Structural and functional characterization of the coxsackievirus B3 CRE(2C): role of CRE(2C) in negative- and positive-strand RNA synthesis

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A stem–loop element located within the 2C-coding region of the coxsackievirus B3 (CVB3) genome has been proposed to function as a cis-acting replication element (CRE). It is shown here that disruption of this structure indeed interfered with viral RNA replication in vivo and abolished uridylylation of VPg in vitro. Site-directed mutagenesis demonstrated that the previously proposed enteroviral CRE consensus loop sequence, R1NNNAAR2NNNNNR3, is also applicable to CVB3 CRE(2C) and that a positive correlation exists between the ability of CRE(2C) mutants to serve as template in the uridylylation reaction and the capacity of these mutants to support viral RNA replication. To further investigate the effects of the mutations on negative-strand RNA synthesis, an in vitro translation/replication system containing HeLa S10 cell extracts was used. Similar to the results observed for poliovirus and rhinovirus, it was found that a complete disruption of the CRE(2C) structure interfered with positive-strand RNA synthesis, but not with negative-strand synthesis. All CRE(2C) point mutants affecting the enteroviral CRE consensus loop, however, showed a marked decrease in efficiency to induce negative-strand synthesis. Moreover, a transition (A to G) regarding the first templating adenosine residue in the loop was even unable to initiate complementary negative-strand synthesis above detectable levels. Taken together, these results indicate that the CVB3 CRE(2C) is not only required for the initiation of positive-strand RNA synthesis, but also plays an essential role in the efficient initiation of negative-strand RNA synthesis, a conclusion that has not been reached previously by using the cell-free system.

INTRODUCTION

Coxsackie B viruses, belonging to the Human enterovirus B cluster of the family Picornaviridae, are small, non-enveloped viruses with a single-stranded 7–5 kb RNA genome containing a 3’ poly(A) tail. The genomic organization consists of one large open reading frame flanked by non-coding regions (NCRs) at the 5’ and 3’ termini. Virus replication is a two-step process, commencing with the synthesis of a complementary negative strand from virion RNA, which then serves as a template for the generation of a large number of progeny positive-strand RNAs (Wimmer et al., 1993). Essential for viral RNA replication are cis-acting replication elements (CREs) located at both the 5’ and 3’ NCRs of the viral genome. The cloverleaf structure present at the 5’ terminus of the virus genome is a multifunctional element that interacts with viral and cellular proteins to form a ribonucleoprotein (RNP) complex that is required for the initiation of both negative- and positive-strand RNA synthesis (Andino et al., 1990; Herold & Andino, 2000). Additionally, this RNA structure has been suggested to be involved in mediating the switch from viral translation to replication (Gamarnik & Andino, 1998) and also plays a role in the uridylylation of
the viral protein VPg (Lyons et al., 2001). Studies have shown that the 3' NCR forms a complex ‘kissing’ RNA interaction that is required for virus replication (Melchers et al., 1997, 2000; Mirmomeni et al., 1997; Filipenko et al., 1996; Wang et al., 1999). Interaction between the RNP complexes formed at the 5' and 3' termini of the viral genome has been proposed to induce a circular conformation that is required for the initiation of negative-strand RNA synthesis (Barton et al., 2001; Herold & Andino, 2001).

The identification of a CRE located within the coding region of the picornaviral genome (Gerber et al., 2001; Goodfellow et al., 2000; Lobert et al., 1999; Mason et al., 2002; McKnight & Lemon, 1998) presented an additional RNA structure that is required for virus replication. The conformation of these CREs, based on computational and biochemical analysis, is a hairpin structure containing a terminal loop, most commonly of 14 nt (Goodfellow et al., 2003a), confirmed recently by nuclear magnetic resonance spectroscopy (Thiviyanathan et al., 2004), although in different picornaviruses the size of the loop may vary (Gerber et al., 2001; Yang et al., 2002; Yin et al., 2003). Sequence similarity within the loop present in all CREs described thus far is consistent with the consensus sequence 5'-AAAACA-3' (Rieder et al., 2000). In poliovirus and human rhinovirus 2, the first two adenine residues within this consensus are the residues essential for the covalent linkage of UMP nucleotides to VPg, resulting in the extended viral peptide primer VPgpU(pU) (Gerber et al., 2001; Paul et al., 2000, 2003; Rieder et al., 2000). By studying the human rhinovirus 14 CRE(VPI), Yang et al. (2002) extended this research and postulated a common R1NNNAAR2NNNNNNR3 motif (R, A/G; N, any nucleotide) for the loop of rhinovirus and enterovirus CREs, a conclusion confirmed for poliovirus (Yin et al., 2003). Because positive as well as negative RNA strands are VPg-linked (Nomoto et al., 1977a; Pettersson et al., 1978), it was proposed that the CRE is required for the initiation of both steps of RNA replication (Paul et al., 2000; Rieder et al., 2000). Recent reports, however, showed that a structurally disrupted CRE mutant retained the capacity to induce negative-strand RNA synthesis in a cell-free translation/repllication system, suggesting that the CRE is only required for positive-strand RNA synthesis (Goodfellow et al., 2003b; Morasco et al., 2003; Murray & Barton, 2003).

