JOURNAL OF VIROLOGY, June 1990, p. 2692–2701
0022-538X/90/062692-10$02.00/0
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Vol. 64, No. 6

Echovirus 22 Is an Atypical Enterovirus

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Received 2 November 1989/Accepted 1 March 1990

Although echovirus 22 (EV22) is classified as an enterovirus in the family *Picornaviridae*, it is atypical of the enterovirus paradigm, typified by the polioviruses and the coxsackie B viruses. cDNA reverse transcribed from coxsackievirus B3 (CVB3) RNA does not hybridize to genomic RNA of EV22, and conversely, cDNA made to EV22 does not hybridize to CVB3 genomic RNA or to molecular clones of CVB3 or poliovirus type 1. EV22 cDNA does not hybridize to viral RNA of encephalomyocarditis virus or to a molecular clone of Theiler's murine encephalomyelitis virus, members of the cardiovirus genus. The genomic RNA of EV22 cannot be detected by the polymerase chain reaction using generic enteroviral primers. EV22 does not shut off host cell protein synthesis, and the RNA of EV22 is efficiently translated in vitro in rabbit reticulocyte lysates. Murine enterovirus-immune T cells recognize and proliferate against EV22 as an antigen in vitro, demonstrating that EV22 shares an epitope(s) common to enteroviruses but not found among other picornaviruses.

The enteroviruses constitute a large genus in the family *Picornaviridae*, and much information is available regarding their biology (3, 31), replication (33, 35, 44), and structure (1, 17, 29, 40, 57). The three serotypes of poliovirus (PV1 to PV3) (30, 37, 54, 70, 74), three of the six coxsackievirus B serotypes (CVB1, CVB3, and CVB4) (23, 28, 38, 79), and two of the coxsackievirus A serotypes (CVA16 and CVA9) (7, 19) have been molecularly cloned and sequenced. Limited sequence information is available as well from various other enteroviruses (15, 82). These data permit description of the prototypic enterovirus (46, 47, 53, 77). The enteroviral genome is approximately 7,450 nucleotides long, consisting of a long (ca. 2,200 codons) open reading frame flanked by nontranslated sequences. The RNA is polyadenylated at the 3′ terminus, terminated at the 5′ end with a covalently linked small, virus-encoded protein, and translates poorly in vitro in rabbit reticulocyte lysates (RRL) (6, 66). Enteroviruses shut off host cell protein synthesis after infection (18, 56). Numerous studies (8, 21, 58, 76) have demonstrated that enteroviral genomes share sufficient nucleotide identities to enable them to be detected by hybridization with probes derived from individual enteroviral genomes. Recent work has demonstrated that enteroviruses share a group-specific antigenic epitope which is recognized by enterovirus-immune T cells (2).

Previous reports suggested that echovirus 22 (EV22) may not be a typical enterovirus. Microscopic studies demonstrated that the cytopathic effects (CPE) induced by EV22 and EV23 replication were distinct from those caused by other echoviruses (65), and Jamison (25) corroborated earlier findings (11, 83) that EV22 affected nuclear pathology. Tamm and Eggers (73) showed that EV22, EV23, and EV28 were not inhibited during replication by guanidine hydrochloride and 2-(alpha-hydroxylbenzyl)benzimidazole. Of 29 enterovirus serotypes examined for growth in RD cells, only EV22 and EV23 failed to replicate (81). Recently, EV22 RNA has been shown to possess what appears to be a large 5′ hairpin on the basis of its ability to resist pancreatic ribonuclease and nuclease P-1 digestion (62). In the course of characterizing enteroviral nucleic acid sequences as generic hybridization probes for the detection of a wide variety of enteroviral genomes, we and others have observed that EV22 RNA cannot be detected by these sequences (9, 58; C.-F. Yang and M. A. Pallansch, unpublished data). We report here the results of a molecular and biologic characterization of EV22 which suggests that EV22 has diverged significantly from the enterovirus paradigm.

MATERIALS AND METHODS

Virus. Two strains of EV22 were used in this study. EV22 (Omaha) is an isolate from human stool (Omaha, Neb.) typed at the University of Nebraska Medical Center as EV22 by using neutralizing antibody pools (42) and confirmed by virus neutralization using two independently prepared (rabbit and horse) polyclonal antisera. EV22 (Harris), obtained from the American Type Culture Collection, Rockville, Md., is the prototypic EV22 isolate. CVB1, -B2, -B4, -B5, -B6, PV1 (Sabin), and encephalomyocarditis virus (EMCV) were obtained from the American Type Culture Collection. CVB3 was described by Tracy et al. (78), and EV34, CVA16, and CVA21 were obtained from T. Smith (Section of Clinical Microbiology, Mayo Clinic, Rochester, Minn.). Poliovirus type 3 (PV3) is an isolate typed at the University of Nebraska Medical Center (Omaha, Neb.). High-titer, purified Mengo virus and Theiler's murine encephalomyelitis virus (TMEV) preparations were obtained from A. Palmenberg (University of Wisconsin, Madison) and H. Lipton (Northwestern University, Chicago, Ill.), respectively. Influenza A/Bangkok/1/79 virus was obtained from Flow Laboratories (McLean, Va.). All picornaviruses, with the exception of Mengo virus and TMEV, were propagated in monolayer HeLa cell cultures in minimal essential medium–10% fetal calf serum–50 μg of gentamicin per ml in 5% CO_2 at 37°C.

