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Molecular Detection and Identification of Enteroviruses Using Enzymatic Amplification and Nucleic Acid Hybridization

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Analysis of enteroviral genomes has revealed that the 5′ nontranslated region is highly conserved, providing consensus sequences for the design of oligonucleotides which should anneal to most, if not all, human enteroviral RNAs. We designed and used a pair of such generic primers to enzymatically amplify cDNA from coxsackievirus group B types 1 through 6, poliovirus types 1 through 3, 4 coxsackievirus A types, and 29 echoviruses. The polymerase chain reaction (PCR) products generated with these enteroviral primers were analyzed by agarose gel electrophoresis, Southern blotting, or slot blot hybridization. A genotype-specific PCR was used to detect coxsackievirus B3, to the exclusion of other enteroviruses, by using a coxsackievirus B3 genome-specific primer pair that was derived from sequences coding for part of a capsid protein. A technique is demonstrated by which individual genotypes, for which no sequence information is known, can be identified by high-criterion hybridization analysis following amplification with generic enterovirus PCR primers.

Human enteroviruses (*Picornaviridae*) are established or suspected etiologic agents in numerous diseases and are widespread in the population (22). The prototypic enteroviral genome has been defined by extensive analyses of poliovirus (PV) (15, 19, 27, 37, 41) and coxsackievirus group B (CVB) (9, 12, 14, 21, 46, 47) genomes: positive sense, single-stranded, polyadenylated at the 3′ terminus, approximately 7.4 kilobases (kb) in length, and nontranslated sequences at both the 5′ and 3′ termini (17, 24, 25, 45). Numerous hybridization studies have demonstrated that enteroviruses share sufficient nucleotide sequence homology so that probes which are capable of detecting many other enteroviral RNAs can be designed from one viral genome (2, 5, 13, 31, 42–44, 48, 49). The polymerase chain reaction (PCR) (32, 33) has made possible the sensitive detection of specific sequences in the presence of many other nucleic acids to an extent that has not been possible by nucleic acid hybridization alone (20).

Because of the significant health hazards posed by enteroviruses and because a rapid and specific detection and identification system would be a useful supplement to clinical enterovirus diagnostic procedures, we designed specific oligonucleotide primers to investigate the potential use of PCR and nucleic acid hybridization as diagnostic approaches for the identification of enteroviruses. Enteroviral diagnosis is performed by using propagation of virus in cell cultures followed by analysis with neutralizing serum pools, a process which is not rapid and which is costly and often futile: many enteroviruses (notably, coxsackieviruses of the A group [CVA]) do not replicate well or at all in cell culture, and mutable genomes enable neutralizing escape mutants to occur at high rates (29–31).

We demonstrate here the utility of three generic enterovirus oligonucleotides for the detection of numerous human enteroviruses through PCR amplification and hybridization analysis, and we evaluate two approaches for the detection and identification of specific enteroviruses. The techniques outlined in this report should be directly applicable to the evaluation of clinical and environmental samples for the presence of these viruses and useful in the design of more rapid enteroviral diagnostic strategies.

(A preliminary report of part of this study was presented at the sixth meeting of the European Study Group on the Molecular Biology of Picornaviruses, September 1989, Bruges, Belgium.)

MATERIALS AND METHODS

Viruses. The viruses (CVBs, CVAs, echoviruses [EVs], and PVs) used in this study were obtained either from the American Type Culture Collection (Rockville, Md.) or from clinical virology laboratories (1). Viruses were propagated in HeLa or RD (human rhabdomyosarcoma) cell monolayer cultures. Cells were infected with a multiplicity of infection of >10 50% tissue culture infective doses per cell and were harvested by trypsinization when cytopathologic effects were severe (≥3+).

Nucleic acids. Viral RNA was obtained by the isolation of total nucleic acids from cell pellets of infected monolayer cultures. Approximately 4×10^6 to 6×10^6 cells (a single T-25 flask) per virus were lysed in 50 mM NaCl–20 mM Tris hydrochloride (pH 7.5)–50 mM EDTA–1% sodium dodecyl sulfate, extracted three times with phenol-chloroform and once with chloroform, and then precipitated from 2.5 M ammonium acetate with ethanol. Nucleic acid pellets were washed with 70 and 95% ethanol, dried, and suspended in 100 μl of sterile distilled water. DNA was not removed. Preparations were stored at −75°C.