This study was undertaken to examine the structural and functional requirements of the coxsackie B3 virus (CVB3) CRE(2C) in RNA replication. CRE(2C) loop mutants were analysed for their capacity to serve as template for VPg uridylylation and to induce negative- and positive-strand synthesis using HeLa S10 cell extracts. Here, we provide evidence that the CVB3 CRE(2C) is involved in the initiation of both negative- and positive-strand RNA synthesis.

**METHODS**

**Cells and media.** Buffalo green monkey (BGM) cells were grown in minimal essential medium (MEM) (Gibco) as described previously (Merkle et al., 2002). HeLa S3 cells (ATCC CCL 2.2) were grown either (i) in tissue-culture flasks in Dulbecco’s modified Eagle’s medium–nutrient mixture F-12 (Ham) (1:1), supplemented with 2 mM L-glutamine, 100 U penicillin and streptomycin ml⁻¹ and 10% fetal calf serum, or (ii) in suspension using suspension MEM (Joklik-modified) supplemented with 2 mM L-glutamine, 100 U penicillin and streptomycin ml⁻¹ and 10% newborn calf serum.

**Construction of CRE mutants.** All CRE mutations were introduced into the CVB3 infectious clone (Klump et al., 1990) by using the Altered Sites in vitro mutagenesis system (Promega). Selected clones were verified for the correct mutation by using sequence analysis and a BshHI/XhoI digestion was used to ligate the mutated CREs into pRibCB3/T7. For details of the construction, see Supplementary Material (available in JGV Online).

**Oligonucleotide site-directed mutagenesis.** Oligonucleotide-directed mutagenesis was performed by using phagemid pALTER-1 as described previously (Melchers et al., 1997). Synthetic oligonucleotides (Biologeo) were used to introduce site-specific mutations (Table 1). The mutated fragments were cloned into the infectious cDNA clone (pRibCB3/T7) and sequence analysis was used to verify the mutated nucleotide sequence.

**RNA transfection and virus growth.** pRibCB3/T7 plasmids were linearized by using MluI and RNA was transcribed in vitro by using T7 RNA polymerase as described previously (Melchers et al., 1997). The transcript RNA was transacted into BGM cells as described previously (Melchers et al., 1997). Virus yields were determined in BGM cells by end-point titrations using eight replicates of serial 10-fold dilutions in 96-well plates containing BGM cell monolayers (van Kuppeveld et al., 1997). TCID50 was calculated according to Reed & Muench (1938).

**Luciferase assay.** BGM cells, grown in six-well plates to a confluence of 80%, were transfected as described previously (Melchers et al., 1997) with 4 µg T7 RNA polymerase-generated cRNA derived from MluI-linearized replicon plasmid, containing the firefly luciferase gene. Ten hours post-transfection, cells were washed twice with PBS, prior to lysis using 200 µl lysis buffer (Promega). Luciferase activity was measured on a BioOrbit 1251 luminometer using the Luciferase Assay system according to the recommendations of the manufacturer (Promega).

**Expression and purification of coxsackie B3 viral 3Dpol and 3CDpro–6His.** Proteins were expressed by using the pET26-Ub-based prokaryotic expression system as reported previously (Gohara et al., 1999), which generates N-terminally linked yeast ubiquitin-fusion proteins. Overexpression of protein is performed in the BL21(DE3)pCG1 strain of *Escherichia coli*. CVB3 3Dpol and 3CDpro–6His were expressed essentially in the same manner as the poliovirus proteins described previously (Gohara et al., 1999) from the polyethylenimine precipitation. The protein concentration of each collected fraction (0.5 ml) was determined by measuring the A280 in the presence of 6 M guanidine hydrochloride and using the following absorbance coefficients: 3D, 69270 M⁻¹ cm⁻¹; 3CD, 82640 M⁻¹ cm⁻¹. Samples were aliquotted and stored at −80 °C.