Viral RNA preparation. HeLa cells were infected with CVB3, EMCV, or either strain of EV22 at a multiplicity of infection (MOI) of ca. 10 to 50 50% tissue culture infective doses ($TCID_{50}$) per cell. After lysis overnight at 37°C, the cultures were frozen and thawed three times and then cleared of cellular debris by centrifugation for 15 min at

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25,000 rpm (85,000 × *g*) in an SW28.1 (Beckman Instruments) rotor. Virus was collected with polyethylene glycol (43) and suspended in 100 mM NaCl–10 mM $MgCl_2$–50 mM Tris, pH 7.5. Boiled RNase A (Sigma Chemical Co.) and DNase I (RQ1; Promega Biotec) were added to each preparation at 0.5 mg/ml and 5 U/ml, respectively, followed by incubation at 37°C for 1 h. Viral preparations were then pelleted through 30% (wt/vol) sucrose–1 M NaCl–50 mM Tris hydrochloride, pH 7.5, in an SW41 rotor (Beckman) at 38,000 rpm (180,000 × *g*) for 16 h at 8°C. The virus was suspended in 100 mM NaCl–10 mM dithiothreitol containing 10 U of the ribonuclease inhibitor Inhibit Ace (5 Prime-3 Prime, Inc.). The virus was pelleted through sucrose as before, suspended in 50 mM NaCl–10 mM Tris hydrochloride, pH 8.3–10 mM EDTA, to which proteinase K (BMB, Inc.) at 200 μg/ml and sodium dodecyl sulfate (SDS) (1% [wt/vol]) were then added. After incubation at 45°C for 1 h, the mixture was phenol-chloroform and chloroform extracted. Highly purified oyster glycogen (30 μg/ml) (75) was added as a carrier, and the viral RNA was ethanol precipitated in the presence of 2.5 M ammonium acetate.

Analysis of virion capsid proteins. HeLa cells were infected with CVB3 or EV22 (Omaha) at an MOI between 0.1 and 1 $TCID_{50}$ per cell. After replacement with medium lacking cysteine and methionine (Select-Amine Kit; GIBCO), the infection was allowed to proceed for 6 h before addition of 50 uCi of [^{35}S]cysteine-methionine (Tran^{35}S-label, >1,000 Ci/mmol; ICN Radiochemicals). When lysis of the cells was complete, cultures were frozen and thawed three times and cleared of cellular debris by centrifugation at 40,000 rpm (150,000 × *g*) in an SW50.1 rotor (Beckman) for 10 min. Labeled virions were pelleted through a 30% (wt/vol) sucrose cushion as described above, suspended in reducing sample buffer (36), and heated to 100°C for 5 min. Virion proteins were analyzed on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels (36) which were stained with Coomassie brilliant blue R-250 (Fisher), treated with Autofluor (National Diagnostics), and dried in vacuo. Fluorography was performed using XAR-5 film (Kodak) at −75°C with intensifying screens.

Radioactive probe synthesis. cDNA was transcribed as described previously (78), using avian myeloblastosis virus reverse transcriptase, [α-^{32}P]dCTP (800 Ci/mmol; Amersham Corp.), viral RNA, and random oligonucleotide primers (Pharmacia). The labeled cDNA was isolated by Sephadex G50 (Pharmacia) chromatography and then collected by ethanol precipitation. Before hybridization, the cDNA was suspended in 100 mM NaOH and incubated at 68°C for 30 min to degrade template RNA.

Oligonucleotide primers (0.01 to 0.05 optical density at 260 nm units) were ^{32}P labeled, using [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Bethesda Research Laboratories) as described elsewhere (41). Labeled oligonucleotides were ethanol precipitated and suspended in water before hybridization.

cDNA clones, previously linearized and heat denatured, were ^{32}P labeled, using [α-^{32}P]dCTP (800 Ci/mmol; Amersham), random oligonucleotide primers (Pharmacia), and 3 to 6 U of the large fragment of DNA polymerase I (Klenow; U.S. Biochemical Corp.) as previously described (16). Labeled DNA was twice precipitated in ethanol. The labeled DNA was suspended in 50 μl of 100 mM NaOH and heated to 68°C for 3 min before hybridization.

Nucleic acid blot preparation and hybridization. Genomic viral RNAs were electrophoresed in neutral agarose gels and transferred to nylon membranes (Nytran 45; Schleicher & Schuell, Inc.) in 20× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate), and the membranes were baked in vacuo for 2 h at 80°C. Blots were prehybridized in 500 mM NaCl–10 mM EDTA–20 mM sodium phosphate buffer (pH 6.8)–50 μg of sheared salmon sperm DNA per ml–0.5% (wt/vol) nonfat dry milk at 65°C. Hybridization of the blots with viral cDNA probes was conducted overnight at 65°C in the same buffer. The blots were washed in 0.2× SSC–0.2% SDS at 65°C and exposed to XAR-5 film (Kodak) at −75°C with intensifying screens.

Identically prepared Northern (RNA) blots were hybridized with either radiolabeled (41) oligo(dT_{12-18}) or oligo (dG_{12-18}) (Pharmacia) in hybridization buffer at room temperature overnight and then washed in 3 M tetramethylammonium chloride (Aldrich)–10 mM Tris, pH 8.0–1 mM EDTA at 30°C (12). The blots were exposed to X-ray film as described above. Alternatively, viral RNAs were denatured in 16.7% formamide at 80°C, frozen in dry ice-ethanol, diluted in 20× SSC, and immobilized on nitrocellulose (BA85; Schleicher & Schuell) using a slot blot apparatus. The RNAs were applied at 1.0 and 0.2 μg per slot, and the blots were baked as before. The slot blots were hybridized with radiolabeled oligo(dT) or oligo(dG), washed, and exposed to X-ray film as described previously.