Oligonucleotides. Oligonucleotide primers were purchased from either Genetic Design (Houston, Tex.) or Operon Technologies (San Pablo, Calif.). Primers were suspended in water and twice precipitated from 2.5 M ammonium acetate with ethanol. Concentrations of primers were determined by spectrophotometry, adjusted to approximately 50 units/ml in water at 260 nm, and stored at −20°C. Oligonucleotides were used without gel or high-pressure liquid chromatography purification. The generic enterovirus oligonucleotides, E1

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through E3, were derived from conserved sequences in the 5'-nontranslated region of the enterovirus genome, while the CVB3 genome-specific primers, B3-1 and B3-2, were derived from the VP2 capsid protein-coding region of CVB3 (see Fig. 1). Enzymatic amplification of enteroviral cDNA with the E1 and E2 primers resulted in the synthesis of a 196-bp fragment, whereas the B3-1 and B3-2 oligonucleotides primed the synthesis of a 91-bp fragment from CVB3 cDNA. Because of the high potential variability in the B3-1- and B3-2-primed fragments (see below), no detection primer was designed for use with this primer pair.

Synthesis of cDNA and enzymatic amplification. cDNA synthesis was performed essentially as described previously (44) in 50-μl volumes containing 1 μl of viral RNA (the equivalent of ca. 2×10^4 cells), 7 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.), and 0.1 unit/ml at an optical density of 260 nm of E1 or B3-1 primer. Reactions proceeded at 40°C for 20 min and then were precipitated directly by adding ammonium acetate to 2.5 M and ethanol. The precipitate was washed in 95% ethanol, dried, suspended in 50 μl of sterile distilled water, and stored at −75°C.

Enzymatic amplification of cDNA was performed in 25-μl volumes containing 1.5 mM $MgCl_2$, 10 mM Tris hydrochloride (pH 7.5), and 0.05% Tween 20 and 0.05% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.); 5 μl of cDNA; 0.2 mM each dATP, dGTP, dTTP, and dCTP (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.); 0.1 unit at an optical density of 260 nm each of either E1 and E2 or B3-1 and B3-2 per ml; and 25 U of *Thermus aquaticus* (*Taq*) DNA polymerase (AmpliTaq; Perkin Elmer Cetus, Norwalk, Conn.) per ml. Amplifications were performed in a DNA thermal cycler (Perkin Elmer Cetus), as follows: following heat denaturation for 1 min at 94°C, 10 cycles (one cycle = 94°C for 1 s; 50°C for 1 s; and 72°C for 1 s) and then 40 cycles (one cycle = 94°C for 1 s and 50°C for 1 s). Lag times in the heating and cooling of the cycler permit approximately 8 to 12 s at each temperature under these conditions. Because of the extreme rapidity of nucleotide incorporation by *Taq* polymerase between 50 and 72°C (10), fragments in excess of 1 kbp can be synthesized with the appropriate primers under these conditions (S. Tracy, unpublished data).

Agarose gel electrophoresis and Southern and slot blots. A total of 5 to 10 μl of a PCR reaction mixture was electrophoresed at 100 V into 1% agarose gels in 0.2× TAE (1× TAE is 40 mM Tris acetate [pH 7.8] and 1 mM EDTA). Gels were stained in ethidium bromide to detect DNA by illumination with UV light (302 nm). Gels were immersed in 0.5 M NaOH–1.5 M NaCl for 30 min at room temperature, and the DNA was transferred (36) onto membranes (Nytran 45; Schleicher & Schuell, Inc., Keene, N.H.) by Southern blotting in 0.05 M NaOH–1 M NaCl. For slot blot analysis, portions of PCR reaction mixtures were denatured in 1 M NaOH, neutralized with 200 μl of 150 mM sodium phosphate buffer (pH 6.8)–3 M NaCl, and blotted onto nitrocellulose membranes (BA-85; Schleicher & Schuell). Membranes were baked at 80°C for 2 h under vacuum.

Blot hybridization with radioactive oligonucleotide probes. Oligonucleotides were labeled with ^{32}P by using T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) (38). Blots were prehybridized and hybridized in 500 mM NaCl–0.1% (wt/vol) dry milk–5 mM EDTA–10 mM sodium phosphate buffer (pH 6.8)–0.1% sodium dodecyl sulfate at 37°C for 2 to 4 h. Blots were washed as described previously (4) in a covered container in 3 M tetramethylammonium chloride. Blots were exposed for various periods of time to X-ray film at −75°C with intensifying screens.

Hybridization analysis with oligo-labeled probes. The 196-bp fragments obtained from amplification reactions with the E1 and E2 primers were excised from 1% agarose gels and purified by using glass powder and potassium iodide (Gene Clean; Bio101, Inc., San Diego, Calif.). Approximately 10 to 50 ng of DNA was labeled with ^{32}P by using the E1 and E2 primers and Klenow DNA polymerase (Pharmacia LKB Biotechnology, Inc.) (6). Labeled DNA was purified by Sephadex G-50 chromatography. Hybridization was performed in the buffer that was used for oligonucleotide hybridization at the temperatures noted in the text. Blots were washed in 30 mM NaCl at 62°C and exposed to X-ray film as described above.

