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Molecular cloning of the genome of a cardiotropic Coxsackie B3 virus: Full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells

(Coxsackie B3 virus RNA/cDNA-derived virus/picornavirus/enterovirus/viral heart disease)

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ABSTRACT **The molecular cloning of double-stranded cDNA synthesized from the single-stranded RNA genome of the cardiotropic Coxsackie B3 virus (Nancy strain) is reported. Full-length reverse-transcribed cloned viral cDNA of ≈7500 nucleotides generated infectious antigenically identical Coxsackie B3 virus upon transfection of recombinant plasmid DNA into mammalian cells, demonstrating the molecular cloning of a biologically active viral cDNA copy. Furthermore, the cloned cDNA is characterized by restriction enzyme analysis and partial nucleotide sequencing of the 5′ end. The Coxsackie B3 virus cDNA described can now be used to study the molecular basis of human enteroviral heart disease, and it provides a valuable diagnostic means for patients with suspected viral heart disease.**

Coxsackie viruses are important human pathogens, causing a remarkable variety of diseases from minor common colds to fatal myocarditis, neurological disorders, and possibly acute-onset diabetes (1, 2). Coxsackie viruses (groups A and B) as well as polioviruses, echoviruses, and hepatitis A virus are enteroviruses of the picornaviridae, whose natural host is man.

We have recently initiated a project to study the role of enteroviruses in acute and chronic cardiac disease. The aim of the study is to understand the pathogenesis of human viral heart disease at the cellular and molecular level. We chose Coxsackie B3 virus (CB3 virus) as a model because of the obvious significance of Coxsackie B viruses in clinical cardiology (3, 4). Knowledge of the life cycle of such a virus in myocardial cells is essential to an understanding of the pathogenesis of viral heart disease. So far, we have chosen two approaches to this problem.

The first approach was to establish a tissue culture system based on human fetal heart cells that allows the simulation of viral heart disease *in vitro*. The second approach is based on molecular cloning of the CB3 virus genomic RNA, which is of utmost medical and biological interest. A specific cDNA probe would allow us to follow the viral genome in myocardial cells, for example, and to study questions concerning the molecular basis of pathogenicity and tissue tropism. In addition, cloned CB3 virus cDNA copies would provide a powerful means of diagnosing myocardial biopsies and blood samples of patients with suspected viral heart disease.

The tissue culture system for the study of Coxsackie myocarditis together with the isolation of a cardiotropic large plaque variant of CB3 virus (Nancy strain) has been recently described by us (5, 6). Cultured human fetal myocytes were found to disintegrate upon infection with this virus, whereas myocardial fibroblasts developed a persistent carrier state infection.

Recently, a cloned partial cDNA copy of the CB3 virus genome has been described (7) that hybridized to enteroviral RNA from infected cells (8). We report here the cloning of a full-length reverse-transcribed CB3 virus cDNA copy that generated replication-competent CB3 virus upon transfection of recombinant viral cDNA into permissive mammalian cells. This finding offers the unique opportunity for *in vitro* site-directed mutagenesis and thus for genetic analysis of this cardiotropic virus.

MATERIALS AND METHODS

Propagation and Purification of CB3 Virus. The Nancy strain (9) of CB3 virus was obtained from The American Type Culture Collection (ATCC, no. VR-30). After two passages of the virus in Vero cells, a large plaque variant was obtained by three successive plaque-purification cycles as described (6). Plaque-purified CB3 virus was further passaged twice in Vero cells for the preparation of seed virus.

For a large-scale production of this virus, Vero cells grown in roller bottles were infected by using a low input multiplicity of 0.5 plaque-forming unit (pfu) per cell. Cultures were harvested 20 hr after infection when 100% of the cells exhibited cytopathologic effects. To avoid contamination with membrane-bound virions (10), only extracellular virus was pelleted from clarified culture medium and purified by two successive bandings in cesium chloride.