Restriction endonuclease digests of full-length molecular clones of poliovirus type 1 Mahoney (63), TMEV (55), and CVB3 (78; N. Chapman and S. Tracy, unpublished data) were electrophoresed in neutral agarose gels, denatured at room temperature in 0.5 M NaOH–1 M NaCl for 1 h, and then Southern blotted (69) to Nytran 45 (Schleicher & Schuell) in 0.05 M NaOH–1 M NaCl. Membranes were neutralized and baked in vacuo for 2 h at 80°C. The blots were hybridized overnight at 65°C in hybridization buffer containing 0.5 M NaCl. The blots were washed in 0.2× SSC–0.2% SDS at 62°C and then exposed to X-ray film.

Polymerase chain reaction (PCR). HeLa cells were infected with CVB1, CVB3, CVB4, CVB5, CVB6, PV1, PV3, CVA16, CVA21, EV22 (Omaha), EV25, EV30, or EV34. At 4 h postinfection, cells were harvested by trypsinization, washed in 100 mM NaCl, suspended in 50 mM each Tris hydrochloride, pH 7.5, EDTA, and NaCl, and lysed with the addition of SDS to a final concentration of 0.5%. The lysates were phenol-chloroform extracted, chloroform extracted, and ethanol precipitated twice in 2.5 M ammonium acetate. Nucleic acids were suspended in a total volume of 100 μl of sterile water.

Two oligonucleotide primers were used for PCR (60, 61) amplifications. The E1 (5′-CACCGGATGGCCAATCCA) and the E2 (5′-TCCGGCCCCTGAATG) sequences are located in the 5′ nontranslated region of the CVB3 genome (79) at nucleotides 623 to 640 and 446 to 460, respectively. cDNA was synthesized to viral RNAs, using the E1 primer, 1 μl of infected-cell RNA, reverse transcriptase, and all four nonradioactive deoxynucleoside triphosphates as described above. The cDNA was ethanol precipitated and suspended in 50 μl of sterile water. PCRs were performed as described elsewhere (9) in 50-μl volumes containing both E1 and E2 primers, 2 μl of the cDNA as template, and 1.25 U of *T. aquaticus* (*Taq*) DNA polymerase (Amplitaq; Cetus/Perkin-Elmer). After denaturation at 94°C for 1 min, amplification was performed in a Thermal Cycler (Cetus/Perkin-Elmer) for 10 cycles (each cycle was 94°C for 1 s, 50°C for 1 s, and 72°C for 1 s) and then for 30 cycles (each cycle was 94°C for 1 s and 50°C for 1 s). Twenty percent of each reaction was analyzed by neutral agarose gel electrophoresis.

Equal portions (2 μl) of PCRs were immobilized on

nitrocellulose membranes, using a slot blot apparatus and hybridized with the radiolabeled oligonucleotide E3. E3 (5'-ACACGGACACCCAAAGTAGTCGGTTCC) is complementary to nucleotides 535 to 561 in CVB3 (79), a region which lies between the two primers used for PCR amplification, and therefore detects amplified product from this region (9). The blots were washed with 3 M tetramethylammonium chloride (12) at 68°C and exposed to film as described above.

Shutoff of host cell protein synthesis. HeLa cell monolayers were infected with EV22 (Omaha), CVB3, or EMCV at an MOI of 50 $TCID_{50}$ per cell. After 1 h at 37°C to permit viral attachment, the medium was removed and replaced with minimal essential medium lacking cysteine and methionine (GIBCO) supplemented with 5% fetal calf serum and 50 μg of gentamicin per ml. At 1 and 5 h postinfection, 50 μCi of [^{35}S]cysteine-methionine was added to the medium. One hour after addition of label, the medium was removed, and the cells were harvested and lysed by addition of SDS-PAGE reducing sample buffer (36). The cell lysates were heated to 100°C for 5 min and each sample was analyzed by SDS-PAGE (36) and fluorography as described above.

In vitro translation of viral RNA. EV22 (Harris), EMCV, and CVB3 viral RNAs, prepared as described above, were used as templates for in vitro translation in RRL (Promega Biotec) according to the manufacturer's recommendation. Brome mosaic virus RNA, supplied by the manufacturer, served as a positive control for RRL activity. Briefly, 1 μg of viral RNA was added to 35 μl of RRL, 1-μl of amino acid mix, 50 μCi of Tran^{35}S-label, and 1 U of Inhibit Ace (5 Prime-3 Prime, Inc.) in a total volume of 50 μl. The reaction mixtures were incubated at 30°C, and 2-μl samples were removed at 0, 5, 10, 20, 30, 45, 60, 75, and 90 min. Each sample was diluted in 1 ml of water and decolorized with the addition of hydrogen peroxide (Fisher) and 1 N NaOH, and the protein was precipitated with the addition of 25% trichloroacetic acid (Sigma) as previously described (24). The precipitated protein was then collected on GF/C filters (Whatman), washed with 8% trichloroacetic acid, and dried, and label incorporation was determined by liquid scintillation counting. The data were evaluated, using analysis of variance. In addition, a sample was removed after incubation for 60 min for analysis on 10% SDS-PAGE gels (36) by fluorography.