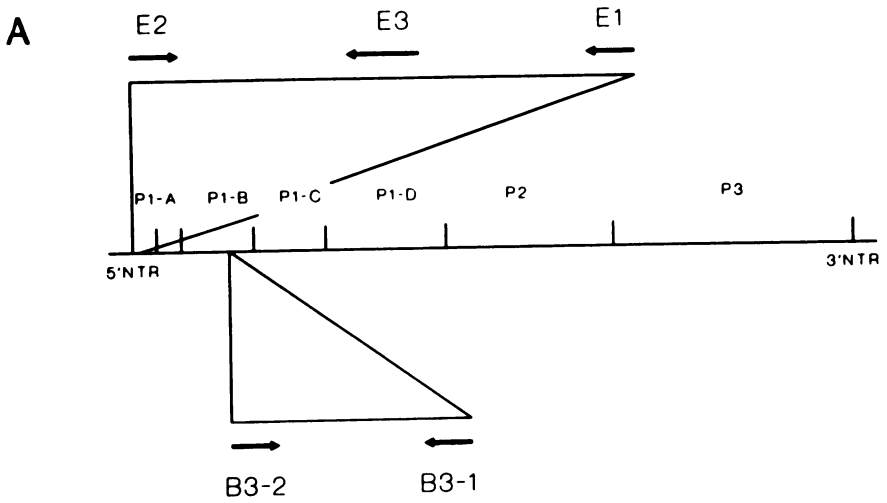
Nucleotide sequence analysis. Enzymatic amplification products from E1 and E2 amplifications of CVAs A16 and A21 and EVs 25 and 34 were electrophoresed into 1% agarose and purified as described above. The 196-bp fragments were ligated into the *Eco*RV site of the pBluescript plasmid vector (Stratagene, San Diego, Calif.) and transformed into an *Escherichia coli* K-12 strain, DH5α, by using the dimethyl sulfoxide-dithiothreitol method (7). Four clones from each transformation were picked, and plasmid DNA was isolated from the minilysates (16). The DNA was sequenced by using modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio) and the dideoxynucleotide method (34, 39) by using only the cytosine reaction to determine those clones that were identical. Two clones for each virus were then fully sequenced.

RESULTS

Characterization of generic enterovirus primers E1 and E2. The sequences of the E1 and E2 oligomers and their locations in the enterovirus genome are shown in Fig. 1A and B. The E1 primer primes cDNA synthesis from the virion (plus) strand of RNA. The location of the E3 oligomer, a hybridization probe without homology to the E1 or E2 primer, is between the E1 and E2 primers. Comparison of the sequences of E1, E2, and E3 demonstrates the extreme conservation among the known enteroviral sequences (Fig. 1B).

Viral RNAs representing the four enteroviral groups were used as templates to synthesize cDNA with the E1 primer and reverse transcriptase. The cDNAs were then amplified as described in Materials and Methods by using both E1 and E2. Results of a typical enzymatic amplification are shown in Fig. 2. All enteroviruses tested, with the exception of EV22, were readily detected by the appearance of the expected 196-bp fragment on electrophoretic analysis. The control from uninfected HeLa cells was clearly negative, demonstrating the specificity of the primers for prototypic enteroviral sequences. The inability of these primers to detect EV22 is consistent with other data (11, 30, 35, 40; B. A. Coller, N. M. Chapman, and S. Tracy, unpublished data) which demonstrate that EV22 is atypical of the enteroviral genomic paradigm.

We screened various enteroviruses for their ability to be detected following E1 and E2 amplification by slot blot hybridization analysis (Fig. 3A). Of 41 enteroviruses, 40 were detectable in this manner; as described above, only the EV22 and the negative control, normal HeLa RNAs were not detected (data not shown). The variable intensity of hybridization of the E3 probe to various enteroviral PCR products likely reflects different levels of amplification in each reaction, as only 1 μl of a 25-μl reaction volume per reaction was blotted.