**In vitro uridylylation assay.** For short RNA transcripts of wild-type and CRE(2C) loop mutants, a PCR product was generated, using wild-type or mutant infectious clones as template, with reverse primer 5'-CACGGCGAAAATACAGGTTCA-3' and forward primer 5'-GCGGAATGAGCATTAATCAGGTTCA-3', containing a T7 polymerase sequence (italic type). For the generation of genomic wild-type and mutant RNA transcripts, infectious clone p53CB3/T7 was linearized by using MluI. Synthesis of VPgpU(pU) was measured by using a reaction mixture (20 µl) containing 50 mM HEPES (pH 7.5), 8% (v/v) glycerol, 3-5 mM magnesium acetate, 0-7 µM 3CDpro, 2 µM 3Dpol, 40 µM synthetic...
Table 1. Oligonucleotides used for mutagenesis

Underlined nucleotides indicate mutations from the wild-type sequence.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>DM</td>
<td>AGGGCTCCCGTGACAGGAAGCAAGCAACACGTTCTATCCTACATT</td>
</tr>
<tr>
<td>DM + A7G</td>
<td>AGGGCTCCCGTGACAGGAAGCAAGCAACACGTTCTATCCTACATT</td>
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<tr>
<td>CRE PM1</td>
<td>AAATACGGCATTCTTGGACATTGAATGTAATTGCAATTTG</td>
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<tr>
<td>CRE PM2</td>
<td>AAATACGGCATTCTTGGACATTGAATGTAATTGCAATTTG</td>
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<td>CRE PM3</td>
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<td>CRE PM16</td>
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</tr>
<tr>
<td>CM</td>
<td>ACAGGTTCAATACGGCATTTGGACTTG</td>
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CVB3 VPg, 0·75 μCi (0·277 MBq) [α-32P]UTP (3000 Ci mmol⁻¹) and 25 μM unlabelled UTP. Amount of template for the reaction was 0·5 μg for CRE(2C) transcript RNA and 2 μg for genomic transcript RNA. Reaction mixtures were incubated for 1 h at 34 °C and the reaction was quenched by adding 5 μl gel-loading buffer. The samples were analysed by Tris/Tricine SDS-PAGE (Bio-Rad) with 13·5 % polyacrylamide. The gels were dried and autoradiographed for visualization of product. Reaction products were quantified by measuring the amount of [32P]UMP incorporated into the product by using a phosphorimager (Storm 860; Molecular Dynamics) and converting it into c.p.m. by using a radioactive marker.

In vitro translation and replication assay. Preparation of HeLa S10 cell extract and initiation factors has been described previously in detail by Barton et al. (1995). Negative- and positive-strand RNA synthesis was analysed as described by Herold & Andino (2000) with some minor modifications: 2 μg coxsackievirus transcript RNA was mixed with 30 μl HeLa S10 cell extract, 2 μl initiation factors, 5 μl TENV/energy mix (Herold & Andino, 2000) and 1 μl 100 mM guanidine hydrochloride in a total volume of 50 μl. After incubation at 30 °C for 4 h and centrifugation, the pre-initiation complexes were resuspended in 50 μl labelling mix, containing 30 μl HeLa S10 cell extract, 5 μl 10× NTP/energy mix and 4 μl [α-32P]UTP (10 μCi μl⁻¹). After incubation at 30 °C for 2 h, the samples were mixed with 150 μl TENSK buffer [50 mM Tris/HCl (pH 7·5), 5 mM EDTA, 100 mM NaCl, 1 % (v/v) SDS, 200 μg proteinase K ml⁻¹] to stop the reaction. RNA was extracted by using phenol/chloroform and RNA was precipitated by using ethanol. The pellet was resuspended in RNA-storage solution (Ambion) and gel-loading buffer. The gel-loading buffer was added prior to loading on a 0·8 % agarose gel. After drying the gel, products were visualized by using autoradiography. In order to quantify the accumulated replicative form (RF) bands, irrespective of loading and RNA-extraction efficiency, the amount of [32P]UMP incorporated in both the RF and the 28S rRNA was determined by using a phosphorimager (Storm 860; Molecular Dynamics). The amount of 28S rRNA in each lane represents the amount of RNA loaded on the gel and was normalized to wild-type. Subsequently, the accumulated RF was corrected for the amount loaded on the gel.

RESULTS

Identification of the CVB3 CRE(2C)

Based on homology with poliovirus, it has been suggested that the CVB3 CRE is located within the 2C-coding region (Fig. 1a) (Goodfellow et al., 2000; Paul et al., 2000; Rieder et al., 2000; Witwer et al., 2001). Experimental evidence, however, has not yet been provided. The corresponding region was predicted by Mfold (Zuker et al., 1999) to form a hairpin structure with a terminal loop region of 14 nt (Fig. 1b). In order to investigate whether this RNA structure is indeed required for CVB3 replication, silent mutations designed to disrupt the stem were introduced [distortion mutant, CRE(2C)-DM, Fig. 1c]. Transfection of this RNA into susceptible BGM cells abolished virus production, an observation indicating the significance of this RNA structure for virus replication (data not shown). Testing CRE(2C)-DM in a construct in which the capsid-coding region was replaced by a luciferase gene showed no increase in luciferase activity above translational levels of the input RNA as determined in the presence of guanidine hydrochloride, thereby indicating a defect in RNA replication (Fig. 2a).