RNA Extraction and Characterization. Suspensions of purified CB3 virus were made 1% in $NaDodSO_4$ and incubated at 37°C for 1 hr with 250 μg of proteinase K per ml. After extraction once with phenol and then twice with chloroform/isoamyl alcohol (24:1, vol/vol) CB3 RNA was precipitated in ethanol, redissolved, and after removal of a portion for analysis, reprecipitated. The structural integrity of the viral RNA was analyzed by agarose gel electrophoresis with 1 M glyoxal/50% dimethyl sulfoxide (vol/vol) (11). The functional integrity of the viral RNA was proved by the induction of an infectious cycle upon transfection into Vero cells as described below. Aliquots of this batch were used to standardize optimal conditions for cDNA synthesis.

Preparation of CB3 Virus cDNA Clones. The polyadenylylated purified CB3 virus RNA (3 μg) served as a template for first-strand cDNA synthesis by using reverse transcriptase (2000 units/ml) for 40 min at 42°C in 50 μl that contained 100 mM Tris·HCl, pH 8.3/10 mM $MgCl_2$/140 mM KCl/1 mM each dATP, [α-^{32}P]dCTP (0.2 Ci/mmol; 1 Ci = 37 GBq), dGTP, TTP/0.4 mM dithiothreitol/oligo(dT)$_{12-18}$ (60 μg/ml)/RNasin (500 units/ml). After addition of EDTA to 20 mM and $NaDodSO_4$ to 0.5%, the RNA·cDNA hybrid mol-

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Abbreviations: kb, kilobase(s); CB3 virus, Coxsackie B3 virus; pfu, plaque-forming units.

ecules were isolated by phenol extraction, spin-column chromatography, and ethanol precipitation.

The RNA strand of the RNA·cDNA hybrids was replaced by RNase H and DNA polymerase I mediated second-strand synthesis (12, 13) in 100 μl of 20 mM Tris·HCl, pH 7.4/7 mM $MgCl_2$/100 mM KCl/0.15 mM β-NAD/bovine serum albumin (50 μg/ml)/100 μM each of the four deoxynucleoside triphosphates/*Escherichia coli* RNase H (5 units/ml)/*E. coli* DNA polymerase I (250 units/ml)/*E. coli* DNA ligase (5 μg/ml). After successive incubation for 60 min at 12°C and 90 min at 22°C, double-stranded CB3 virus cDNA was isolated as described for the RNA·cDNA hybrid molecules.

Homopolymer tails of 10–15 residues of dGMP were added directly to double-stranded cDNA (13) by using terminal deoxynucleotidyl transferase (833 units/ml) for 20 min at 37°C in 15 μl of 140 mM sodium cacodylate/30 mM Tris·HCl, pH 6.8/0.1 mM dithiothreitol/1 mM $CoCl_2$/0.1 mM [^{3}H]dGTP (7.4 Ci/mmol).

Plasmid vector p2732B was linearized at its cloning site by digestion with *Cla* I and *Bgl* II. After sedimentation through a sucrose gradient, the plasmid vector was tailed with dCMP, hybridized to oligo$(dG)_{10-15}$-tailed double-stranded CB3 virus cDNA, and used to transform competent *E. coli* BJ5183 (14) by standard procedures (15). The cloning and growth of recombinant bacteria were carried out according to the L3B1 safety regulations as advised by the German Control Committee for Recombinant DNA Research.

Analysis of CB3 Virus cDNA Clones. Ampicillin-resistant clones were analyzed by colony filter hybridization (15, 16) using in a first step ^{32}P-labeled CB3 virus cDNA as a probe, prepared under conditions of reduced dCTP in order to enrich for sequences corresponding to the 3′ region of the viral RNA (17). Clones displaying strong hybridization signals were screened by cleaving recombinant plasmid preparations (15) with *Eco*RI and sizing of the released cDNA by electrophoresis. cDNA inserts were isolated from agarose gels, nick-translated (18), and used as probes in hybridization (*i*) to electrophoretically resolved CB3 virus RNA (19) to establish the specificity of cloned cDNA, (*ii*) to restriction fragments of insert cDNA resolved by electrophoresis to confirm tentative restriction maps constructed on the basis of double digests, and (*iii*) to recombinant plasmid DNA bound to nitrocellulose filter after lysis of bacterial colonies for further screening. Sequence determination was done by the method of Maxam and Gilbert (20).