B

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          5' TCCGGCCCCTGAATG 3' E2
CVB3  446 TCCGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCACACACCCTCAAGCCAGAGGGCAGTGTGTCGTAACGGGCAACT
CVB1  449 ---------------------------------------T------A-----G-------------------------
CVB4  451 ---------------------------------------GTT-G---------C-A-TG---------------------
PV1   446 ---------------------C---CT-------GGTGGT-A---A-----T-ATTG-CC---------C-----G-
PV2   447 ---------------------CA-------GG---GTGG---T-----C-AC---CC---------C-----G-
PV3   449 ---------------------CAT------GG---G-TG---C-----CA-C---CC---------C-----G-

CVA16     ---------------------------------G...---T-G---GA-----C-A-----CT----------T----------
CVA21     ---------------------CA---------AC-G-T-A---C-----T-A-----GT----------T-C-T---G-
EV25      ---------------------------------..GT---------A-----G-----TG----C-----------------
EV34      ---------------------C---CA-------AGT-TT-------A-----T-AAAT-CT----------C-A---G-

          E3  3' CCTTGGCTGATGAAACCCACAGGCACA 5'
CVB3      CTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCATTTTATTCCTATACTGGCTGCTTATGGTGACAATTGAGAGAT
CVB1      ------------------------------------------------------------------------------C---G-
CVB4      ---------G---------------------C---------T---CCT-------------------------------A-----
PV1       -C-TG--------------------------C---------.T-----TG-----------------------.C-C-----
PV2       TC-TG--------------------------C---------TT----C-A-----------------------CAT.T-----
PV3       -C-TG--------------------------C---------T-GA-.-----------------------CAT.-----

CVA16     -C-----------------------------------C-A---TCCTT---C-------------------------------A---
CVA21     ------GCT----------------------------CC------ATTC-------------------------------T-C-A---
EV25      ---------------------------------C---------C-T-----T-------------------------------A-AGA---
EV34      ------TG-----------------------------C---------T-TATA-----A-----------------------.C-T-----

                E1  3' ACCTAACGGGTAGGCCAC 5'
CVB3      TGTTACCATATGC.TATTGGATTGGCCATCCGGTG  640
CVB1      ----------T.-------------------------  643
CVB4      ----------.---------------A----------  645
PV1       ------T-----A-----GA-----------------  639
PV2       ------T-----A-----AA-----------------  641
PV3       ------T-----A-----GAG----------A-----  642

CVA16     ----------.-------------------------
CVA21     ----------.-------------------------
EV25      ----------.-------------------------
EV34      ------T-----A-----G-----------------
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C

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B3-2 5 'AACG          CTAGACAACACCCCA          TC 3'                                    B3-1 3'GGCCAGCGTAGGCCCAGGTTG 5'
CVB3 1350 AACG.....CTAGACAACACCCCA.....TCCAGTGCAGAATTGCTGGGGGGCGATACGGCAAAAGAGTTTGCGGACAAACCGGGTCGCATCCGGGTCCAAC 1440
CVB1 1353 --AT.....---A----------.....AAGTT---T---C-TTCC---A---A-----AT---T-G-AT-----CA-T-----CGGAA---G-GGA-ATCCAA-G-T 1443
CVB4 1355 C-AT.....GC---A---G-A---C.....G-GTA---GT---T---TGT---A---A---G---A-------GAGT---C...--AC-GAAT-CA---CA-A---T...---G 1439
PV1  1354 T-GCAACACCACTACCATGC-------AGCTATCAAAATG----A-C-T-GCGA-AAA---A----AC-TTCA-GGGTAC-----CA-TCCTG-CAACAA-CAGA-ATCAC-TGC- 1452
PV2  1356 CAGC...ACAACC-ACATGTTT---AAA-TATGAGAATG-A-A-C-G-GTGA-AAA-------T---ATTCAA-GGGAGT-----A-TCTGG-TA-TAA-----TA---AACC-TGCA 1461
PV3  1354 C-GTGAC...AAG-A-AGGT-----TAGTTATGCAAATG-G-A-C---GTGAAAGA-------AA-ATTTTACTCCC-A---CAACA-GG-TAAC-CA-T-A-ATCCC-A---A 1459
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FIG. 1. Amplified sequences and oligonucleotides used in this study. (A) Location in the CVB3 genome of the oligonucleotides used in this study. NTR, Nontranslated region. (B) Sequences of E1, E2, and E3 primers and comparison of nucleotide sequences from CVB1 (9), CVB3 (47), CVB4 (12), and PV1-3 (15, 19, 37) in the region amplified by the E1 and E2 primers with cloned CVA16-, CVA21-, EV25-, and EV34-amplified DNA. Because the genomes of CVA16, CVA21, EV25, and EV34 have not yet been sequenced, the location of the amplified region is not precisely known. (C) Sequences of B3-1 and B3-2 primers and comparison of nucleotide sequences in the region of the CVB3 genome amplified by these primers. Nucleotide sequence alignments are derived from the amino acid sequence alignments of Palmenberg (26). A hyphen indicates that the nucleotide at that position is identical to that in the CVB3 genome, and a period was used to maintain the alignment in genomes in which bases were deleted relative to the bases in other genomes.