Poliovirus and rhinovirus CREs have been described as the primary templates required for VPg uridylylation (Gerber...
et al., 2001; Goodfellow et al., 2003a; Paul et al., 2000; Yang et al., 2002). This prompted us to establish whether the CVB3 CRE(2C) is also the primary site for VPg uridylylation. Full-length genomic RNA transcripts, containing either wild-type CRE(2C) or the DM mutant, were used as templates together with purified CVB3 3Dpol, 3CDpro, VPg and Mg\(^{2+}\) in an in vitro uridylylation assay. Using full-length genomic RNA transcripts, wild-type RNA was able to support VPg uridylylation in the presence of both 3Dpol and 3CDpro, whereas no detectable amount of uridylylated VPg was observed upon distortion of the CRE(2C) structure (Fig. 2b). These results suggest that the CVB3 CRE(2C) is indeed the primary template for VPg uridylylation.

**Disrupting the CVB3 CRE(2C) stem–loop structure does not affect negative-strand RNA synthesis**

A cell-free translation/replication system, originally described for poliovirus (Molla et al., 1991), has been used to analyse the effect of gross distortions of the CRE structure on negative-strand RNA synthesis (Goodfellow et al., 2003b; Morasco et al., 2003; Murray & Barton, 2003). To ascertain whether CVB3 transcript RNA is able to function as a template for RNA replication using the cell-free system, CVB3 transcript RNA containing different 5′ ends was used (Fig. 2c). RNA containing the 5′ hammerhead ribozyme (HR) sequence (Herold & Andino, 2000), which, after
transcription, produces RNA with authentic 5' termini, efficiently produced both negative-stranded (RF) and positive-stranded [replicative intermediate (RI) and ssRNA] RNA. Absence of this HR sequence produced CVB3 transcripts that contained two non-viral guanosine residues at their 5' terminus, which only produced RF RNA (Herold & Andino, 2000) and therefore efficiently blocked the accumulation of RI and ssRNA during the 2 h incubation period (Fig. 2c). No bands were observed when CVB3 HR transcripts were incubated in the presence of guanidine hydrochloride, indicating that the cell-free system only displays viral RNA production (Fig. 2c). It has previously been described that rearrangements of the CRE structure did not interfere with the accumulation of negative strands (Goodfellow et al., 2003b). In order to quantify the amount of complementary strands produced by coxsackievirus RNA using HeLa S10 cells extracts, we used RNA transcripts containing two non-viral guanosine residues at the 5' terminus (GG). (d) In vitro replication assay using RNA derived from ribozyme-negative constructs containing wild-type or CRE(2C)-DM as described in Methods. The mutants are indicated above the autoradiograph and RF accumulation was quantified by measuring \(^{32}\text{P}\text{UMP}\) incorporation by using a phosphorimagager. All values were normalized to wild-type RNA. (e) In vitro replication assay using wild-type (WT) or CRE(2C)-DM (DM) HR transcript RNA. As a control, wild-type CVB3 RNA incubated in the presence of guanidine hydrochloride is shown (WT + GuHCl).

**Fig. 2.** Identification of the CVB3 CRE(2C). (a) Replicons in which the capsid-coding region is replaced by a luciferase gene, incubated in the absence or presence (+GuHCl) of guanidine hydrochloride or containing the CRE(2C) distortion mutant, CRE(2C)-DM, were transfected into BGM cells. Ten hours post-transfection, cell lysates were harvested and assayed for luciferase activity as described in Methods. Each column represents the mean of five independent experiments and SD is indicated. (b) Uridylation of VPg in vitro by using genomic CVB3 RNA transcripts containing wild-type (WT) or CRE(2C)-DM as template. Where indicated, 3CDpro was omitted from the reaction mixtures. The in vitro uridylation products were separated on a Tris/Tricine SDS-PAGE gel and visualized by using autoradiography. (c) In vitro replication assay using wild-type CVB3 RNA containing a 5' hammerhead ribozyme sequence incubated in the absence (HR) or in the presence (+GuHCl) of guanidine hydrochloride and a ribozyme-negative construct that, after RNA transcription using T7 RNA polymerase, gives rise to transcript RNA containing two non-viral guanosine residues at the 5' terminus (GG). (d) In vitro replication assay using RNA derived from ribozyme-negative constructs containing wild-type or CRE(2C)-DM as described in Methods. The mutants are indicated above the autoradiograph and RF accumulation was quantified by measuring \(^{32}\text{P}\text{UMP}\) incorporation by using a phosphorimagager. All values were normalized to wild-type RNA. (e) In vitro replication assay using wild-type (WT) or CRE(2C)-DM (DM) HR transcript RNA. As a control, wild-type CVB3 RNA incubated in the presence of guanidine hydrochloride is shown (WT + GuHCl).
RF, indicating that negative-strand RNA synthesis in this mutant is not inhibited (Lane DM, Fig. 2d). Quantification indicated CRE(2C)-DM RNA to accumulate amounts of RF at the measured time point comparable to those of wild-type (Fig. 2d). In contrast to wild-type transcript containing a cis-acting HR sequence, pre-initiation replication complexes programmed with CRE(2C)-DM RNA containing a HR sequence produced only the RF band, but no progeny positive-strand RNA accumulation was observed (Fig. 2e).

