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# Complete Nucleotide Sequence of Infectious Coxsackievirus B3 cDNA: Two Initial 5′ Uridine Residues Are Regained during Plus-Strand RNA Synthesis

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**A full-length reverse-transcribed, infectious cDNA copy of coxsackievirus B3 (CVB3) was used to determine the nucleotide sequence of this cardiotropic enterovirus. Comparison of the nucleotide sequence and the deduced amino acid sequence of the viral precursor polyprotein with the sequences of other group B coxsackieviruses (CVB1 and CVB4) demonstrates a high degree of genetic identity. They share about 80% homology at the nucleotide level and about 90% when the amino acid sequences of the polyproteins are compared. The potential processing sites of the coxsackievirus polyproteins, as deduced from alignment with the poliovirus sequence, are conserved among these enteroviruses with the exception of the cleavage sites between VP1 and $2A^{pro}$ and between polypeptides 2B and 2C. Comparison of the 5′ termini of the enteroviral genomes reveals a high degree of identity, including the initial 5′ consensus UUAAAACAGC, suggesting essential functions in virus replication. An important finding concerning the molecular basis of infectivity was that both recombinant CVB3 cDNA and in vitro-synthesized CVB3 RNA transcripts are infectious, although two initial 5′ uridine residues found on the authentic CVB3 RNA were missing. Here, we report that cDNA-generated CVB3, as well as CVB3 generated by in vitro-synthesized RNA transcripts, regains the authentic initial 5′ uridine residues during replication in transfected cells, indicating that the picornaviral primer molecule VPg-pUpU may be uridylylated in a template-independent fashion. The generation of virus or virus mutants with infectious recombinant CVB3 cDNA and in vitro-synthesized infectious CVB3 transcripts should provide a valuable means for studying the molecular basis of the pathogenicity of this cardiotropic enterovirus.**

Enteroviruses of the human *Picornaviridae*, such as the group B coxsackieviruses (types 1 to 5), are the most common agents known to cause viral myocarditis (1, 12, 24, 34, 53). Other human enteroviruses, comprising at present over 70 serotypes (e.g., various group A coxsackieviruses and echoviruses), have also been associated with human heart disease. These agents are capable of producing dilated cardiomyopathy of acute onset or leading to life-threatening arrhythmias. Particularly intriguing is the concept of enterovirus persistence in chronic dilated cardiomyopathy evolving from acute or subacute infections of the human heart (13, 17).

The structure and molecular genetics of the enteroviruses are well understood, chiefly by analogy with the extensively studied poliovirus (for a review, see reference 52). The coxsackieviruses possess a single-stranded RNA genome of about 7,500 nucleotides (nts) of positive polarity, which is covalently linked at the 5′ end to a small virus-encoded protein, VPg (3B, according to the systematic nomenclature of picornavirus proteins [36]). The viral RNA, which is infectious (22, 39), is polyadenylylated at the 3′ end and functions as an mRNA in the cytoplasm of infected cells. It is translated into a large precursor polyprotein, which is processed by virus-encoded proteinases to yield the mature structural and nonstructural proteins (19). Replication of picornaviral RNA has been studied extensively (52, and references therein), although the individual steps in RNA replication, e.g., initiation of plus-strand RNA synthesis, have not yet been elucidated unambiguously. Distinct proteins may be involved in the formation of a replication complex, including the virus-encoded RNA-dependent RNA polymerase ($3D^{pol}$), VPg (3B) or its precursor molecule 3AB, and possibly other virus- or host cell-encoded factors (2, 27, 52, 55).

In order to study the molecular basis of pathogenicity and to introduce in situ hybridization as a diagnostic tool in patients with a clinical suspicion of enteroviral heart disease, we have previously cloned the genome of the cardiotropic coxsackievirus B3 (CVB3), which had been propagated in cultured human myocardial cells (14–16). Full-length reverse-transcribed recombinant cDNA generated infectious virus antigenically identical to CVB3 upon transfection into mammalian cells, demonstrating the biological intactness of the cloned cDNA copy. The isolation of cDNA fragments representing the genomic RNA of CVB3 has also been described by others (20, 48), and a nucleotide sequence of CVB3 based on subgenomic cDNA fragments has been reported by Lindberg et al. (20). We now present the entire nucleotide sequence of infectious, full-length reverse-transcribed recombinant CVB3 cDNA and have compared this with that of other human enteroviral genomes.

In this context, we report on transfection experiments with circular recombinant CVB3 cDNA and in vitro-synthesized CVB3 RNA transcripts, demonstrating that inherent infectivity of these CVB3 constructs is independent of the presence of two initial 5′ uridine residues found on the authentic viral RNA. Remarkably, the authentic viral 5′ terminus is restored in the RNA genome of both cDNA-

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derived CVB3, as well as in vitro-synthesized RNA-derived virus progeny.

## MATERIALS AND METHODS

**Sequence determination.** Full-length reverse-transcribed, infectious CVB3 cDNA (16) was used to determine the nucleotide sequence of CVB3. For this purpose, subgenomic CVB3 cDNA fragments were inserted into the *Sma*I site of pUC19 (54) and sequenced by the chain termination method (3, 37). Fragments from the cloned CVB3 cDNA were generated by digestion with *Bgl*I or *Pvu*II and subsequent incubation with nuclease Bal 31 or by digestion with one of the frequently cleaving restriction endonucleases, *Hae*III, *Alu*I, and *Rsa*I. A total of 93% of the CVB3 nucleotide sequence was obtained from both strands, and the remainder was obtained from multiple determinations on one strand of at least two independent subclones.

To determine the sequence of the very 5′ end of the viral genome, 1 μg of purified CVB3 RNA (16) was primed with an 18-base-long synthetic oligonucleotide, complementary to a region of viral RNA comprising nucleotide positions 62 to 78. Single-stranded cDNA was synthesized with reverse transcriptase as previously described (16). The primer-extended cDNA was separated on an 8% denaturating polyacrylamide gel and sequenced by the method of Maxam and Gilbert (23). Analogously, the 5′ sequence of cDNA-generated CVB3, as well as the 5′ sequence of viruses generated by in vitro-synthesized CVB3 RNA, was obtained.

**Computer analysis.** The program library of the University of Wisconsin Genetics Computer Group (6) was used for sequence analysis on a Microa VAX II computer system. Alignments were performed by the GAP program at a gap weight penalty of 5 and a length weight penalty of 0.3. Secondary structures and free energies for the 5′ noncoding (5′ nc) region (bases 1 to 741) were calculated by the folding program of Zuker and Stiegler (56).