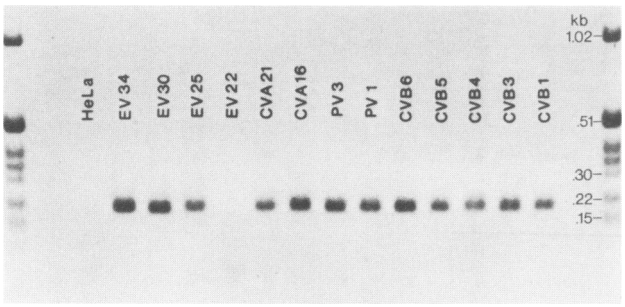

FIG. 2. E1 and E2 amplification products of E1-primed cDNAs. Portions of amplification reactions were electrophoresed into an agarose gel, stained with ethidium bromide, and photographed under UV light. Molecular size standards (in kilobase pairs [kb]) were electrophoresed in the outer lanes.

The amplification products of a similar experiment were electrophoresed into agarose and then Southern blotted and probed with the radiolabeled E3 oligomer (Fig. 3B and C). In each case in which the 196-bp fragment was observed, the E3 probe hybridized to the same band. The EV22-infected HeLa cell reaction was negative, which is consistent with previous results (Fig. 2), demonstrating that at the higher sensitivity (ca. 0.1 to 10 pg of DNA per band) of hybridization analysis, no amplification products from EV22-infected cells could be detected.

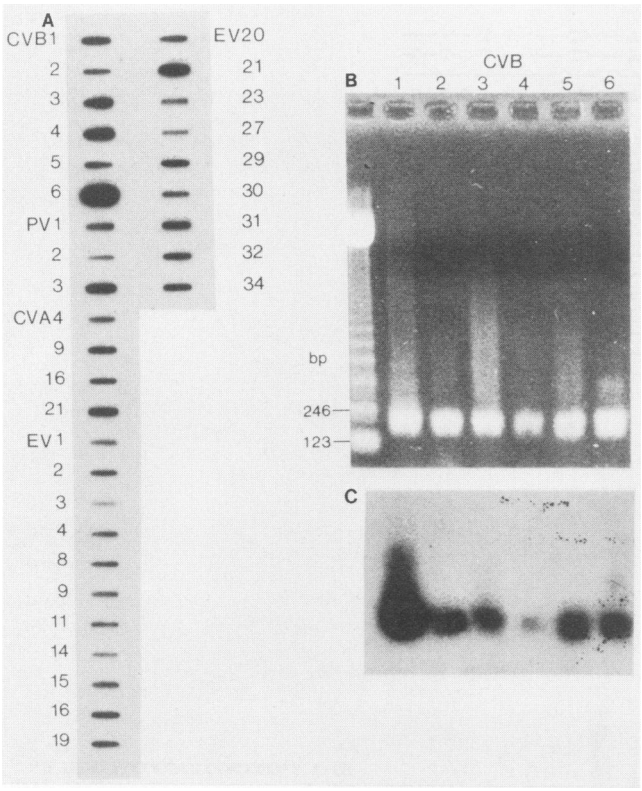

FIG. 3. Detection of E1 and E2 amplification products by hybridization with the E3 oligonucleotide. (A) Autoradiogram of a slot blot of E1 and E2 amplification products from E1-primed cDNAs hybridized with radiolabeled E3 oligonucleotide. Exposure was for 15 h at −75°C with intensifying screens. (B) E1 and E2 amplification products were electrophoresed into agarose, stained with ethidium bromide, and photographed under UV light. The left lane is a molecular size standard (123-bp DNA ladder; Life Technologies, Inc., Gaithersburg, Md.). (C) Autoradiogram of the Southern blot of the same gel shown in panel B hybridized with radiolabeled E3 oligonucleotide. Exposure was for 17 h at −75°C with intensifying screens.

To examine whether the variability in hybridization intensity observed when E3 was used as the probe (Fig. 3A) might also reflect significant sequence divergence in the region with identity to the E3 probe, we randomly chose two CVAs (CVA16 and CVA21) and two EVs (EV25 and EV34) for sequence analysis. Sequences were derived from two clones of each virus and were compared with similar sequences of other known enteroviruses (9, 12, 15, 19, 37, 47) (Fig. 1B). The 27-nucleotide sequence corresponding to E3 was almost completely conserved in the four enteroviral genomes, although significant nucleotide changes were observed elsewhere in the sequence. In each case, the two independently derived sequences of each virus were identical. Therefore, while it is possible that the E3 sequence may not be entirely conserved in other enteroviral genomes, thereby giving rise to variable hybridization signals (Fig. 3A), the sequence analysis data from four randomly chosen enteroviruses are consistent with a high degree of, if not perfect, conservation in this nucleotide tract.