The results described above suggest that, under the conditions of the experiments, CRE(2C)-derived VPgpU(pU) is required for the initiation of positive-strand, but not negative-strand, RNA synthesis.

**Effect of CRE(2C) point mutations on replication efficiency**

A functional analysis was undertaken to determine whether the consensus sequence for the apical loop postulated by Yang et al. (2002) is also applicable to the CVB3 CRE(2C). Subsequently, every nucleotide involved in this consensus sequence was substituted for almost all other nucleotide possibilities and, additionally, the coxsackie B-specific A₅AAUG₉ loop sequence was substituted for the consensus AAACA motif (consensus mutant, CM) to ascertain the relevance of the CVB3-specific U₆G₉ residues (Fig. 3b) (Rieder et al., 2000). As point-mutational analysis of the CRE(2C) at its original 2C location faces complications, due to amino acid changes in the 2C protein, and the CRE function is described to be position-independent (Goodfellow et al., 2000; Yin et al., 2003), a HR-CRE(Art) construct was devised containing the CRE(2C) distortion mutant at the original 2C position and a second, artificial CRE at the junction of the P2 region and the luciferase gene, which replaces the capsid-coding region (Fig. 3a). Also, a cis-acting hammerhead ribozyme sequence was introduced in order to generate RNA transcripts containing authentic 5' ends (Herold & Andino, 2000). This construct enabled a mutational analysis of the CVB3 CRE(2C) for its function in RNA replication, without altering the amino acid sequence of the CVB3 2C protein. In vitro-transcribed RNA was transfected into susceptible BGM cells and, at 10 h post-transfection, luciferase activity was measured as described previously (van Kuppeveld et al., 1995). Substituting the coxsackievirus-specific AAAUG₉ sequence for the consensus AAACA motif showed wild-type-like luciferase activity (Lane CM, Fig. 3b). Alterations affecting the adenosine triplet showed that A₇ can only be replaced by another purine, whilst for A₅ and A₆, no substitutions were permitted (Fig. 3b). Substituting A₅ for a uridine residue, which results in the introduction of a stop codon (UAA), is included as a negative control for further studies described below. In this assay, however, no conclusions can be drawn for this mutation with respect to virus replication. Purine residues at the extreme ends of the CRE(2C) loop sequence were proposed by Yang et al. (2002) to be required for rhinovirus and enterovirus replication. Substitutions regarding G₁ were in accordance with this consensus, as only a transition to an adenosine conferred the ability to replicate. Interestingly, residue A₁₄ can be substituted by both a guanosine and a uridine, although the uridine substitution displayed reduced replication efficiency compared with the guanosine substitution. This difference in replication efficiency, when comparing the A₁₄U with the A₁₄G transition, might be explained by alternative folding of the loop as a result of non-canonical base-pair formation (MFOLD prediction), as opposed to changes in the primary CRE sequence (data not shown).

In summary, the structure of the CVB3 CRE(2C) loop resembles previously examined picornavirus CRE elements and is in accordance with the consensus sequence proposed by Yang et al. (2002), with the exception of nucleotide A₁₄, which can be substituted by a guanosine or a uridine residue.

**Effect of CRE(2C) point mutations on VPg uridylylation efficiency**

The capacity of CVB3 CRE(2C) mutants to support VPg uridylylation was tested by using short mutant CRE(2C) transcript RNAs, representing only the CRE(2C). Uridylylation efficiencies were quantified as described in Methods. Consistent with the results obtained by using genomic RNA transcripts, a short transcript containing only the CRE(2C) distortion mutant (Fig. 1c) was unable to support uridylylation above detectable levels (Lane DM, Fig. 3c). Substituting the AAAUG₉ sequence for the AAACA₉ consensus motif showed only a slight decrease in uridylylation efficiency compared with wild-type (Lane CM, Fig. 3c). In general, mutants that were able to support RNA replication (Fig. 3b) also showed efficient VPg uridylylation (G₁A, A₇G, U₆C/A, A₁₄G/U and CM, Fig. 3c). Interestingly, lower levels of uridylylation did not always correlate with reduced levels of RNA replication, as shown for mutants U₆C and U₆A (compare Fig. 3b with Fig. 3c). The reason for this might be that the uridylylated products are made in large excess over what is used for RNA synthesis (Murray & Barton, 2003). No VPgpU(pU) synthesis above background levels, however, could be detected for G₁C, A₁₄C/U or A₁₄C substitutions or for any of the A₅ or A₆ mutants (Fig. 3c), which were also defective for RNA replication (Fig. 3b). Therefore, it can be concluded that uridylylation of VPg is essential for viral RNA replication.