**Plasmid construction.** A 7.5-kilobase *Eco*RI fragment obtained by partial digestion of the infectious CVB3 cDNA plasmid pCB3-M1 (16) was inserted in both orientations into the dual-promoter plasmid pSPT18 (constructed at Pharmacia) containing both the SP6 and T7 promoter sequences, diametrically opposed and separated by the multiple cloning site of pUC-18 (54). The resulting recombinant plasmids were designated pCB3/T7 and pCB3/SP6 (Fig. 1) and allowed the in vitro synthesis of plus-strand CVB3 RNA under the control of the T7 and SP6 promoter, respectively, whereas minus-strand transcripts are synthesized under the control of the opposite promoter. The nucleotide sequences at the ligation junctions were determined directly from plasmid DNA (3). The T7 promoter of pCB3/T7 directs synthesis of plus-strand transcripts with 34 nonviral nts at the 5′ end. pCB3/SP6-derived SP6 transcripts contain 82 nonviral nucleotides at the 5′ end (see legend to Fig. 1). Both plasmids, pCB3/T7 and pCB3/SP6, were found to be infectious upon transfection into HeLa cells.

**In vitro transcription.** Plasmid pCB3/T7 was digested with *Sal*I [cuts 65 nts after the 3′ poly(A) tail], and the resulting linear DNA was isolated by phenol extraction and ethanol precipitation. RNA transcription was carried out essentially as described previously (4, 50). Briefly, 1 μg of *Sal*I-digested pCB3/T7 DNA served as a template for plus-strand RNA synthesis with T7 polymerase (400 U/ml) for 60 min at 37°C in 50 μl that contained 40 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine hydrochloride, 10 mM dithiothreitol, 500 μM each of ATP, CTP, UTP, and

FIG. 1. Plasmids containing infectious CVB3 cDNA (16) under the control of the T7 and SP6 promoters. Plasmids pCB3/T7 and pCB3/SP6 were constructed by insertion of infectious CVB3 cDNA (open bars) in both orientations into the dual-promoter plasmid pSPT18 as described in Materials and Methods. Infectious plus-strand CVB3 RNA was synthesized in vitro from plasmid pCB3/T7 following linearization with *Sal*I or from plasmid pCB3/SP6 following linearization with *Pvu*I. Plus-strand CVB3 RNA transcripts synthesized in vitro from pCB3/T7 under the control of the T7 promoter contain 34 nonviral nts at the 5′ end: 5′GGGAGACCC GAAUUCUCCAAGACAUCCCCCCCCCAAAACAGC. . .. Plus-strand CVB3 RNA transcripts synthesized from pCB3/SP6 under the control of the SP6 promoter contain 82 nonviral nts at the 5′ end: 5′GAAUACAAGCUUGCAUGCCUGCAGGUCGACUCUAGAG GAUCCCCGGGUACCGAGCUCGAAUUCUCCAAGACAUCCC CCCCCCAAAACAGC. . .. Nucleotides underlined represent the 5′ terminal region of in vitro-synthesized CVB3 RNA. Note that two initial 5′ uridine residues of the authentic CVB3 RNA are missing that are not contained in the infectious CVB3 cDNA (16). kb, Kilobases; amp, ampicillin resistance.

GTP, bovine serum albumin (100 μg/ml), and RNasin (500 U/ml). After the DNA template was digested with DNase I (20 U/ml) for 15 min at 37°C, RNA transcripts were isolated by phenol extraction and ethanol precipitation. Analogously, plus-strand RNA was synthesized from plasmid pCB3/SP6 after digestion with *Pvu*I [cuts 1,652 nts after the poly(A) tail], except that transcription was performed with SP6 polymerase.

Synthesis of minus-strand RNA was identical to that described above, except that plasmid pCB3/T7 was linearized with *Pvu*I while plasmid pCB3/SP6 was linearized with *Sal*I and transcription was performed under the control of the opposite promoter.

**Transfection of cells and detection of CVB3.** At 18 h before being transfected, HeLa cells were plated onto 60-mm petri dishes at about $5 \times 10^5$ cells per dish. RNA transfections were performed with DEAE-dextran as a facilitator (49). A total of 10 to 15 μg of in vitro-synthesized CVB3 RNA in 500 μl of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM glucose, 20 mM HEPES adjusted to a pH of 6.98 with 0.5 M NaOH) was mixed with 0.5 ml of DEAE-dextran (1 mg/ml in HEPES-buffered saline). The RNA-DEAE-dextran mixture was added to HeLa cells (about 80% confluent) and was incubated at room temperature for 30 min; then, 4 ml of Dulbecco modified Eagle minimal minimal medium was added and incubation was continued for 2.5 h at 37°C. At the end of the incubation, the cells were washed twice with Dulbecco modified Eagle minimal medium and maintained in medium supplemented with 10% fetal bovine serum and kanamycin at 50 μg/ml. Complete lysis of cell cultures transfected with plus-strand RNA transcripts was observed at 48 to 96 h posttransfection. The supernatant medium of lysed cultures was taken and submitted to a specific antibody neutralization assay (16) to examine the identity of transfection-derived viruses. In addition, plaque assays (15) were carried out to determine

specific infectivities in dilution experiments and to assess the plaque morphology of transfection-derived first-passage virus.

DNA transfections of circular recombinant CVB3 plasmids were performed with HeLa cells as described previously (16).

**Materials.** Sources were as follows: plasmid pSPT18, Pharmacia; avian myeloblastosis virus reverse transcriptase, J. W. Beard, Life Sciences (St. Petersburg, Fla.); Bal 31, Bethesda Research Laboratories, Inc.; RNasin and all other enzymes, Boehringer Mannheim Biochemicals; dideoxynucleotide sequencing kit, New England BioLabs, Inc.; chemical sequencing kit, Dupont, NEN Research Products; [α-$^{32}$P]dCTP and [γ-$^{32}$P]ATP, Amersham Corp.