Characterization of the CVB3 genome-specific oligonucleotides B3-1 and B3-2. The locations in the CVB3 genome of oligomers B3-1 and B3-2 and their sequences are shown in Fig. 1A and C, respectively. A computer search of the closely related CVB1 and CVB4 genomic sequences (9, 12, 47) as well as those of PV1-3 (15, 19, 37) detected only partial identity in this region (Fig. 1A). The sequence amplified by this primer pair coded for amino acids 136 to 165 of the VP2 capsid protein (P1-B) of CVB3. In this region, which is part of the VP2 "puff," there is little conservation of the amino acid sequence among the three sequenced CVB serotypes; and the sequence varies between groups of picornaviruses (8, 26, 28).

We synthesized cDNA from the RNA genomes of five CVB3 isolates, as well as from four other enteroviruses, using B3-1 as the primer, and then amplified the cDNA using both B3-1 and B3-2 primers. Electrophoresis of the PCR products in an agarose gel demonstrated that (i) only the CVB3 genomes (Fig. 4A, lanes 1 through 5) permitted generation of the expected 91-bp fragment and (ii) the four non-CVB3-infected and uninfected HeLa cell nucleic acids did not contain sequences which could be amplified and detected by this method (Fig. 4A, lanes 6 through 10). Although the amplification products in lanes 1 and 2 in Fig. 4 were from laboratory strains of CVB3, the amplification products in lanes 3 through 5 in Fig. 4 represent independent human CVB3 isolates from the years 1956, 1962, and 1965 which were not routinely passaged in cell culture. Similar amplifications were performed with E1-primed cDNA and both E1 and E2 primers in order to ascertain that all of the enteroviral samples contained amplifiable RNA (Fig. 4B). This control demonstrated that enteroviral RNA is present in all viral samples, and therefore, the inability of B3-1 and B3-2 to prime the synthesis of a detectable DNA product in the non-CVB3 genomes was due to the lack of sequence homology between the primers and non-CVB3 RNA.

Detection of specific genotypes following amplification with generic enteroviral primers by high-criterion hybridization. Amplification of many, if not all, enteroviruses with the E1 and E2 primers permitted the detection of an enteroviral genome but provided no information on the identity of the

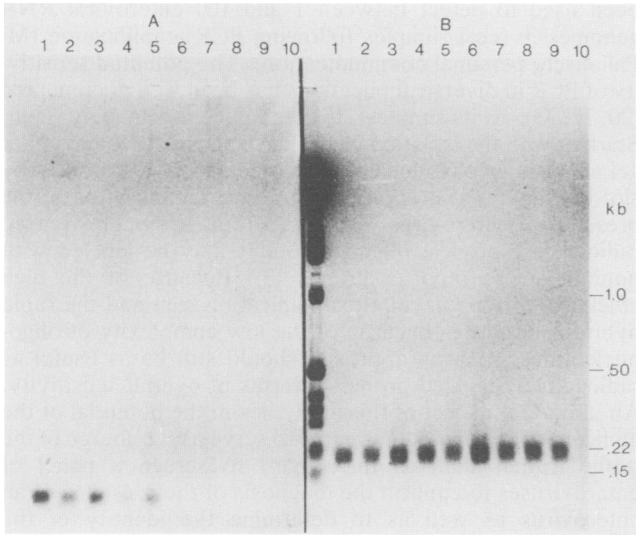

FIG. 4. B3-1 and B3-2 primers can be used for priming cDNA and enzymatic amplification from CVB3 genomes to the exclusion of other enteroviruses. Amplification products were electrophoresed into agarose gels, stained with ethidium bromide, and photographed under UV light. (A) B3-1- and B3-2-primed amplification products from B3-1-primed cDNAs. (B) E1- and E2-primed amplification products from E1-primed cDNAs. Lanes 1, CVB3-M (myocarditic variant in mice); lanes 2, CVB3-ST (amyocarditic variant in mice); lanes 3, CVB3-GA (human isolate, 1956); lanes 4, CVB3-RE (human isolate, 1962); lanes 5, CVB3-OL (human isolate, 1965); lanes 6, CVB4; lanes 7, PV1; lanes 8, CVA16; lanes 9, EV25; lanes 10, uninfected HeLa cells. Molecular size standards appear in the center, with lengths (in kilobase pairs [kb]) given on the side.

specific enterovirus (genotype) detected. Genome-specific primers such as B3-1 and B3-2 are limited in their usefulness to special cases and, in any event, require sequence information for their design. Therefore, we asked whether a target blot containing the E1 and E2 PCR products from many different enteroviral genomes, when probed with the E1 and E2 PCR product from a known enterovirus under stringent hybridization conditions, could identify the specific (probe) virus.