Transfection of Cells. Transfections were carried out essentially as described (21, 22). Briefly, 10 μg of recombinant plasmid DNA in 6 mM Tris·HCl, pH 7.4/6 mM NaCl/0.2 mM EDTA was diluted into 500 μl of Hepes-buffered saline (137 mM NaCl/6 mM glucose/5 mM KCl/0.7 mM Na_2HPO_4/20 mM Hepes adjusted to pH 6.98 with 0.5 M NaOH). The DNA was precipitated by addition of 2.5 M $CaCl_2$ to a final concentration of 125 mM, and the mixture was incubated at room temperature for 25 min and then added to 8×10^5 HeLa cells or Vero cells maintained in Dulbecco's modified Eagle's minimal medium (DME medium) supplemented with 10% fetal bovine serum and 50 μg of kanamycin per ml. After at least 4 hr of incubation at 37°C, cells were treated with 15% (vol/vol) glycerol in DME medium for 45 sec and then washed twice with DME medium. Cultures were maintained in supplemented DME medium or overlaid with 1% agarose in complete DME medium as described (6).

Materials. The sources used were as follows: reverse transcriptase, J. W. Beard, Life Sciences (St. Petersburg, FL); terminal deoxynucleotidyl transferase, *E. coli* RNase H, large fragment of *E. coli* DNA polymerase I, *E. coli* DNA ligase, and certain restriction enzymes, P-L Biochemicals; other restriction enzymes, Boehringer Mannheim; proteinase K, Merck (Darmstadt, F.R.G.); pancreatic ribonuclease, Worthington; [α-^{32}P]dCTP, Amersham; [^{3}H]dGTP, New England Nuclear; RNasin, Bethesda Research Laboratories; plasmid vector p2732B, a derivative of pBR322 containing the ampicillin-resistance gene, the plasmid origin of replication, and a cloning site array between two *Eco*RI sites, was constructed by J. D. Monahan (Roche Institute, Nutley, NJ) and kindly provided by K. Müller (Max Planck Institute for Biochemistry, D-8033 Martinsried, F.R.G.).

RESULTS

Isolation and Characterization of CB3 Virus RNA. Viral RNA was isolated from purified virions after treatment with proteinase K. The purity and structural integrity of the RNA was shown by agarose gel electrophoresis. CB3 virus RNA migrated as a single band (Fig. 1, lane 2). The biological integrity of this RNA could be demonstrated by generating infectious CB3 virus upon transfection of the RNA into permissive Vero cells. This characterized RNA was the starting material for the cloning of the viral genome.

Molecular Cloning of the CB3 Virus Genome. Single-stranded cDNA was synthesized from the genomic viral RNA using oligo$(dT)_{12-18}$ as a primer for reverse transcriptase under optimized conditions. Without further sizing of the reaction products, cDNA·RNA hybrids were subjected to second-strand cDNA synthesis making use of the RNase H and DNA polymerase I mediated method (12, 13), adapted to the purposes of the $\approx$7500-base-long genomic RNA. After second-strand cDNA synthesis, the CB3 cDNA was directly tailed with dGMP by using terminal deoxynucleotidyl transferase without the necessity of prior trimming of double-stranded cDNA molecules with S1 nuclease. Oligo$(dG)_{10-15}$-tailed CB3 virus double-stranded cDNA was annealed to oligo$(dC)_{10-15}$-tailed plasmid vector p2732B, previously linearized with *Cla* I and *Bgl* II in its cloning site. On transformation of competent *E. coli* BJ5381, at least 4×10^4 bacterial colonies resistant to ampicillin were obtained per μg of viral RNA used in the initial reverse transcription.