T-cell proliferation assay. The T-cell proliferation assay was performed as described previously (2). Briefly, C3H/HeJ male mice (25 days of age) were inoculated intraperitoneally with 10^5 PFU of CVB3. At 5, 10, 14, 21, and 28 days postinoculation, spleens from five infected mice were removed, and single-cell suspensions from individual spleens were placed in culture. In addition, at days 35 and 42 postinoculation, spleens from 10 mice were removed, and single-cell suspensions were prepared from two pools of five spleens each. Cells were added in 0.1-ml volumes to round-bottom microdilution plates at 2×10^5 cells per well. Five additional C3H/HeJ mice were inoculated intraperitoneally with 10^5 $TCID_{50}$ of EV22 (Omaha). At day 28 post-EV22 inoculation, the spleens were harvested individually and cultured as described above. Viral antigens were prepared by infecting HeLa cells with the appropriate virus [CVB2, CVB3, CVB6, CVA16, PV1, EV22 (Omaha), EMCV, or Mengo virus] and harvesting the cells at 3+ CPE (75 to 90% of cells demonstrating cytopathology). Preparations were frozen and thawed three times and centrifugally cleared of large cellular debris. The viral antigen preparations were then pelleted, suspended, and added to triplicate wells at 1.0 μg per 2×10^5 cells. Influenza virus antigen was prepared by inoculating embryonated hen eggs with a 10^{-2} dilution of stock virus, incubating the eggs for 48 h at 34°C, and collecting the allantoic fluid, which was subsequently clarified by low-speed centrifugation. Control (mock) antigen consisted of uninfected HeLa cells or embryonated hen eggs processed in an identical manner as the virus-infected cells or eggs. After 5 days of antigen exposure, splenocyte cultures were exposed to 1.0 μCi of [^{3}H]thymidine (6.7 Ci/mmol; Amersham) per well and then harvested 4 h later onto glass fiber filters, using a cell harvester (PHD; Cambridge Technology, Inc.). Radioactivity was measured in a liquid scintillation counter. The Student *t* test was used to evaluate the significance of the results.

RESULTS

Growth characteristics of the EV22 strains. The appearance of CPE in cell monolayers infected with either strain of EV22 (Harris or Omaha) at an MOI of 10 to 50 $TCID_{50}$ per cell was slower by several hours than monolayers similarly infected with EV34, CVB3, or PV1. As reported previously (11, 25, 83), the appearance of CPE was clearly differentiable from that of CVB3, PV1, or EMCV (data not shown). EV22 (Omaha), in contrast to EV22 (Harris), plaqued poorly on HeLa cell monolayers, consistently demonstrating a $TCID_{50}$/PFU ratio of approximately 1,000:1. Neither strain of EV22 produced CPE upon initial infection of RD cells, as reported by von Zeipel (81); however, both strains of EV22 were adapted to grow and lyse RD cells upon multiple passages. EV22 (Omaha) banded at 1.34 g/cm^3 in cesium chloride, as described for both enteroviruses and cardioviruses (59; data not shown).

Comparison of EV22 RNA to other picornavirus genomes. We assessed average nucleotide identity between EV22 RNA and the enteroviral CVB3 genome and the cardioviral EMCV genome, using Northern blot hybridization. Radioactive EV22 and CVB3 cDNAs were synthesized by using random primers and then used to probe Northern blots of CVB3, EV22, and EMCV RNAs. The RNA genome of EV22 has been reported to possess an unusually large region of secondary structure (62). To control for the possibility that reverse transcriptase might not be able to transcribe through the snap-back region, we prepared cDNA from both native and denatured RNA. In the denatured reaction, the genomic EV22 RNA was heated at 96°C for 5 min in the presence of the Mg^{2+} ion-containing reverse transcriptase buffer (see Materials and Methods) and random oligonucleotide primers, following which the mixture was rapidly chilled in ice water and the remainder of the reagents were added for transcription. No difference in yield of total cDNA counts per minute was observed between heated and unheated samples (data not shown).

Viral RNAs were electrophoresed in neutral agarose gels and stained with ethidium bromide (Fig. 1A) before being blotted onto Nytran membranes. Although EV22 (Harris) cDNA hybridized to homologous RNA, neither the prototypic enteroviral (CVB3) nor the cardioviral (EMCV) RNAs were detected by the EV22 probe (Fig. 1B). Probing of an identically prepared Northern blot with CVB3 cDNA showed that the CVB3 cDNA hybridized only with the CVB3 RNA, not with the EV22 (Harris) or EMCV RNAs (Fig. 1C).

Southern blot experiments, in which restriction digests of molecular clones of CVB3, TMEV, and PV1 (Fig. 2A) were probed with EV22 (Harris) cDNA, demonstrated that radiolabeled EV22 cDNA failed to hybridize with either CVB3,

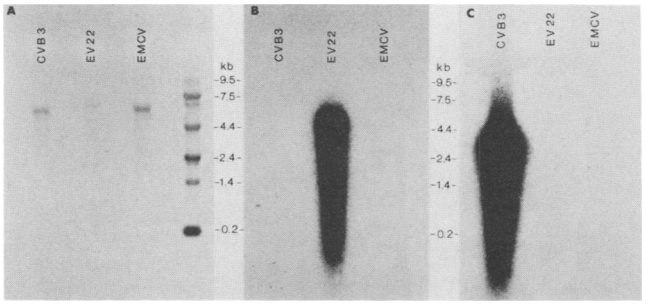

FIG. 1. Northern blot analysis to assess nucleotide identity between EV22 and other picornaviruses. (A) Ethidium bromide-stained gel of CVB3, EV22, (Harris), and EMCV viral RNAs. The hybridization pattern obtained upon probing identical Northern blots of viral RNA gels with cDNA made to EV22 (Harris) RNA (B) or CVB3 RNA (C) at 65°C is shown. The blots were exposed to Kodak XAR-5 film at −75°C for 15 h. Numbers indicate sizes in kilobases (kb) of RNA markers.