## RESULTS

**Nucleotide sequence of the CVB3 genome.** The nucleotide sequence of the CVB3 genome was determined from subcloned fragments of infectious recombinant CVB3 cDNA. The sequence of the two initial 5′ uridine residues of the genome that was not contained in the infectious CVB3 cDNA was obtained from primer-extended cDNA with a synthetic oligodeoxynucleotide corresponding to positions 62 to 78 of the authentic viral RNA. The complete nucleotide sequence of CVB3 is shown in Fig. 2, including an alignment with the genomes of CVB1 (10) and CVB4 (11). The CVB3 genome consists of 7,399 nts in addition to a 3′ terminal poly(A) stretch. The 5′ nc region comprises 741 nts, followed by an open reading frame that encodes the viral polyprotein consisting of 2,186 codons with a start codon (AUG) at position 742 and a stop codon (UAG) at position 7297. The 3′ nc region consists of 100 nts. Results from independently obtained CVB3 cDNA clones revealed that up to 59 adenine residues constitute the 3′ poly(A) stretch. The base composition of the CVB3 genome excluding the poly(A) stretch is A (28.8%), C (23.3%), G (24.5%), and U (23.4%).

Comparison of the sequence of the infectious CVB3 cDNA with the CVB3 nucleotide sequence reported by Lindberg et al. (20) showed 65 nucleotide changes throughout the genome that result in 41 amino acid changes. Regarding the partial CVB3 sequence reported by Tracy et al. (48) comprising the 5′ part of the genome (nts 1 through 3822), 41 nts were found to be changed corresponding to 24 amino acid changes. Some of these are likely to be changes which occurred when the virus was propagated in different cell systems, reflecting a selective adaption process to myocardial cells. At two positions within the gene region coding for the 2A proteinase (2A$^{pro}$), the infectious CVB3 cDNA exhibits differences which cause significant alterations in the amino acid sequence. One nucleotide insertion after position 3299 together with 1 nt deletion at position 3333 leads to a frameshift within the previously reported CVB3 sequences (20, 48) (Fig. 3). This frameshift results in an altered peptide sequence of 12 amino acids. The majority of amino acids in the immediate vicinity of the cleavage site between the structural protein VP1 and 2A$^{pro}$ are changed in a nonconservative fashion. In addition, insertions after positions 3486 (1 nt) and 3502 (2 nts) within the partial sequence reported by Tracy et al. (48) lead to three amino acid changes and the appearance of an additional amino acid (leucine) within 2A$^{pro}$. Alignment of these two regions of the infectious CVB3 cDNA sequence with other enterovirus sequences reveals a high degree of sequence identity with CVB1 (100%) (10), CVB4 (83%) (11), and, less pronounced, with poliovirus type 1 (PV1) (50%) (18, 32), whereas the previously reported CVB3 sequences (20, 48) clearly deviate (Fig. 3).

**Sequence comparison with other group B coxsackieviruses.** Comparison of the nucleotide sequence of the CVB3 genome with the sequences of the potentially cardiotropic CVB1 and CVB4 (compare the sequences in Fig. 2) revealed high degrees of overall identity, 80.7 and 78.3%, respectively. The nc regions are highly conserved among the coxsackieviruses: CVB3-to-CVB1 identity, 94.1% (5′ nc) and 89.4% (3′ nc); CVB3-to-CVB4 identity, 83.4% (5′ nc) and 92.3% (3′ nc). One insertion (CVB4, 1 nt after position 32) and three deletions (CVB3, 1 nt after position 115; CVB1, 1 nt each at positions 118 and 7399) were observed within the 5′ nc and 3′ nc regions, respectively.

The majority of nucleotide differences among group B coxsackieviruses appear to be almost evenly distributed over the entire open reading frame encoding the viral polyprotein and occur frequently (71.3%) at third-nucleotide positions. As expected, the regions coding for the structural proteins VP1, VP2, and VP3 are less conserved than the regions coding for VP4 and the nonstructural proteins. Notable deletions and insertions are observed in the coding regions of VP1 and VP2. A total of 15 nts are inserted within the gene region of VP1 of CVB4 (after nt 2703), 6 nts are deleted within VP1 (nt 2673 and nts 2680 through 2684) and VP2 of CVB4 (nt 1404 and nts 1414 through 1418), and 9 nts are inserted within VP1 of CVB3 (nts 3283 through 3291).

Alignment of the deduced CVB3 polyprotein sequence with those of CVB1, CVB4, and PV1 (Fig. 4) allows the identification of putative cleavage sites. In comparison to those of PV1, cleavage sites are conserved, with the exception of the cleavage sites between polypeptides 2B and 2C (2B/2C) and between VP1 and 2A$^{pro}$ (VP1/2A$^{pro}$). In PV1, 2B/2C is a target of the 3C proteinase (3C$^{pro}$) (8). However, a Q-N pair is found to be present in CVB3, CVB1, and CVB4 at a position corresponding to the Q-G cleavage site of PV1. Since no alternative Q-G site is found at a proximal position, we suggest that the Q-N pair is cleaved by the coxsackievirus 3C$^{pro}$, generating termini of polypeptides 2B and 2C at equivalent positions with respect to PV1.

In the case of poliovirus, the cleavage between VP1 and 2A$^{pro}$ is mediated by 2A$^{pro}$, leading to the proper release of the structural precursor protein P1 (46). In comparison to the Y-G cleavage site of PV1 comprising the cleavage between VP1 and 2A$^{pro}$, the corresponding amino acids are not conserved among the group B coxsackieviruses (Fig. 4). Assuming cleavage sites with properties similar to the poliovirus Y-G pair (aromatic amino acid-G), the F (or Y)-G pairs located two residues downstream with respect to PV1 would represent suitable cleavage sites in the group B coxsackieviruses. Alternatively, the T-G pair (two residues upstream) has been suggested (10, 30). To verify the proposed cleavage site for CVB3, protein sequence studies are required.

The alignment of the polypeptides of CVB1, CVB3, CVB4, and PV1 (Fig. 4) indicates that identity among the coxsackieviral polyproteins is clearly higher compared with that of PV1. Taking into consideration the deduced cleavage sites of the polyproteins, the degree of identity among the viral proteins has been determined and is summarized in Table 1. This comparison reveals that the structural protein VP4 and all nonstructural polypeptides are highly conserved among the coxsackieviruses (90.9 to 99.0% identity) and that polypeptides 2B and 2C with yet unknown functions are almost identical (98 to 99%). In contrast, the structural proteins VP2, VP3, and VP1 exhibit a pronounced diversity (69.4 to 82.9% identity) which is restricted to clusters of heterologous sequences alternating with highly conserved regions (compare sequences in Fig. 4). Highly homologous

FIG. 2. Nucleotide sequence of infectious CVB3 cDNA (top) in comparison with the sequences of CVB1 (middle) (Iizuka et al. [10]) and CVB4 (bottom) (Jenkins et al. [11]). The CVB3 sequence is shown in its entirety together with the corresponding nucleotide changes of CVB1 and CVB4. The gene regions of the distinct viral proteins are defined by vertical lines by analogy to the poliovirus genome (18, 32). The interrupted line after nucleotide position 3303 indicates the proposed border between the CVB3 gene regions of VP1 and $2A^{pro}$. Dots represent nucleotide deletions.