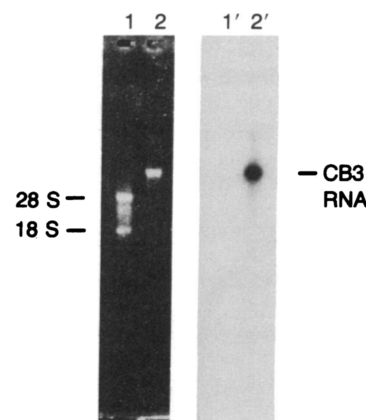

FIG. 1. Hybridization of cloned CB3 virus cDNA probe to RNA. Nucleic acids were denatured (11) and electrophoresed on a 1% agarose minigel (6 × 4 cm); marker lanes 1 and 2 were removed and stained with ethidium bromide. Lanes 1′ and 2′ were transferred to nitrocellulose paper and hybridized for 20 hr at 37°C with ^{32}P-labeled pCB3-M1 (50 ng/ml; 4×10^8 dpm/μg) in 50% formamide (vol/vol)/2× NaCl/Cit (0.3 M NaCl/0.03 M trisodium citrate)/0.12 M sodium phosphate, pH 6.8/0.2% $NaDodSO_4$/1% *N*-lauroylsarcosine/denatured sheared salmon sperm DNA (10 μg/ml)/0.08% each of bovine serum albumin, polyvinylpyrollidone, and Ficoll. Lanes 1 and 1′ contained 1 μg of total RNA isolated from cultured human heart cells (5, 6); lanes 2 and 2′ contained purified CB3 virus RNA at 250 ng and 125 ng, respectively. Autoradiograph exposure was at −80°C for 36 hr.

Identification of CB3 Virus cDNA Clones. To detect virus-specific sequences in transformed bacteria, colony filter hybridizations were carried out using in a first step ^{32}P-labeled CB3 virus cDNA as a probe, synthesized under conditions of reduced dCTP in order to enrich for cDNA copies representing the 3′ region of the viral RNA. Of 2304 colonies tested, at least 60% hybridized using stringent conditions. Ninety-six clones displaying strong hybridization signals were screened by cleaving recombinant plasmid preparations with *Eco*RI and sizing the released CB3 virus cDNA inserts by agarose gel electrophoresis. The 5′-terminal *Pvu* II fragment of the 4.2-kilobase (kb) clone pCB3-23E1 (corresponding to the region from 3.2 to 3.6 kb of the viral genome) was nick-translated and used as a probe for further screening of ampicillin-resistant bacterial colonies. Of 4800 colonies tested, including those from the previous screening experiment, 468 clones hybridized. Recombinant plasmid DNA was analyzed as described above until CB3 cDNA inserts were found approaching the length of the viral genome of ≈7500 nucleotides. The identity of various inserted DNAs in recombinant plasmids was established by specific hybridization to CB3 virus RNA bound to nitrocellulose paper after gel electrophoresis (Fig. 1, lane 2′). One of 96 cDNA clones analyzed by agarose gel electrophoresis appeared to be full-length and generated infectious cDNA-derived CB3 virus upon transfection of recombinant plasmid DNA into mammalian cells.

Infectivity of Cloned CB3 cDNA. The infectivity of the full-length reverse-transcribed CB3 virus cDNA copy, designated pCB3-M1, was shown by transfection of closed circular forms of recombinant plasmid DNA into semiconfluent monolayers of permissive HeLa cells and Vero cells. From day 3 after transfection, lysis of cells started as a focal event of 5–17 plaques (mean, 10 ± 3 plaques; n = 16 different experiments) in transfected cultures overlaid with agarose (Fig. 2). Total lysis of cells overlaid with fluid medium was observed within 4–6 days.

The supernatant medium of lysed cultures was taken and submitted to specific antibody neutralization assay (23) to examine the identity of the cDNA-derived virus. Neutralization tests were done with rabbit antiserum of each of the six Coxsackie B subtypes (B1–B6). Neutralization of the cDNA-derived virus was achieved only with CB3 virus antiserum. About 50 pfu of the cDNA-derived virus as well as 50 pfu of authentic CB3 virus were neutralized 50% by a 1:30,000 dilution of the CB3 virus antiserum, indicating that the cDNA-derived virus was authentic Coxsackie B3 virus. With respect to cardiotropism, the cDNA-derived CB3 virus is also capable of inducing a persistent carrier state infection in cultured human myocardial fibroblasts, as was described for the authentic CB3 virus (5, 6).