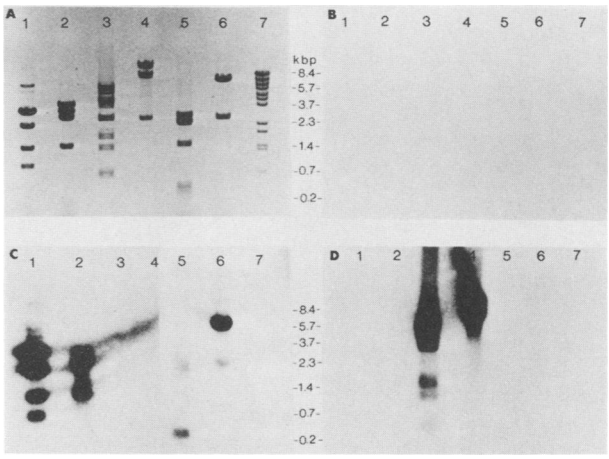

FIG. 2. Southern blot analysis to assess sequence identity between EV22 and CVB3, PV1, and TMEV. Genomic clones of CVB3, PV1, and TMEV were digested with restriction endonucleases before electrophoresis in neutral agarose gels and staining with ethidium bromide (A). N, nucleotide; N1 is the 5′-terminal nucleotide in the viral genome. Lanes: 1, CVB3 clone digested with *Cla*I and *Sca*I (3.35 kilobase pair [kbp], N 24 to 3369 pES131 vector; 3.33 kbp, N 1 to 2814 CVB3 and N 3370 to 3884 pES131 vector; 2.32 kbp, N 2815 to 5134 CVB3; 1.36 kbp, N 6033 to 7394 CVB3,; 0.90 kbp, N 5135 to 6032 (CVB3); 2, CVB3 clone digested with *Cla*I and *Eco*RI (3.86 kbp, N 24 to 3882 pES131 vector; 3.22 kbp, N 4172 to 7394 CVB3; 2.76 kbp, N 1 to 2761 CVB3; 1.41 kbp, N 2762 to 4171 CVB3); 3, TMEV clone digested with *Cla*I, *Xba*I, and *Hin*dIII (3.68 kbp, N 1731 to 5408 TMEV; 2.76 kbp, pSKII- vector; 1.73 kbp, N1 1 to 1730 TMEV; 1.32 kbp, N 6772 to 8093 TMEV; 0.72 kbp, N 5409 to 6127 TMEV; 0.64 kbp, N 6128 to 6771 TMEV); 4, TMEV clone digested with *Cla*I and *Xba*I (8.09 kbp, N 1 to 8093 TMEV; 2.76 kbp, pSKII- vector); 5, PV1 clone digested with *Eco*RI and *Bst*I (2.84 kbp, N 4601 to 7440 PV1; 2.50 kbp, N 2100 to 4600 PV1; 2.39 kpb, N 376 to 2768 pNT4 vector; 1.43 kbp, N 671 to 2099 PV1; 0.45 kbp, N 221 to 670 PV1; 0.38 kbp, N 1 to 375 pNT4 vector; 0.22 kbp, N 1 to 220 PV1); 6, PV1 clone digested with *Eco*RI (7.44 kbp, N 1 to 7440 PV1; 2.77 kbp, N 1 to 2768 pNT4 vector); 7, lambda phage DNA digested with *Bst*EII. Details of vectors pES131 and pNT4 have been described elsewhere (63). The pSKII- vector is a Bluescript vector (Stratagene, La Jolla, Calif.). Approximate sizes of DNA markers in kilobase pairs are indicated. Identically-prepared blots were hybridized with radiolabeled EV22 (Harris) cDNA (B), cloned CVB3 DNA (C), or cloned TMEV DNA (D) at 65°C. The blots were exposed to Kodak XAR-5 film at −75°C for 16 h (B), 10 min (C, lanes 1 through 4), 3 h (C, lanes 5 through 7), and 10 min (D).

TMEV, or PV1 cloned DNAs (Fig. 2B). The same EV22 cDNA hybridized with slot-blotted homologous EV22 RNA, however (data not shown). Under identical hybridization criteria, radiolabeled CVB3 hybridized to both homologous DNA and to DNA from a PV1 clone but not to TMEV DNA (Fig. 2C). Upon a longer exposure (16 h), all PV1 fragments were detected by the labeled CVB3 DNA (data not shown). As expected on the basis of nucleotide sequence analysis (45, 51), radiolabeled TMEV hybridized only to homologous DNA, failing to detect either CVB3 or PV1 cloned DNA (Fig. 2D).

Enzymatic amplification of enteroviral genomic sequences. Two generic enterovirus PCR primers were used to examine whether reverse transcription of EV22 RNA into cDNA followed by PCR amplification could promote the synthesis of a diagnostic enteroviral fragment. Both primers are derived from consensus sequences in the highly conserved enteroviral 5′ nontranslated region (79) and prime the synthesis of a 195-base-pair (bp) fragment, the nucleotide sequence of which we have confirmed by sequence analysis for several enteroviruses (9). cDNAs were synthesized to various enteroviral RNAs, using the E1 (complementary to positive strand viral RNA) primer, and then enzymatic amplification of the cDNA products was carried out, using both E1 and E2 primers and *Taq* polymerase. The reactions were analyzed by electrophoresis in neutral agarose gels, for which visualization of a 195-bp fragment demonstrated successful detection (Fig. 3A). Although representatives of the PV, EV, CVA, and CVB genera were detected in this manner, EV22 (Omaha) was not detected.