FIG. 2—*Continued.*

peptide sequences are found corresponding to the antiparallel oriented beta strands and short alpha helices which determine the core structure of PV1 capsid proteins (9). Diverging sequences correlate to distinct loops connecting the conserved core elements of VP2, VP3, and VP1. CVB3 peptide sequences at positions 75 through 86 of VP1 (site 1), 197 through 210 of VP1 and 135 through 174 of VP2 (site 2), and 54 through 66, 75 through 80 of VP3, 72 through 76 of VP2, and the C terminus of VP1 (site 3) correspond to PV sequences containing the main immunodominant antigenic sites of the three poliovirus strains PV1, PV2, and PV3 (25, 29). These regions are part of the highly divergent sequences

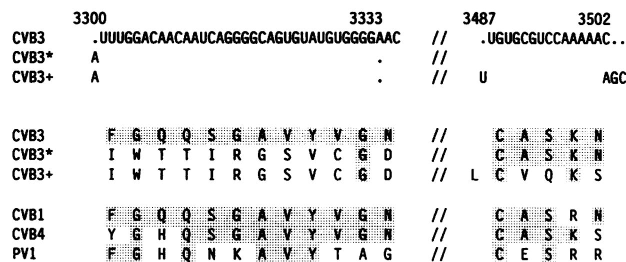

FIG. 3. Alignment of two regions within $2A^{pro}$ of infectious CVB3 cDNA with various enterovirus sequences. The deduced amino acid sequence of infectious CVB3 cDNA presented here (CVB3) reveals a high degree of identity to CVB1 (10) and CVB4 (11) but lower homology to PV1 (18, 32). The CVB3 sequences determined by Lindberg et al. (20) (CVB3*) and Tracy et al. (48) ($CVB3^+$) clearly deviate from the CVB3 sequence presented here as a consequence of the frameshift between nts 3300 and 3333 (indicated). Note that the majority of amino acids in this region are nonconservatively changed. A second frameshift between nts 3487 and 3502 was only found to be present in the partial sequence reported by Tracy et al. (48) ($CVB3^+$), leading to a different peptide sequence with conserved amino acid changes. Shaded areas depict identical amino acids.

among the coxsackieviruses, suggesting that they play a major role in the formation of the antigenic determinants.

**The 5′ terminus of the CVB3 genome contains structural features conserved among the picornaviruses.** The 5′ terminal sequence is highly conserved among different enteroviruses. Enteroviruses have the first 10 nts, 5′ UUAAAACAGC, in common (Fig. 5). In addition, two pairs of inverted repeats are found to be present within the first 90 nts at corresponding positions (nts 2 through 9 and 79 through 86, 10 through 18 and 26 through 35, in the case of CVB3). These features are common to other human pathogenic picornaviruses such as rhinoviruses, and to a lesser extent, hepatitis A virus (Fig. 5). Computer-calculated secondary structures (data not shown) indicate that inverted repeats facilitate the formation of unique secondary structures presumably serving essential functions in virus replication. In addition, it is of interest that all picornaviral genomes sequenced so far contain two initial 5′ uridine residues. However, their relevance and function are still obscure.

**Two initial 5′ uridine residues missing in infectious recombinant CVB3 cDNA are present in cDNA-derived virus.** Sequencing of the 5′ terminus of infectious recombinant CVB3 cDNA revealed that two 5′ uridine residues are missing that were found to be encoded in the authentic viral RNA. Instead, deoxycytidine residues have taken their position corresponding to the oligo(dC) tract generated during cloning of the CVB3 genome (16). This finding raised the question of how the recombinant CVB3 cDNA containing a modified 5′ terminus is capable of inducing an infectious cycle. To examine whether this modification is conserved during transfection of mammalian cells, genomic RNA was isolated from cDNA-derived CVB3. The 5′ nucleotide sequence of the genome of cDNA-generated viruses was determined by primer extension and chemical sequencing, as performed for the determination of the very 5′ end of the authentic wild-type RNA. Strikingly, the 5′ terminus of the cDNA-generated CVB3 genome was found to be identical to that of the wild-type RNA containing two 5′ uridine residues. These residues must have been provided during the initiation of an infectious cycle upon transfection of recombinant CVB3 cDNA into permissive cells (Fig. 6).

**Restoration of the authentic CVB3 5′ end takes place in the cytoplasm of transfected cells.** To prove whether the authentic 5′ terminus is restored through a cytoplasmic event, transfection experiments were performed with in vitro-synthesized RNA transcripts, which should initiate an infectious cycle independent of a nuclear event. For this purpose, the recombinant CVB3 cDNA was set under the control of both the T7 and SP6 promoters and was used for the synthesis of full-length CVB3 RNA transcripts of plus-strand polarity (compare structures shown in Fig. 1). These RNA molecules also lacked the two 5′ uridine residues and contained additional nonviral nucleotides at both ends. A specific infectivity of 14 to 30 PFU/μg of T7- and SP6-derived transcripts was observed in transfected HeLa cells ($n = 3$ different experiments each), whereas virion RNA showed an infectivity of $2 \times 10^5$ PFU/μg. Total lysis of cells overlaid with fluid medium was observed within 2 to 4 days upon transfection of in vitro-synthesized plus-strand RNA. As expected, no infectious cycle was initiated upon transfection of minus-strand RNA transcripts. To examine whether the authentic CVB3 5′ terminus had been restored in plus-strand RNA-transfected cells, the 5′ nucleotide sequence of the virus progeny was determined. The analysis revealed the presence of two initial 5′ uridine residues identical to the 5′ sequence 5′UUAAAA. . . in cDNA-derived CVB3 (Fig. 6). This finding demonstrates that the authentic CVB3 5′ terminus has been restored in the cytoplasm upon transfection of in vitro-synthesized RNA transcripts.