FIG. 2. Plaques produced by cDNA-derived CB3 virus upon transfection of HeLa cells with recombinant plasmid DNA. HeLa cells were grown in 60-mm Petri dishes and transfected as described using 10 μg of closed circular forms of recombinant plasmid DNA of the full-length reverse-transcribed CB3 virus cDNA clone, pCB3-M1. Immediately upon transfection, cultures were overlaid with DME medium containing 1% agarose and incubated at 37°C. To count plaques produced by cDNA-derived CB3 virus, cultures were fixed after 4 days with 5% trichloroacetic acid and stained with 1% crystal violet.

In addition, the medium from cells transfected with pCB3-M1 or unsubstituted plasmid vector p2732B was analyzed for quantitation of infectious cDNA-derived virus. A high titer of infectious virus was found in the medium from cells transfected with pCB3-M1 (Table 1), but no virus was released from p2732B transfected cells. Following the experimental design as performed by Racaniello and Baltimore with a cloned poliovirus cDNA construct (24), no infectious CB3 virus was generated when cells were transfected with cDNA copies lacking 156 or 400 nucleotides at the 5′-terminal end (Table 1). Infectivity due to contaminating viral RNA of pCB3-M1 DNA was excluded by RNase treatment which did not lower the infectivity of the cDNA. However, treatment with DNase I or complete digestion of pCB3-M1 with *Hin*fI generating multiple fragments, resulted in total loss of infectivity (Table 1). When pCB3-M1 was assayed directly for contaminating virions on HeLa cells or Vero cells, no infectivity was detected. These results indicate that the infectivity of pCB3-M1 is inherent to the recombinant plasmid DNA.

Restriction Map of Cloned CB3 Virus cDNA. To establish the restriction map of the viral genome presented in Fig. 3, we used a set of cDNA inserts of different lengths, which included the 3′ end of the viral genome as confirmed by hybridization of labeled inserts to fractionated cloned cDNA or by demonstration of the 3′ poly(A) tract through partial sequencing (data not shown). Comparing digests of cDNA inserts of different sizes that are released from the plasmid vector by *Eco*RI (Fig. 4), specific cleavage sites could be easily located by the length of new appearing fragments in a cDNA insert of a given size (compare legend to Fig. 4).

Partial Sequence of the CB3 Virus Genome. A sequence of 350 nucleotides of cloned CB3 virus cDNA corresponding to the 5′ end of CB3 virus RNA was aligned with the corresponding poliovirus type I sequence (25). A comparison of the two sequences (Fig. 5) revealed a remarkably high degree of 70% homology among these different members of the human enterovirus group. In addition, the 5′ sequence of CB3 virus is in agreement with the published 5′-terminal 20 nucleotides of Coxsackie B1 virus, obtained by RNA sequencing (26). Two initial uridine residues are missing in the infectious CB3 cDNA that are present in Coxsackie B1 virus RNA as well as in poliovirus RNA.

Table 1. Transfection of cultured mammalian cells with various plasmid DNAs

Nucleic acid	pfu of cDNA-derived CB3 virus per ml of medium	
	HeLa cells	Vero cells
pCB3-M1	2.1 ± 1.5 × 10^7 (7)	3.6 ± 1.4 × 10^7 (7)
pCB3-M1/RNase A	1.9 ± 0.7 × 10^7 (3)	2.6 ± 1.3 × 10^7 (3)
pCB3-M1/DNase I	0 (3)	0 (3)
pCB3-30A11	0 (4)	0 (4)
pCB3-41B12	0 (3)	0 (3)
p2732B	0 (4)	0 (4)

HeLa cells or Vero cells were grown in 60-mm Petri dishes. About 8 × 10^5 cells were transfected with 10 μg of closed circular forms of plasmid DNA. After incubation of the transfected cells at 37°C for 4–6 days, the supernatant medium was assayed for infectious CB3 virus on confluent monolayers of Vero cells. pCB3-30A11 and pCB3-41B12 are lacking 156 and 400 nucleotides, respectively, at the 5′-terminal end of the viral genome. Values are expressed as mean pfu per ml of supernatant medium ± SEM; numbers in parentheses indicate number of different transfection experiments.

FIG. 3. Restriction map of cloned CB3 virus cDNA. Sites for rarely cutting restriction enzymes are indicated with symbols. The corresponding viral RNA is shown with the 3′ poly(A) (thicker line) at the right. Restriction endonuclease analysis was done by comparing digests of seven CB3 virus cDNA inserts of different lengths, starting precisely with the 3′ end of the viral genome as described in the legend of Fig. 4.