Enzymatic amplifications were performed by using the E1 and E2 primers and E1-primed cDNAs from CVB1-, CVB3-, CVA21-, PV3-, EV25-, and EV31-infected cell nucleic acids. Two microliters from each PCR reaction mixture were applied to each blot. Identical slot blot panels were then probed with the E1 and E2 amplification product of CVB1 (Fig. 5A), CVB3 (Fig. 5B), or CVA16 (Fig. 5C) in 100 mM NaCl (see Materials and Methods) and at 70, 76, 80, or 83°C. Some differences in the intensity of the signal were due to unequal amounts of DNA in the slot blot or in the probe because PCR amplification products were not matched with regard to the quantity of DNA that was used. As expected, both CVB1 and CVB3 probes hybridized to other viral sequences at 70 and 76°C. However, only genotype-specific hybridization reactions were observed when hybridization was performed at 83°C; the CVB1 probe detected only the CVB1 PCR product, and the CVB3 PCR product detected only the CVB3 sequences. Because the CVB1 and CVB3 E1 and E2 sequences differed from each other by only 6 nucleotides (3.1%; Fig. 1B), these data indicate that this approach is capable of differentiating between highly related sequences. Hybridization of a similarly prepared CVA16 probe demonstrated the expected sequence identity at lower

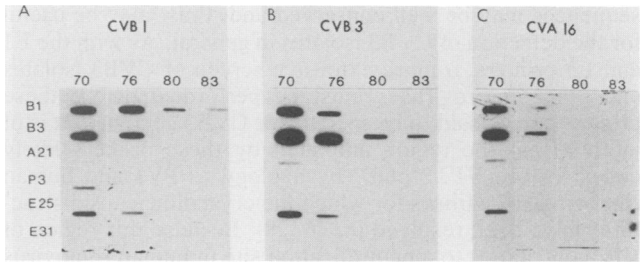

FIG. 5. High-criterion hybridization identification of enteroviral sequences. Autoradiograms of slot-blotted E1 and E2 amplification products of CVB1 (B1), CVB3 (B3), CVA21 (A21), PV3 (P3), EV25 (E25), and EV31 (E31) hybridized with the radiolabeled E1 and E2 amplification product of CVB1 (A), CVB3 (B), and CVA16 (C) at 70, 76, 80, and 83°C. Exposure was for 14 h at −75°C with intensifying screens.

hybridization temperatures but no detectable hybridization to the different serotype sequences on the blot at 83°C (Fig. 5C), further demonstrating the specificity of this approach.

DISCUSSION

We examined two sets of primers for use in the identification of enteroviruses using PCR (Fig. 1). One set, E1 and E2, was derived from a highly conserved region of the 5′ nontranslated region of the enterovirus genome. These primers were capable of amplifying viral cDNAs from the six CVB serotypes, all three PV serotypes, four (of four tested) CVA serotypes, and 28 of 29 EV serotypes tested (Fig. 2 through 4). That these primers were able to anneal to and prime cDNA from a broad spectrum of enteroviral genomes strongly suggests that these primer sequences can be useful for the detection of all human enteroviruses. Because this method does not require virus propagation in cell culture, the application of these techniques to the detection of those enteroviruses which can only be propagated in mice (such as many CVAs) is an important new tool.

Our observation that EV22, the only one of the 41 enteroviruses examined, was not detected by the E1 and E2 primers is consistent with other data which suggest that EV22 does not correspond to the enteroviral paradigm (11, 35, 40). EV22 RNA is not detectable with either CVB3 or PV1 sequences, both of which are prototypic of the enteroviral genome (30). Work from our laboratory also suggests that the biology of EV22 is quite different from that of other human enteroviruses, in that EV22 does not shut off host cell protein synthesis and the viral RNA is efficiently translated in vitro (B. A. Coller, N. M. Chapman, and S. Tracy, unpublished data). Thus, the PCR data suggest that these group-specific amplification primers may also be useful in enteroviral taxonomic studies.