**Biological properties of transfection-derived CVB3.** The supernatant medium of transfected cultures was tested after complete lysis of the cells in a specific antibody neutralization assay to examine the identity of transfection-derived viruses. Neutralization was achieved with CVB3 serotype-specific antiserum but not with CVB1 or CVB5 antiserum, indicating that the cDNA-generated viruses, as well as viruses generated through in vitro-synthesized RNA transcripts, were authentic CVB3. With regard to tissue tropism, transfection-derived viruses were found to induce myocarditis in mice and are also capable of establishing a persistent carrier state infection in cultured human myocardial fibroblasts, as was previously described for the parental CVB3 (14, 15). In addition, transfection-derived viruses were found to grow to titers of about $2 \times 10^8$ to $4 \times 10^8$ PFU/ml upon passage in HeLa cells, which is typically observed for wild-type stocks. cDNA-derived virus, as well as virus generated through transfection of in vitro-synthesized CVB3 RNA, clearly revealed wild-type virus plaque morphology (Fig. 7).

## DISCUSSION

Picornavirus research is in a very dynamic phase following the demonstration that cloned cDNA copies of poliovirus (33) and CVB3 (16) and in vitro-synthesized RNA transcripts of poliovirus (50), human rhinovirus 14 (26), and hepatitis A virus (4) are infectious. In addition, recombinant DNA techniques have contributed to an improved diagnosis of picornavirus infections. We have previously developed an in situ hybridization assay capable of detecting enterovirus RNA in myocardial tissue with full-length reverse-transcribed recombinant CVB3 cDNA as an enterovirus group-specific probe (14). By this approach, the presence of enterovirus RNA has been assessed in endomyocardial biopsy samples of a significant number of patients with myocarditis or dilated cardiomyopathy or both (13, 17).

By comparison of the CVB3 genome to those of other

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CVB3 1    MGAQVSTQKTGAHETRLNASGNSIIHYTNINYYKDAASNSANRQDFTQDPGKFTEPVKDIMIKSLPALN|SPTVEECGYSDRARSITLGNSTITTQECANVVVGYGVWPDYLKDSEATAEDQPTQ   VP4
CVB1                G                                                          M  | SA      V                                    E   N  G
CVB4                S S                          S        V                       |         V                                      S E
PV1          S  V   NSNR Y G T N  T     R S   A SK   S   S     I  VL   TA M  | NI A      VLQL          A  S  A  R  E  R   NPV    E

125   PDVATCRFYTLDSVQWQKTSPGWWWKLPDALSNLGLFGQNMQYHYLGRTGYTVHVQCNASKFHQGCLLVVCVPEAEMGCATLDNT..PSSAELLGGDTAKEFADKPVASGSNKLVQRVVYNAGMG   VP2
                     E   M N A              QM                 I                       SN N    KF   S    N RM T TE GTSND K  TA W
                     N  K EMQ A      F      EM               S   I                     TNAE A   AYGD C  E    S EQN.. ATGKTA  TA C
         A          T S T E R            RDM          Y       S               A G FA   MCLAGDSNTT MHT YQNANP EKGGT TGTFTPDNNQTSPA RFCPVDYL

248   VG....VGNLTIFPHQWINLRTNNSATIVMPYTNSVPMDNMFRHNNVTLMVIPFVPLDYCPGSTTYVPITVTIAPMCAEYNGLR...LAGHQ|GLPTMNTPGSCQFLTSDDFQSPSAMPQYDVTP
                                      I        Y    L   I      N SE  SP                   SS |  V T      T                     F
              Y                       I                F    I     A     VT ASS I       V   S   |      L       T                     F
      L NGTLL  AFV      I         C  L  L   V  LSI S  VK     WGIAIL  LA    NFASE  SPEI    L       C F      NIT PRL |   V         N Y  A N     C L EF

365   EMRIPGEVKNLMEIAEVDSVVPVQNVGEKVNSMEAYQIPVRSNEGSGTQVFGFPLQPGYSSVFSRTLLGEILNYYTHWSGSIKLTFMFCGSAMATGKFLLAYSPPGAGAPTKRVDAMLGTHVIWD   VP3
       Q    R  N              N TDNN   GLK          Q   SDNRR                ANN LN                                        V KN R
       N     Q R               IN LKANLMT      RVQ    TDEM G I              A    LQ                   L      V                  DS KN
      PID          M   L   I  TMI  FDLSAT K  T   M  RVRLSDKPHTDDPILCLS  S  ASDPRL  H  M                A   L  F   L     FM       L VS A      DP  K   KE

490   VGLQSSCVLCIPWISQTHYRFVASDEYTAGGFITCWYQTNIVVPADAQSSCYIMCFVSACNDFSVRLLKDTPFISQQNFFQ|G...........PVEDAITAAIGR..VADTVGTGPTNSEAIPA
               V              Y VE       A YV        I        V  T D L                 M          R D  Y |                ESVER MV           SSK      S
               V              Y VD K     S   S       VI      E   K                    M R   Q   K T  Y |                T ESVER M           IAR  S     Q
      I       TMVV     N  T   QTID SF E   Y SVF     R        LSTPREMD LG                R    TH E KALA |  LGQMLESMIDNT RETVG   TS DALPN EAS    H KE

601   LTAAETGHTSQVVPGDTMQTRHVKNYHSRSESTIENFLCRSACVYFTEYKNSGA.....KRYAEWVLTPRQAAQLRRKLEFFTYVRFDLELTFVITSTQQPSTTQNQDAQILTHQIMYVPPGGPV   VP1
                       S                  S               YAT N NSE          G        INT  V    L RKLE   L                   A  E     ATSV  PVQ Q
         V           D S            H          S              IY..I Y S ESNNL        INT  V           M M     I C M               H EM   AT S VPVQ
         V     A NPL   S   V        VQHR       S    S FA G     TIMTVD  PASTTNKD  LF V KI  YKDTV              S     M           V A..NFTE N GH LNQVY            A

721   PDKVDSYVWQTSTNPSVFWTEGNAPPRMSIPFLSIGNAYSNFYDGWSEFS........RNGVYGINTLNNMGTLYARHVNAGSTGPIKSTIRIYFKPKHVKAWIPRPPRLCQYEKAKNVNFQPSG
      T  TD A                           I          M          C          TQ                         M        EAGQ           V                 V                   Q       N T
      TS ND                  I                     M          TM        N                 D  I  Y S          I          DS P GLT                       YV                 K    S   DVEA
      E W D T        S      I Y Y T   A I V  YVG S        H        F KVPLKDQSAALGDSL  AAS    DF I AV V    DHNPTKVT K  V L        IRV C          AVA Y.GPG DYKDGT