DISCUSSION

Being interested in the role of enteroviruses in the pathogenesis of human viral heart disease, we cloned the genome of a large plaque variant of the CB3 virus (Nancy strain), which had been found to be infectious for cultured human myocardial cells (5, 6). Making use of the RNase H and DNA polymerase I mediated second-strand cDNA synthesis (12, 13), we designed and followed an efficient cloning strategy for large RNA molecules, which comprises an improvement on earlier methods used in the cloning of viral RNA genomes. The efficiency of this cloning approach is given by the fact that full-length reverse-transcribed CB3 virus cDNA of ≈7500 nucleotides was cloned. Moreover, full-length reverse-transcribed CB3 virus cDNA yielded infectious antigenically identical Coxsackie B3 virus upon transfection of recombinant plasmid DNA into permissive cells. This finding together with the partial nucleotide sequence of the 5′ end indicates that cloned CB3 cDNA was a faithful transcript of the original viral RNA.

FIG. 4. Strategy in restriction endonuclease analysis of CB3 virus cDNA clones. Seven different cDNA clones, all starting precisely with the 3′ end of the viral genome were submitted to digestion with *Eco*RI to release the cDNA inserts (*Left*) and to double digests with *Eco*RI and another restriction endonuclease—e.g., *Bgl* II (*Right*)—and electrophoresed on agarose gels. The cDNAs are lined up from lane 1–7 with respect to their lengths (3.0–7.5 kb), starting with the smallest cDNA insert (upper band in lane 1). The first internal *Eco*RI site is observed in lane 4 (*Left*), generating a fragment of ≈1.1 kb. This fragment is shifting to 2.3 kb in lane 5, to 2.5 kb in lane 6, and, finally, to 2.7 kb in lane 7. Based on this result the *Eco*RI site could be located on a linear restriction map of the viral genome (corresponding to 2.7 kb in Fig. 3). Analogously, the *Bgl* II site was mapped with respect to the *Eco*RI site. The single *Bgl* II site appears in lane 4 (right) generating a fragment of 0.65 kb, which remains constant in size in the following larger cDNA clones. Following this strategy, the restriction map of the CB3 viral genome could be easily constructed. Lanes: M, λ*Hin*dIII fragments: p, nonrecombinant plasmid p2732B; 1, pCB3-319; 2, pCB3-374; 3, pCB3-23E1; 4, pCB3-33H6; 5, pCB3-41B12; 6, pCB3-30A11; 7, pCB3-M1.

In agreement with the finding of Racaniello and Baltimore (24), who reported on the infectivity of a cloned poliovirus cDNA copy that had been constructed from subgenomic viral cDNA clones, no infectious virus was generated when cells were transfected with cloned CB3 cDNA copies lacking the 5′-terminal end of the viral genome (Table 1). Although the 5′ end of picornaviruses does not encode viral proteins (27), it is obviously required for infectivity.

Because there is no DNA intermediate in the life cycle of picornaviruses, the molecular mechanisms involved in the initiation of an infectious cycle by a cloned cDNA will be important to understand. We must assume that the first event

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CB3   1 ..AAAACAGCCTGTGGGTTGATCCCACCCACAGGGCCCA..TTGGGC
PV1   1 TTAAAACAGCTCTGGGGTTGTACCCACCCCAGAGGCCCACGTGGCGG

