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

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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). TCID50 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
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 SalI 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 TCID₅₀ 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-GACCCTCCCCCG-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
The infectious coxsackie B3 virus cDNA clone pCB3/T7. The mutations were verified by sequence analysis and introduced into structures were generated by site-directed mutagenesis. The mutations either to disrupt or to retrieve the higher order RNA examined by a genetic analysis. A series of constructs, containing ′UTRs available (Fig. 2) showed that the kissing interaction always contains 6 bp, and a single nucleotide is used to gap the major groove.

**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/A7354 → U; vCB3–3′UTR/A7353 → U; vCB3–3′UTR/A7349 → U; vCB3–3′UTR/U7390 → A; vCB3–3′UTR/U7391 → A; and vCB3–3′UTR/U7395 → 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/ACCGAA7349–7354 → UGGCUU and pCB3–3′UTR/UUCGGU7390–7395 → AAAGCCA, 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 A7389-residue into a uracil in mutant pCB3–3′UTR/A7389 → U or a guanine pCB3–3′UTR/A7389 → 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...
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 A7349–A7395, A7353–A7391, A7354–A7390, U7349–U7395, U7353–U7391, U7354–U7390; Fig. 1) resulted in viruses which exhibited a temperature sensitive phenotype while mispairing of either one of the three G•C base pairs (mutants C7350–C7394, C7351–C7393, C7352–C7393, G7350–G7394, G7351–G7393, G7352–G7393; Fig. 1) resulted in lethal phenotypes. The results obtained by mispairing C7350•G7394 and C7351•G7393 confirmed the importance of maintaining the C•G base pair as previously described for the coxsackie B3 virus C7352•G7393 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 C7350•G7394 and C7351•G7393 in coxsackie A9 virus were shown likewise to be important (4). The importance of the kissing A•U base pair extremities (A7349•U7395 and A7354•U7390) 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 A7353•U7391 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 G7352•C7392 base pair in coxsackie B virus into
The kissing interaction itself. Interestingly the canonical A7391 kissing rather than on the thermodynamic stability of the kissing mainly dependent on the right orientation of all strands nearby the orientation. From this position in the different enteroviruses (Fig. 2), it appears phylogenetically correct since both a guanine and a uracil yielded viruses with wild type growth characteristics. This appears to be true for all 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 foundation of the nucleotide that gap the major groove of the kissing domain to fold back the kissing domain to domain X, A7389, is of minor importance as shown by mutants vCB3–3'UTR/A7389 → U and vCB3–3'UTR/A7389 → 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 the 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 kissing (H.J.Bruins Slot, E.V.Filipenko, V.I.Agol and W.J.G.Melchers, in preparation). The stability seems to be due to the structural fitness of the complex kissing 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 A7391•U7434 and U7392•A7433 base pairs converted 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).

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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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