838   VTTTRQSITTMTNTG AF:GQQSGAVYVGNYRVVNRHLATSADWQNCVWESYNRDLLVSTTTAHGCDIIARCQCTTGVYFCASKNKHYPISFEGPGLVEVQESEYYPRRYQSHVLLAAGFSEPGD   2A
         SN       ...   :                             RE    R     D                                    R            V                        K
       AE A LI  ...    PY: H              K             HV         D                      T                    S            V                        K                  T
      L PLSTKDL  ... YIG    H  NK      TAG KIC Y         QD L    A  NVMMS          TESR Q T S          N  NA        Y E  RR Y   V  V   TFQYMEANN       A          M  IGH     AS

961   CGGILRCEHGVIGIVTMGGEGVVGFADIRDLLWLEDDAMEQ|GVKDYVEQLGNAFGSGFTNQICEQVNLLKESLVGQDSILEKSLKALVKIISALVIVVRNHDDLITVTATLALIGCTSSPWRWL   2B
                  V          V                               |                                             V                                       V
                  L          V                              |
               H      I A        L A S        YAY EE     | ITN I S  A        Q    SDKITE  TNMVTS..T T   L    N I        S       IT    YE  TT   L        L   DA      Q

1085  KQKVSQYYGIPMAERQ|NNSWLKKFTEMTNACKGMEWIAVKIQKFIEWLKVKILPEVREKHEFLNRLKQLPLLESQIATIEQSAPSQSDQEQLFSNVQYFAHYCRKYAPLYAAEAKRVFSLEKKM
                      | SG                              I                      K
      H                | G                                                     K           S
      RK ACDVLE   YVIK |GD            AC  A  L  VSN   S    D     E   I  QA D L    VTK R   EM    N   S    H    C      EH    I    N    RWLSIQSKRF        V          IQK    HTI

1209  SNYIQFKSKCRIEPVCLLLHGSPGAGKSVATNLIGRSLAEKLNSSVYSLPPDPDHFDGYKQQAVVIMDDLCQNPDGKDVSLFCQMVSSVDFVPPMAALEEKGILFTSPFVLASTNAGSINAPTVS   2C

      N          H              V          T                A AI    RE T T             S                 G                 N             A MK                  T E I            S                           NY             SSR SP     A

1334  DSRALARRFHFDMNIEVISMYSQNGKINMPMSVKTCDDECCPVNFKKCCPLVCGKAIQFIDRRTQVRYSLDMLVTEMFREYNHRHSVGTTLEALFQ|GPPVYREIKISVAPETPPPPAIADLLKS   3A
                                                            E                                                               A                 |        I               I
                                                            E             R                         K                       A                 |                        T           V
      H D        A    D Q MNE    RD    L    A ATEM  KNCHQ  A     R                     LM  KSSR        I  QIT  MIIN  R  R  SNI  NCM      |    LQ  KDL    DI.KTS       EC  N       QA

1458  VDSEAVREYCKEKGWLVPEINSTLQIEKHVSRAFICLQALTTFVSVAGIIYIIYKLFAGFQ|GAYTGVPNQKPRVPTLRQAKVQ|GPAFEFAVAMMKRNSSTVKTEYGEFTMLGIYDRWAVLPRH   3B
                                                                  |      M       K             |   V
            I                 D I                                    |      M       K             |
         QE     D    EK       I .N T QV  T  RNIN    MTI       V      AA        VV VM           H |     L      K       N      I T        |  G DY        A        IV AT SK               VH  NV I     T

1581  AKPGPTILMNDQEVGVLDAKELVDKDGTNLELTLLKLNRNEKFRDIRGFLAKEEVEVNEAVLAINTSKFPNMYIPVGQVTEYGFLNLGGTPTKRMLMYNFPTRAGQCGGVLMSTGKVLGIHVGGN   3C
                    I
                                             I R                                                                      R   D
      S    ES  VIDGK     EI        A E  QA         I   IIT K               PHIPTQIT  T  DG      IV          Y         V       A       Q  Y            RQ  A  T                              ITC           I  M

1706  GHQGFSAALLKHYFNDEQ|GEIEFIESSKDAGFPVINTPSKTKLEPSVFHQVFEGNKEPAVLRSGDPRLKANFEEAIFSKYIGNVNTHVDEYMLEAVDHYAGQLATLDISTEPMKLEDAVYGTEG
                         |              E                                                       N                      R                                                                                  R
                         |                               R                       H                        N                      V                     F                                                     N
      SH   A      KRS    TQS |      QMMRP      EV Y  I   A                  A   Y          V                   TKN               TD                        V  KI  E            K                       MS      N   Q  C          M     D

1830  LEALDLTTSAGYPYVALGIKKRDILSKKTKDLTKLKECMDKYGLNLPMVTYVKDELRSIEKVAKGKSRLIEASSLNDSVAMRQTFGNLYKTFHLNPGVVTGSAVGCDPDLFWSKIPVMLDGHLIA   3D
                                         R                                                             A                                                                      I                                             V
                                                                                                        A                                                   A        I                       V
                    S          M K          N  Q  R   TKEMQKLL  T    I        L                               KT        EQ                                 MA           AA    K          I                                                LMEEK   F

1955  FDYSGYDASLSPVWFACLKMLLEKLGYTHKETNYIDYLCNSHHLYRDKHYFVRGGMPSGCSGTSIFNSMINNIIIRTLMLKVYKGIDLDQFRMIAYGDDVIASYPWPIDASLLAEAGKGYGLIMT
                                          L            S                                                                                                                                                                                    RDC
                                           L                                                                                                                                                                                                    D
            T                 A   EA     V        I  FGDR.VD            NH              KN  T  C  K                                        L           L    T                 HLK                               HEV             QS     D     T

