order RNA structure, similar to the kissing interaction present in the 3′UTR suggesting that both 5′- and 3′-ends might be very similar in overall structure. However, experimental data to support this possibility are lacking.

Despite the fact that any mutation affecting the complementary of the kissing element resulted either in temperature sensitive, quasi-infectious or even lethal phenotypes (2–4), substantial deletions, including the complete deletion of the X or Y domain (23) or even the entire 3′UTR (29), yielded viable viruses. These mutants, however, exhibited a poor replicative potential, again demonstrating the requirement of *oriR* for efficient replication. A residual replicative activity of such mutants might be due to (i) secondary (internal) *cis*-elements similar to those found in the rhinovirus capsid-coding region (30) and/or (ii) suppressive

effects of second-site mutations in the viral replicative proteins as described previously for some 5′UTR mutations (25). Remarkably, the above gross 3′UTR alterations might result in a less severe functional defect compared with certain mutations disturbing the *oriR* kissing element. Thus, the structural defects in the *oriR* did not appear to be compensated as readily as the complete *oriR* deletions.

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