We designed another primer set (B3-1 and B3-2) which appeared to be specific to the CVB3 genome when it was compared by computer analysis with known enteroviral sequences (Fig. 1C). We tested the specificity for CVB3 genomes by synthesizing cDNA with the B3-1 primer from CVB3 RNAs and from other enteroviral RNAs and then amplifying the cDNA products with both B3-1 and B3-2 primers (Fig. 4). The B3-1 and B3-2 primer set was unable to prime the expected 91-bp product from non-CVB3 CVB RNAs. The fact that these primers promoted the synthesis of the 91-bp fragment from two laboratory strains of CVB3 and from three naturally occurring human CVB3 strains isolated over a decade (1956 to 1965) suggests that the primer

sequences may be well conserved and, thus, may be useful for the detection of CVB3 isolates in general. As with the E1 and E2 primers, a more extensive screen of CVB3 isolates and other enteroviruses must be performed before these primers can be said to be specific for CVB3 genomes. It is of interest that the region amplified by these primers likely codes for the VP2 "puff" by analogy to PV1 and human rhinovirus 14, viruses for which the crystalline capsid structures have been resolved (8, 26, 28). Because this region of VP2 contributes to a neutralization site in human rhinovirus 14 and PV1, it is likely that sequences in this region are conserved within a serotype and vary between serotypes. Similar comparisons of sequences of other enteroviruses might provide candidate genotype-specific primers for specific amplifications of other selected enteroviruses.

The advent of enzymatic amplification techniques (32, 33) has made possible the potential detection of one molecule of a specific nucleotide sequence (20). It has been demonstrated (32) that enzymatic amplification can detect RNA molecules that are present at a frequency of as low as 0.01% in a cDNA preparation. Murakawa et al. (23) showed that cycling with reverse transcriptase and DNA polymerase I can detect as little as 10^{-21} mol of RNA (approximately 100 copies) after only 21 amplification cycles. Recently, another enzymatic amplification procedure was described which uses both reverse transcriptase and bacteriophage RNA polymerase to achieve high-efficiency amplification of an RNA sequence (18). Enzymatic amplification techniques will permit the detection of virus at far lower concentrations than was previously possible.

We focused our attention, therefore, on devising a means by which the power of the sensitivity of a PCR could be used simultaneously to detect and identify any enterovirus by using generic enterovirus PCR primers. The generic enterovirus PCR primers described here permit the detection of enteroviral RNA but do not specifically identify the virus. Although the sequence amplified with the E1 and E2 primers was similar among many enteroviral genomes (Fig. 1B), significant differences existed as well. Thus, we reasoned that by exploiting sequence differences and maximizing these differences under stringent hybridization conditions, we might observe the specific and exclusive hybridization of the E1 and E2 fragment from a specific virus to its homolog on a slot blot. In this manner, a slot blotted panel of enteroviral sequences derived from E1- and E2-primed amplifications potentially could be used to identify an unknown clinical isolate. To test this approach, radiolabeled E1 and E2 fragments of CVB1 and CVB3 were used to probe identical slot blot panels containing the E1 and E2 amplification products of CVB1, CVB3, and four other enteroviruses at a range of temperatures (Fig. 5). The CVB1 and CVB3 sequences were specifically chosen for this test, as they differ in this region by only 6 nucleotides (6 of 196 nucleotides, or 3%). The data demonstrate that each probe was specific for its homolog at 83°C, but, as expected, each probe could detect other viruses in addition to its homolog at lower temperatures. These experiments are reminiscent of previous work in which manipulation of hybridization criteria was used to examine the genomic identity of closely related enteroviruses (2, 42, 44). As a first approximation, these experiments suggest a practical approach to the simultaneous detection of enteroviral RNA and the identification of the viral genotype.

We demonstrated in this report molecular approaches for enterovirus detection and identification which should be useful for clinical specimens. A similar set of primers has been used to detect between 1 and 100 enteroviral RNA genomes in fecal samples following PCR amplification (M. Pallansch, personal communication). The potential sensitivity of PCR in diverse applications has been well documented (20, 32, 33). At its simplest, the technique is relatively rapid. Starting with the isolation of nucleic acids by PCR and either gel analysis or oligonucleotide probing of PCR products by slot blot analysis, it is possible to have confirmation of the presence of enterovirus within 8 to 12 h. Although we used radiolabeled probes, oligonucleotides may be labeled with nonradioactive reporter groups (3). Because of the high efficiency of the enzymatic amplification step and the rapid hybridization rates because of the low complexity of oligonucleotides, such an approach should still be as useful as radioactively labeled probes in terms of overall sensitivity. An important aspect of this study lies in the potential of the clinical or environmental sample to serve as the source of the probe (rather than as the target) to screen a panel of enteroviruses to confirm the diagnosis of the presence of an enterovirus as well as to determine the identity of the enterovirus(es) in the sample. Because a single sample containing DNA, RNA, or both can be used as a template for multiplex amplification (the simultaneous amplification of several different sequences by using different primer sets [20]), it is intriguing to consider the possibilities of this approach for the simultaneous detection and identification of different viruses and microorganisms in a single specimen.

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