**Effect of CRE(2C) point mutations on negative-strand RNA synthesis**

To ascertain the effect of CRE(2C) point mutations on RF synthesis, we used a construct from which RNA transcripts containing two non-viral guanosine residues at their 5’ termini, termed GG-CRE(Art), are derived (Fig. 4a), allowing us to specifically evaluate and quantify the effect of CRE(2C) mutations on RF synthesis (Herold & Andino, 2000). A very good correlation was observed between the [³²P]UMP labelling of the 28S rRNA band and the ethidium bromide
staining of the gel, which allowed us to quantify RF accumulation irrespective of loading by determining the ratio between the 28S rRNA band (Herold & Andino, 2000) and synthesized viral RF.

As a completely distorted CRE(2C) structure produced wild-type-like RF levels, and therefore was not or could not be used as a negative control (Fig. 2c) (Morasco et al., 2003; Murray & Barton, 2003), it was rather unexpected that the

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**Fig. 3.** RNA replication and uridylylation efficiency of CVB3 CRE(2C) point mutants. (a) HR-CRE(Art) was generated to assess replication capabilities of CRE(2C) mutants. The P1 region was substituted for the firefly luciferase gene. Wild-type or mutant CREs were introduced between the luciferase gene and the 2A junction by using a cloning cassette. Simultaneously, an additional 3CD<sup>pol</sup> cleavage site was introduced to ensure release and activity of the luciferase polypeptide from the polyprotein. The CRE(2C) at its original 2C position was disrupted by using silent mutations as depicted in Fig. 1(c). Additionally, a hammerhead ribozyme (HR) sequence was inserted downstream of the promoter sequence to generate RNA transcripts containing authentic 5' ends. (b) Luciferase activity of the HR-CRE(Art) RNA transcripts described above, transfected into BGM cells. Each column represents the mean of five independent experiments and SD is indicated. A luciferase replicon that solely contained the CRE(2C)-DM was used as a negative control (DM); mutant CM represents the substitution of the CVB3 AAAUG sequence for the picornaviral consensus sequence AAACA. The sequence above the graph represents the loop region of CRE(2C) and the arrows indicate the nucleotides that have been substituted. The adenosine characters in bold type could not be substituted for any other nucleotide. (c) Determination of uridylylation efficiency, using short RNA transcripts of wild-type and mutant CRE(2C) as templates. The amounts of [α-<sup>32</sup>P]UMP incorporated into the VPgpU and VPgpU(pU) products were quantified by using a phosphorimager. An autoradiograph of the reactions is shown above.
majority of the CRE(2C) substitutions regarding the enterovirus consensus sequence did not accumulate wild-type-like RF levels. Mutants positive for both luciferase activity (Fig. 3b) and VPg uridylylation (Fig. 3c) accumulated, albeit with different efficiencies, the highest levels of RF synthesis (G1A, A7G, U8C/A and A14G/U, Fig. 4b). Only the U8 mutants, a residue not belonging to the consensus sequence, showed wild-type RF production (U8C/A, Fig. 4b). In general, a good correlation was observed between the luciferase activity and the efficiency of RF synthesis using these mutants (compare Fig. 3b with Fig. 4b). VPgpU(pU)-defective point mutations accumulated RF synthesis only to approximately 20% of that of wild-type CVB3 RNA (G1C, A5C, A6G/C/U, A7C and A14C, Fig. 4b), whereas the VPgpU(pU)-defective A5G mutant was even unable to support RF synthesis above detectable levels (Fig. 4b). The fact that point mutants reduced in VPgpU(pU) accumulation were affected in RF accumulation already suggests that CRE(2C)-derived VPgpU(pU) is also utilized to initiate negative-strand RNA synthesis. Because inhibiting RF synthesis by introducing a stop codon (A5U, Fig. 4b) displayed results similar to those for the A5G transition, it was concluded that this latter mutant was defective for negative-strand RNA synthesis. This, again in contrast to previous reports (Goodfellow et al., 2003b; Morasco et al., 2003; Murray & Barton, 2003), implies the necessity for the CRE(2C) in positive-strand, but also in negative-strand, RNA synthesis. Additionally, GG-CRE(Art) transcript RNA, containing a completely distorted CRE(2C) with an additional A5G mutation, still produced wild-type RF levels, concluding that the dominant-negative effect of the A5G transition is only functional in the context of a wild-type CRE(2C) structure (Fig. 4c).

In summary, all CRE(2C) point mutants affecting the enteroviral CRE consensus sequence, so excluding U8A/C, showed a marked decrease in their capacity to support negative-strand RNA synthesis. Therefore, it can be concluded that these results ascribe an important role to the coxsackievirus CRE(2C) in the initiation of both positive- and negative-strand RNA synthesis.