2080  PADKGECFNEVTWTNATFLKRYFRADEQYPFLVHPVMPMKDIHESIRWTKDPKNTQDHVRSLCLLAWHNGEHEYEEFIRKIRSVPVGRCLTLPAFSTLRRKWLDSF     2185
             D            A V                                                                                                                   A
                          V                                                                    Q
         SAT ET     E V                F             K          I                E                       R                               E   NK  LA                  I   A L    EY       Y R
```

FIG. 4. Deduced amino acid sequence of infectious CVB3 cDNA in comparison with that of the polyproteins of CVB1 (Iizuka et al. [10]), CVB4 (Jenkins et al. [11]), and PV1 (18, 32). The CVB3 amino acid sequence is shown in its entirety, and deviating amino acids are depicted for CVB1, CVB4, and PV1. Dots represent amino acid deletions. The proposed cleavage sites of the polyproteins of the coxsackieviruses are marked by vertical lines by analogy to the biochemically established sites of proteolytic processing of PV1 (18). Note that only the proposed cleavage sites between polypeptides 2B and 2C and between VP1 and $2A^{pro}$ differ between the coxsackieviruses and poliovirus. To determine the exact position of the cleavage site of CVB3 between VP1 and $2A^{pro}$, direct sequencing of the termini of these polypeptides will be required.

TABLE 1. Amino acid sequence identity among the proteins of CVB3 and three other enteroviruses[a]

| Protein | CVB3[b] | Identity between: | | |
|---|---|---|---|---|
| | | CVB3 and CVB1 | CVB3 and CVB4 | CVB3 and PV1 |
| VP4 | 69 | 97.1 | 94.2 | 65.2 |
| VP2 | 263 | 82.9 | 79.8 | 51.0 |
| VP3 | 238 | 80.7 | 79.0 | 55.5 |
| VP1 | 281 | 77.2 | 69.4 | 38.8 |
| 2A$^{pro}$ | 150 | 94.0 | 92.0 | 58.0 |
| 2B | 99 | 98.0 | 99.0 | 49.5 |
| 2C | 329 | 98.2 | 97.9 | 62.3 |
| 3A | 89 | 97.8 | 93.3 | 51.7 |
| 3B | 22 | 90.9 | 90.9 | 77.3 |
| 3C$^{pro}$ | 183 | 98.9 | 97.8 | 55.2 |
| 3D$^{pol}$ | 462 | 96.3 | 97.0 | 79.7 |

[a] Sequence identities are expressed as percentages. Details are taken from Iizuka et al. (10) for CVB1, Jenkins et al. (11) for CVB4, and Kitamura et al. (18) for PV1.

[b] Number of amino acids of CVB3 proteins based on the cleavage sites proposed in the legend to Fig. 4.

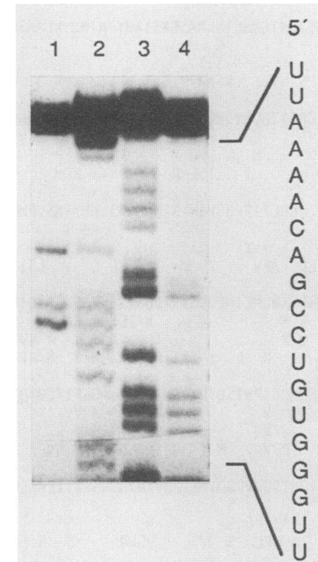


FIG. 6. 5′ Terminal sequence of CVB3 generated through transfection of recombinant CVB3 plasmid DNA into HeLa cells. To obtain the sequence of the 5′ terminal region of the genomic RNA of cDNA-derived CVB3, primer-extended cDNA was synthesized from purified viral RNA and sequenced chemically as described in Materials and Methods. Lanes 1 to 4, Labeled DNA fragments generated from cleavage at G (lane 1), G and A (lane 2), T and C (lane 3), and C (lane 4). The complementary 5′ terminal sequence of the corresponding viral RNA is shown at the right. Note the presence of the two initial 5′ uridine residues which are not contained in the recombinant infectious CVB3 cDNA (16). An identical sequence was obtained by sequencing genomic RNA from CVB3 generated upon transfection of in vitro-synthesized viral RNA.

potentially cardiotropic group B coxsackieviruses, the high degree of identity among nucleotide and amino acid sequences clearly demonstrates their close genetic relationship, which is considerably lower with respect to poliovirus (compare Table 1 with Fig. 4). Most pronounced is the degree of identity within the 5′ and 3′ nc regions and, regarding the polyprotein sequences, within the nonstructural proteins and VP4. In the case of the structural proteins VP1, VP2, and VP3, identity is confined to distinct regions interspersed between nonhomologous sequences (Fig. 4). As is evident from the crystallographic structures of various picornaviruses (9, 21, 35), conserved regions have an impor-

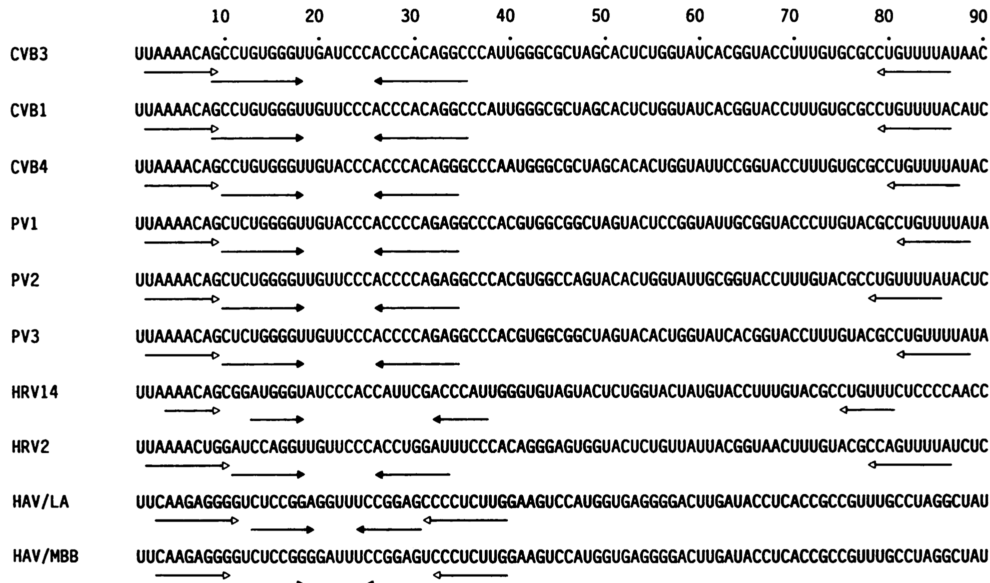


FIG. 5. Common structural features of the 5′ terminus of different picornaviruses. The first 90 5′ terminal nts of CVB3, CVB1 (10), CVB4 (11), PV1 (18, 32), PV2 (45), PV3 (41), human rhinovirus type 14 (HRV14) (40), human rhinovirus type 2 (HRV2) (38), and hepatitis A viruses (HAV/LA) (28) and HAV/MBB (31) are presented. Note that the first 10 nts at the 5′ end are identical among the enteroviruses. Arrows indicate perfect repeats of inverted symmetry, 6 to 10 nts in length, which are found at corresponding positions for the human enteroviruses and rhinoviruses (nts 9 to 20 are complementary to nts 23 to 37, and nts 2 to 11 are complementary to nts 75 to 88). In the case of hepatitis A virus, the inverted repeat that is complementary to nts 2 to 11 is shifted to nts 31 to 39. Computer-calculated secondary structures (data not shown) suggest an important function for these inverted repeats during plus- and minus-strand RNA replication.

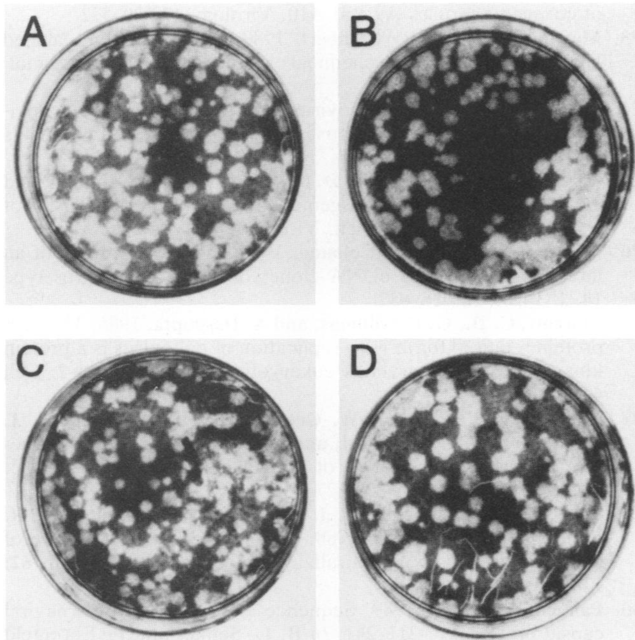


FIG. 7. Plaque morphology of CVB3 generated through transfection of wild-type RNA (A), recombinant pCB3-M1 plasmid DNA (B), recombinant pCB3/T7 plasmid DNA (C), and in vitro-synthesized CVB3 RNA (D). HeLa cell monolayers were infected with transfection-derived first-passage stocks, as described for plaque assays in Materials and Methods, indicating that viruses generated in the absence of two initial 5' uridine residues exhibited wild-type plaque morphology.