Products from similar reactions were immobilized on nitrocellulose membranes and probed with E3, a highly conserved 27-mer located between the E1 and E2 primers which has been shown to detect all enteroviruses except EV22 (C.-F. Yang and M. A. Pallansch, unpublished data). All enteroviruses except EV22 (Omaha) were detected (Fig. 3B). Similarly, EV22 (Harris) could not be detected in identical reactions (data not shown). Neither could complementary EV22 DNA, transcribed by using random primers, be amplified by using E1 and E2 in the PCR (data not shown). PCR amplifications are not equally efficient (Fig. 3A); thus, slot blotting of equivalent volumes of different reactions gives rise to the differences in extent of hybridization observed in Fig. 3B.

Shutoff of host cell protein synthesis. We examined the protein products produced by virus-infected cells at specific time points after infection to determine whether EV22 shut off host cell protein synthesis. HeLa cell monolayers were infected with CVB3, EMCV, or EV22 (Omaha) at an MOI of 50 $TCID_{50}$ per cell. ^{35}S-labeled cysteine-methionine was added at 1 and 5 h postinfection. After 1 h in label, the cells were harvested and lysates were examined by SDS-PAGE and fluorography. SDS-PAGE analysis of virus-infected and uninfected cell lysates revealed no significant differences in Coomassie blue-stained banding patterns between infected and uninfected cells (data not shown). Although fluorography of SDS-PAGE gels demonstrated that CVB3 had shut off host cell protein synthesis by 5 h postinfection (Fig. 4A, compare lane 4 with lane 5), EV22 (Omaha) had failed to shut off host cell protein synthesis by this time (Fig. 4A, lane 6). However, EV22 proteins were prominently labeled by 5 h postinfection. Comparison of the banding pattern obtained in these infected cells with that obtained upon analysis of

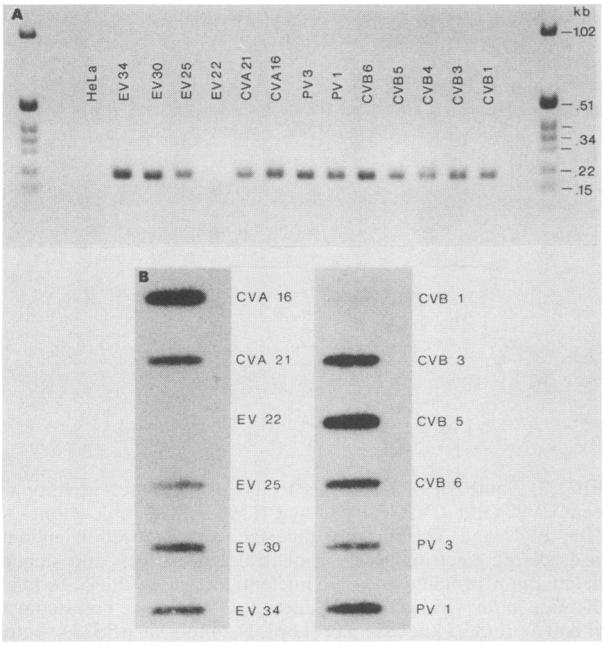

FIG. 3. PCR amplification of a sequence in the 5′ nontranslated region of numerous enteroviruses. (A) Analysis of PCR E1 and E2 amplification products by neutral agarose gel electrophoresis. The gel was stained with ethidium bromide. Sizes in kilobase pairs (kb) are indicated. (B) Analysis of similar E1 and E2 PCR amplification products by slot blot hybridization with radiolabeled E3 probe. The blot was exposed to XAR-5 film at −75°C with screens for 14 h.