**DISCUSSION**

This study describes the presence of a CRE within the CVB3 2C gene, termed CRE(2C), that functions as the template for VPg uridylylation in vitro and that is required for the initiation of both positive- and negative-strand RNA synthesis. The CRE(2C) is predicted to fold into an imperfect stem with a large loop region of 14 nt, containing a
coxsackie B cluster-specific 5′-AAAUUG-3′ sequence. Substituting this sequence for the consensus sequence found in all CREs described thus far, 5′-AAACA-3′ (Paul et al., 2000; Yang et al., 2002), did not affect VPg uridylylation efficiency or RNA replication significantly. Characterization of the CVB3 CRE(2C) loop showed that the proposed entero- and rhinoviral consensus sequence (R1NNNAAR2NNNRR3), extrapolated from sequence-alignment studies and extensive mutational analysis of the rhinovirus 14 CRE(VP1) loop region (Yang et al., 2002; Yin et al., 2003), is also applicable to the CVB3 CRE(2C) loop sequence, because mutations that interfered with this consensus sequence affected CVB3 VPg uridylylation and RNA replication to a similar extent.

Consistent with previous reports, a mutant in which the CRE(2C) structure was disrupted completely still supported efficient negative-strand RNA synthesis in vitro (Goodfellow et al., 2003b; Morasco et al., 2003; Murray & Barton, 2003). This suggested that, under the conditions of the experiment, CRE-mediated VPg uridylylation is required for positive-strand, but dispensable for negative-strand, RNA synthesis. However, we also found a direct correlation between CRE(2C) point mutants producing lower, but detectable, amounts of VPgpU(pU) and reduced RF levels, affected to a similar extent. These latter results suggest that CRE(2C)-derived VPgpU(pU) is also involved in the initiation of negative-strand RNA synthesis. In correlation, CRE(2C) point mutants unable to support VPg uridylylation to a detectable level also showed a severe defect in RF accumulation. In addition, the A5G transition that affects the first templating adenosine residue was found to abolish RF synthesis completely (Fig. 4b).

How can we explain these observations? Very recently, Crowder & Kirkegaard (2005) reported, by using poliovirus CRE(2C) mutants, that a dominant-negative effect is observed on wild-type poliovirus replication using CRE(2C) point mutants, but not when the CRE(2C) has been disrupted completely. This suggests that RNA containing a complete CRE(2C) distortion mutant, as opposed to point mutants, utilizes an alternative mode of RNA replication that is so distinct from wild-type poliovirus replication that it does not interfere. As RF synthesis, in the absence of detectable levels of VPgpU(pU), was observed in the cell-free system using RNA containing a completely distorted CRE(2C) structure, it was suggested previously that this alternative mode of replication might involve the 3′ poly(A) tail. This homopolymeric stretch might serve as the alternative template for the covalent linkage of uracil residues to VPg (Goodfellow et al., 2003b; Morasco et al., 2003; Murray & Barton, 2003), which was based on the findings that negative-strand RNA synthesis is VPg-dependent (Nomoto et al., 1977a, b) and that poly(A) templates supported VPg uridylylation in vitro (Paul et al., 1998).

A wild-type CRE(2C) structure sequesters replication proteins (Yin et al., 2003) and the formation of such protein–RNA interactions is most probably affected upon distortion of the CRE(2C) structure. Absence of this RNP complex in a genome containing a completely distorted CRE(2C) structure might favour the 3′ poly(A) tail as the alternative template for the covalent linkage of UMP to VPg in vitro. The inability to detect free VPgpU(pU) within purified replication complexes might imply that VPg is elongated immediately into complementary negative RNA strands (Murray & Barton, 2003). A number of CRE mutants unable to accumulate detectable levels of uridylated VPg still retained the ability to induce RF synthesis (e.g. G1C, A6C/G/U, A7U/C and A14C). Therefore, it can be speculated that these mutants also utilize the proposed alternative, 3′ poly(A) tail-dependent mechanism to induce negative-strand RNA synthesis. However, whereas the CRE(2C) distortion mutant showed wild-type levels of RF synthesis at the measured time point, RF accumulation was reduced severely in CRE(2C) mutations affecting the enterovirus consensus sequence. Also, in contrast to the CRE(2C) distortion mutant, the G1C, A6C/G/U and A7U/C substitutions all maintained a wild-type CRE(2C) conformation, which we propose still enabled them to sequester the proteins required for VPg uridylylation. As shown previously for poliovirus, A6 substitutions still produced VPgpU(pU), albeit with reduced efficiency, which might support this speculation (Paul et al., 2003). Although the A14C mutation is predicted by MFOLD to adopt an alternative conformation due to a GC base-pair formation between the G1 and C14 residue, this mutant was found in poliovirus not to be reduced in protein binding (Yin et al., 2003) and might therefore still be able to produce VPgpU(pU). Additionally, Nayak et al. (2005) showed that A6 and A7 mutations displayed higher uridylylation activities in the context of full-length RNA compared with short hairpin RNAs, concluding that the template used in the uridylylation reaction determines both the specificity and efficiency of the assay. In summary, we speculate that the CRE-dependent uridylylation mechanism prior to negative-strand synthesis is strongly favoured when the CRE structure is intact.