tant function in maintaining polypeptide chains of the structural proteins in the barrel-like core structure. Structurally conserved loops and terminal extensions have been suggested to be involved in dynamic viral processes, e.g., assembly, receptor binding, and uncoating (7), whereas highly divergent sequences may participate in the formation of antigenic determinants. This pattern of conserved and heterologous peptide sequences is shared by various genera of picornaviruses, as demonstrated by a comprehensive alignment of the capsid proteins of 33 picornavirus strains (30). Peptide sequences, which can be identified by correlation to poliovirus sequences containing antigenic determinants, are highly divergent among the coxsackieviruses and probably exhibit serotype-specific antigenic properties. In addition, peptides with sequences common to a subset of serotypes should provide useful targets for broad-spectrum clinical diagnosis of potentially cardiotropic enteroviruses. In this context, we have recently demonstrated that polyclonal antibodies generated against bacterially synthesized fusion proteins of various CVB3 structural proteins clearly cross-react with a wide variety of group A and B coxsackieviruses, as well as echoviruses (51).

The mechanism by which the recombinant cDNA of a picornavirus initiates an infectious cycle in transfected cells is currently poorly understood. Presumably, the recombinant cDNA is transcribed in the nucleus of transfected cells to produce plus-strand RNA transcripts, which serve as messengers for the synthesis of virus-directed proteins and as templates for replication. Regardless of whether these transcripts are initiated from cellular promoters after integration or from cryptic promoters present in the vector DNA, they probably represent oversized transcripts with extra nonviral sequences at both ends. The in vitro-synthesized CVB3 RNA transcripts also represent oversized transcripts. These RNAs, while about 20 times more infectious than the plasmid DNAs, are only about 0.01% as infectious as RNA isolated from the virus. This may be caused by the presence of oversized nonviral sequences (50) and possibly also by the absence of two initial 5' uridine residues. Nonetheless, transfection-derived viruses have biological properties indistinguishable from those of parental CVB3.

The 5' termini of the transfection-derived viruses and that of parental CVB3 were found to be identical, demonstrating that recombinant plasmid DNA, as well as in vitro-synthesized RNA transcripts, has regained two initial 5' uridine residues during initiation of an infectious cycle. Presumably, these uridine residues are provided by the picornaviral nucleotidyl protein VPg-pUpU, which has been proposed as a component of the initiation complex of poliovirus RNA synthesis (5, 43, 44, 52). Free VPg-pUpU is known to be present in poliovirus-infected cells (5) and has been synthesized in vitro in a membrane fraction of poliovirus-infected cells (43). It was suggested that a precursor of VPg, most likely polypeptide 3AB, is uridylylated and subsequently cleaved by the 3C protease ($3C^{pro}$) to yield the primer molecule VPg-pUpU (44, 52). In vitro pulse-chase analyses have supported a model in which VPg-pUpU can function as a primer in the elongation reaction (42), and evidence was obtained by an in vitro genetic approach that implicates $3D^{pol}$ in the formation of VPg-pUpU (47). Thus, a very attractive model for the initiation of picornaviral RNA synthesis is provided by implying synthesis of VPg-pUpU by $3D^{pol}$ without a template, by its hybridization to template RNA, and by elongation by $3D^{pol}$.

Our finding of a correct 5' end of transfection-derived CVB3 strongly supports uridylylation of VPg in a template-independent fashion. This implies a unique mechanism allowing the correct initiation of plus-strand RNA synthesis independent of the presence of two complementary adenines of the minus strand. Presumably, distinct sequences of the minus-strand RNA template are involved in this process by interacting with components of the replication complex. The inverted repeats identified near the 5' terminus of the viral genome (compare structures shown in Fig. 5) might play an important role. It is tempting to assume that these sequences, as part of the minus strand, might adopt a distinct three-dimensional arrangement serving as a signal element for the interaction with protein entities of the putative replication complex.

The availability of the nucleotide sequence of an infectious cDNA clone of a cardiotropic CVB3 should prove to be useful for further studies on the structure and function of the viral RNA and provides a basis for a molecular genetic approach not previously available for the analysis of enterovirus-induced cardiomyopathy.

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