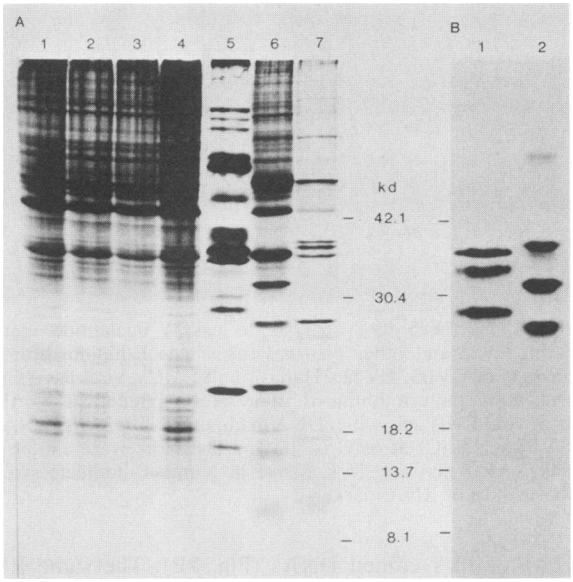

FIG. 4. SDS-PAGE analysis of host cell protein synthesis shutoff by EV22. (A) HeLa cells were infected with CVB3, EV22 (Omaha), or EMCV, pulsed with [^{35}S]cysteine-methionine at 1 and 5 h postinfection, and harvested after 1 h of labeling. Equivalent portions of each sample were analyzed on 12.5% SDS–PAGE gels by fluorography. Lanes: 1, 2, and 3, HeLa cells infected with CVB3, EV22 (Omaha), and EMCV, respectively, at 1 h postinfection; 4, uninfected HeLa cells labeled for 1 h; 5, 6, and 7, HeLa cells infected with CVB3, EV22 (Omaha), and EMCV, respectively, at 5 h postinfection. Locations of molecular mass standards in kilodaltons (kd) are indicated. The fluorograph was exposed to Kodak XAR-5 film at room temperature for 16 h. (B) Purified virions labeled with [^{35}S]cysteine-methionine electrophoresed on 12.5% SDS–PAGE gels and examined by fluorography. Lanes: 1, CVB3 virion preparation; 2, EV22 (Omaha) virion preparation. Fluorograph was exposed to XAR-5 film for 3 h at −75°C.

purified, radiolabeled CVB3 and EV22 virions (Fig. 4B, lanes 1 and 2, respectively) suggest which of the viral proteins are the putative capsid proteins VP1, VP2, and VP3. Other experiments demonstrated that even very late in infection (9, 12, and 17 h postinfection), when EV22-infected monolayer cell cultures exhibited advanced CPE, EV22 failed to shut off host cell protein synthesis (data not shown). This lack of shutoff was observed repeatedly to be as severe as that exhibited by EMCV (Fig. 4A, lane 7), a virus which does not shut off host cell protein synthesis directly but is believed to outcompete host cell RNA for translational machinery (27). The lack of host cell shutoff was confirmed using both strains of EV22 and in both HeLa and BGMK cell lines (data not shown).

In vitro translations. We determined whether EV22 RNA differed in the efficiency of in vitro translation in rabbit reticulocyte lysates relative to a prototypic enteroviral RNA and a cardioviral RNA. Equal amounts (1 μg) of EV22 (Harris), CVB3, and EMCV viral RNA were used as templates for in vitro translation in RRL using ^{35}S-labeled cysteine-methionine. Samples were removed at various time points, and the amount of label incorporated was determined after trichloroacetic acid precipitation of protein onto glass fiber filters and counting in a liquid scintillation counter. The level of label incorporation obtained upon translation of EV22 RNA (Fig. 5A) was similar to the level obtained for EMCV RNA and significantly higher (ca. threefold) at all time points after 30 min than the level obtained for CVB3 RNA. In another experiment, equivalent samples were removed after 60 min of incubation and examined by SDS-PAGE and fluorography (Fig. 5B). The level of label incorporation in the EV22 RNA translation (Fig. 5B, lane 3) was qualitatively superior to that for CVB3 RNA (Fig. 5B, lane 4) and is consistent with the incorporation kinetics (Fig. 5A).

Hybridization of EV22 RNA with oligonucleotide homopolymers. Northern blots and slot blots of EV22 (Harris) RNA were hybridized with oligo(dG) to determine whether EV22 contains a poly(C) tract typical of the cardioviruses EMCV and Mengo virus (5, 10, 47). Viral RNAs were electrophoresed in neutral agarose gels and stained with ethidium bromide (Fig. 6A) before being blotted onto nylon membranes or blotted directly onto nitrocellulose membranes, using a slot blot apparatus. Radiolabeled oligo(dG) hybridized only with the control, poly(C)-containing EMCV RNA (Fig. 6B and C) and failed to detect either CVB3 or EV22 RNA. Hybridization of an identically prepared slot blot panel with radiolabeled oligo(dT) (Fig. 6D) demonstrated that EV22 RNA is polyadenylated, a genomic characteristic of all members of the *Picornaviridae* (59).

Cell-mediated immune response to EV22. In order to determine whether CVB3-immune T cells, which recognize and proliferate against a conserved enterovirus group antigen(s) (2), would also recognize and respond to EV22, we exposed CVB3-immune spleen cells to EV22 antigen in vitro. Spleen cells from CVB3-inoculated mice proliferated in vitro in response to stimulation with various enteroviral antigens in addition to CVB3 (CVB2, CVB6, and CVA16, and PV1; Fig. 7). Although CVB3-immune T cells proliferated well against EV22 (Omaha) antigen, the initial response occurred 7 days later (day 28 postinoculation) than the peak response generated against the coxsackieviruses or PV1 (Fig. 7). Prolifera-