By using purified components [CRE(2C), VPg, 3DP and 3CDP], poliovirus VPg nucleotidylylation has been found not to be stringently dependent upon an AAACA motif in the CRE loop if one of the NTPs other than UTP was present. For example, VPgGPG is formed by CAACA and GTP, as the first nucleotide, in this case a C residue, functions as a template (Gerber et al., 2001; Paul et al., 2003). Similarly, an A6C transversion or an A6G transition in the CVB3 A6AAUG motif yielded VPgpG and VPgpU, respectively, in the CVB3 CRE(2C) nucleotidylylation reaction (unpublished results). Nucleotidylylation is aborted at the stage of VPgpG or VPgpU because of the ‘slide-back’ mechanism to A6 (Gerber et al., 2001; Paul et al., 2003; unpublished results). If dual CRE(2C) CVB3 transcripts carry a 5′-terminal ppgG group (Fig. 4a), only the A6C transversion mutant produced some RF, albeit in greatly reduced yield (Fig. 4b). The rationale for employing the ppgGG transcript was to reduce positive-strand synthesis significantly.
RNA synthesis in the cell-free assay, thereby shifting the complex to synthesize, if possible, predominantly negative-strand RNA (Herold & Andino, 2000). Virological characterization of both the CVB3 A5C and A5G mutants using a single CRE(2C) construct showed that the A5G mutation was quasi-infectious and reverted to a wild-type CRE sequence, whereas, in contrast to previous reports (Morasco et al., 2003; Yin et al., 2003), the A5C mutant proved to be a lethal mutation, probably caused by a dysfunctional 2C protein (unpublished results). On the basis of these data, we suggest that initiation of negative-strand RNA synthesis requires the A5 residue, but that, at least in assays performed using cell-free extract, this requirement is leaky (see below).

The stringency of sequence requirement does not appear to apply to the A6A AUG residue, as all mutations in A6 still produce RF, albeit at very reduced yield (Fig. 4b). This corresponds to data from poliovirus, which have revealed that mutations in the corresponding adenosine residue (AAACA) yielded quasi-infectious phenotypes (Paul et al., 2003). These phenotypes were also found for the analogous point mutants in CVB3 CRE(2C) (unpublished data). Perhaps VPgpU formed on ANAC CRE(2C) mutants of the poliovirus or ANAUG CRE(2C) mutants of CVB3 can still function as primer for negative-strand RNA synthesis on the 3′ poly(A) template.

RNA replication of human enterovirus genomes proceeds on membranes in RNP complexes of which the structures have not yet been entirely deciphered. Available evidence suggests that the 5′-terminal cloverleaf and the 3′-terminal poly(A) tail induce the formation of a circular RNP complex via protein–protein interaction (Barton et al., 2001; Herold & Andino, 2001). Moreover, it has been suggested that the cloverleaf structure is also involved in VPg uridylylation (Lyons et al., 2001). Thus, all three elements, the cloverleaf, the CRE and the 3′ poly(A) tail, may interact for initiation of genome replication to occur. Elimination of the CRE by destroying its stem–loop structure may allow the system to bypass CRE requirement for negative-strand RNA synthesis and, by a default mechanism, make use of the genetically encoded 3′ poly(A) tail as template for uridylylation in a cell-free in vitro system (Goodfellow et al., 2003b; Morasco et al., 2003; Murray & Barton, 2003; Paul et al., 1998). As viral negative-strand RNA was found to be absent in cells transfected with the complete CRE(2C) disruption mutant (Goodfellow et al., 2000), it is sensible to conclude that utilization of the 3′ poly(A) tail for uridylylation can only be seen in the in vitro translation/replication system. In stark contrast, if the replication proteins are presented with a CRE element whose A5 has been mutated to a G residue, the aberrant nucleotidylylation product VPgpG might operate as a dominant-negative element. The nucleotidylylation complex, however, is not ‘frozen’ on the mutated CRE(2C) itself, as the addition of a second wild-type CRE restored the ability to initiate RNA replication (unpublished results; Yin et al., 2003). An alternative mechanism could be envisaged in which the replicase complex containing VPgpC is positioned on the 3′ poly(A) tail and thereby might block the initiation of negative-strand RNA synthesis. We speculate that the A5C transition mutant, accumulating a VPgpG nucleotidylylation product, may form a less-stable RNP complex on the 3′ poly(A) tail, allowing a severely reduced yield of complementary RNA strands via the alternative 3′ poly(A) tail-dependent mechanism, although further investigation is required to elucidate the exact mechanism.

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REFERENCES