 44   CTAG.CCTCTGGTATCACGGTACCTTTGTGCGCCTGTTTTATACCCCCT
 48   CTAGTACTCCGGTATTGCGGTACCCTTGTACGCCTGTTTTATACTCCCT

 92   CCCCCAACTGTAACTTAGAAGTAACACACACCGATCAACAGTCAGCGTG
 97   TCCC.....GTAACTTAGACGCACAAAACCAAGTTCAATAGAAGGGGGT

141   GCACACCAACCACGTTTTGATCAAGCACTTCTGTTACCCCGGACTGAGT
141   ACAAACCAGTACCACCACGAACAAGCACTTCTGTTTCCCCGG..TGATG

190   ATCAATAGACTGCTCACGCGGTTGAAGGAGAAAGCGTTCGTTATCCGGC
188   TCGTATAGACTGCTTGCGTGGTTGAAAGCG.ACGGATCCGTTATCCGCT

239   CAACTACTTCGAAAAACCTAGTAACACCGTGGAAGTTGCAGAGTGTTTC
236   TATGTACTTCGAGAAGCCCAGTACCACCTCGGAATCTTCGATGCGTT..

288   GCTCAGCACT..ACCCC..AGTGTAGATCAGGTCGATGAGTCACCGCAT
283   GGTTAGCACTCAACCCCAGAGTGTAGCTTAGGCTGATGAGTCTGGACAT

333   TCCCCACGGGCGACCGTG
332   CCCTCACCGGTGACGGTG
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FIG. 5. Nucleotide sequence of cloned cDNA corresponding to the CB3 virus 5′ terminus. The sequence was compared with the corresponding sequence from poliovirus type 1 (PV1) (25). Matching nucleotides are indicated with vertical lines. Deleted nucleotides are indicated with points. Sequence was determined for the insert from pCB3-M1 (20).

is the synthesis of a plus strand transcript, which then serves as a messenger for the synthesis of virus-directed proteins—e.g., viral replicase (for a review, see ref. 27). However, it is not known how the cloned cDNA gains a promoter that allows the start of transcription by cellular DNA-dependent RNA polymerase. It has been proposed that RNA synthesis may initiate at one or more areas in the plasmid DNA, which may function as a promoter *in vitro* (24). Alternatively, promoter activity could be provided by integration next to a cellular promoter. The RNA molecules made in this way would probably represent oversized transcripts with extra nonviral sequences at both ends. Precisely how these molecules could replicate remains of focal interest.

Conventional methods used in enteroviral diagnostics—e.g., neutralization assays—have a serious drawback caused by the unusual high mutation rate of enteroviral genomes, which was described to be as high as 10^{-4} (28). It has been observed that certain variants of the same strain are only poorly neutralized by the antiserum of the originally characterized (prototype) strain (23). This could be a general problem in diagnosing any of the so far known 72 antigenically different human enteroviruses. To bypass this problem it appears feasible to use cloned CB3 virus cDNA as a probe for rapid detection of enteroviral-related sequences from a variety of clinical specimens. Cross hybridization experiments (data not shown) using a slot blot assay (19) indicate that the complete CB3 virus cDNA can be used as a sensitive probe for the detection of the main etiologic agents of human viral heart disease—e.g., Coxsackie B, Coxsackie A, and echoviruses. Because of the remarkably high degree of sequence homology among different members of the large human enterovirus group, detection of enteroviral infection appears to be possible by using just one cloned enteroviral cDNA as a probe. Thus, the use of cloned CB3 cDNA should provide a valuable diagnostic tool because from the clinical point of view the exact typing of an etiologically implicated enteroviral strain appears to be of secondary importance and can be carried out later by using standard virological techniques. Moreover, the cloned CB3 virus cDNA described here can be used for the study of the pathogenesis of human enteroviral heart disease. We are especially interested in the question of whether or not persistence of viral or viral-related sequences is etiologically implicated in chronic and progressive cardiac disorders—e.g., dilated cardiomyopathy.

The nucleotide sequence of the infectious CB3 virus cDNA described here will be completed in the near future. This information will enable us to specifically mutagenize the cloned CB3 virus cDNA, which will allow genetic analysis of the virus—e.g., with respect to virulence and attenuation or cardiotropism. Moreover, new constructions involving eukaryotic expression vectors and *in vitro* mutagenized viral sequences can be designed for production systems of distinct viral proteins, which might ultimately lead to the development of polyvalent enteroviral vaccines. Creating picornavirus chimeras by *in vitro* recombination of this cloned CB3 cDNA with parts of other cloned picornavirus genomes (17, 25, 29–31) may explain species and tissue specificity of structurally and functionally related viral proteins. For example, it will be of interest to learn whether the RNA replicase or viral protease—e.g., of poliovirus (17, 25, 30)—can substitute the RNA replicase or protease of CB3 virus.

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