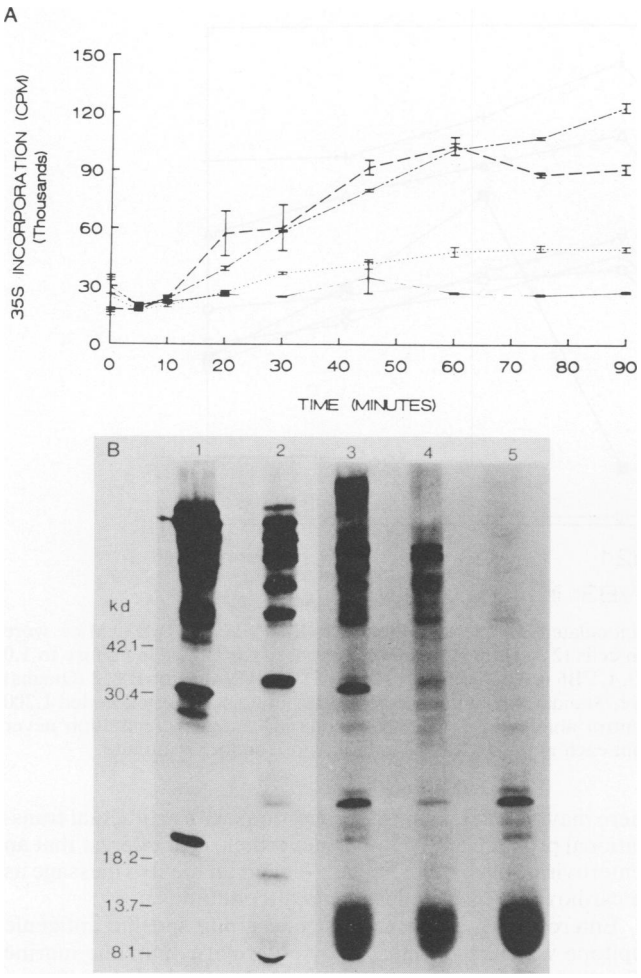

FIG. 5. In vitro translation of viral RNAs. (A) Label incorporation into trichloroacetic acid-precipitable protein with no RNA template (———) or with CVB3 RNA (·····), EV22 (Harris) RNA (— -- —), or EMCV RNA (— — —) as a template is shown. Error bars indicate the standard error of the mean. (B) Samples removed after 60 min of in vitro translation examined by fluorography of 10% SDS–PAGE gels. Equivalent amounts were loaded for all samples except Brome mosaic virus (BMV) for which only 20% as much was loaded. Lanes: 1, BMV; 2, EMCV; 3, EV22 (Harris); 4, CVB3; 5, no RNA. The locations of molecular mass standards in kilodaltons (kd) are indicated. The fluorograph was exposed to XAR-5 film at −75°C with intensifying screens for 24 h (lanes 1 and 2) or 53 h (lanes 3 through 5).

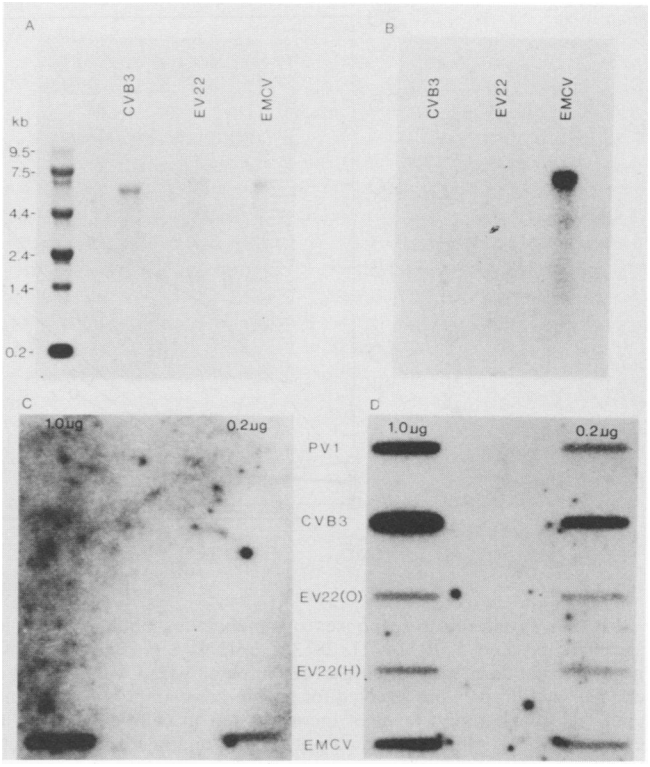

FIG. 6. Northern and slot blot analysis to detect poly(rA) and poly(rC) tracts in viral RNAs. (A) An ethidium bromide-stained gel of CVB3, EV22 (Harris), and EMCV viral RNAs. (B) Hybridization pattern of Northern-blotted viral RNAs with radiolabeled oligo (dG_{12-18}). Slot-blotted viral RNAs were hybridized with radiolabeled oligo(dG_{12-18}) (C) or oligo(dT_{12-18}) (D). The blots were exposed to XAR-5 film at −75°C with intensifying screens for 15 (B) or 17 h (C and D).

tive responses against the coxsackieviruses, PV1, and EV22 antigens remained elevated 42 days post-CVB3 inoculation, the last time point sampled. No significant proliferation occurred in response to the cardiovirus (EMCV) antigen. Additionally, spleen cells, obtained from EV22 (Omaha)-inoculated mice 28 days postinoculation, were exposed in vitro to the various viral antigens as before. EV22 immune spleen cells proliferated in vitro against all of the enteroviruses tested (Table 1). As before (Fig. 7), no response was generated against the cardioviruses EMCV, Mengo virus, or TMEV.

DISCUSSION

In this report, we confirm and greatly extend observations of others (11, 25, 58, 62, 65, 73, 81, 83) that EV22 is an atypical enterovirus. Through nucleic acid hybridization and PCR analyses, we were unable to demonstrate genomic identity between EV22 RNA and the well-characterized, prototypic enteroviral (46, 47, 77) genomes of CVB3 (38, 76, 79), PV1 (30, 74), and the cardioviruses EMCV (48) and TMEV (45, 55) (Fig. 1 through 3). Hybridization of the CVB3 DNA probe to PV1 DNA (Fig. 2C) demonstrated that nucleotide identities of 65% or greater could have been detected under the hybridization criteria used. The inability of EV22 genomic sequences to hybridize to the genomes of either CVB3, PV1, EMCV, or TMEV therefore demonstrate that EV22 RNA shares less than 65 to 70% identity with these sequences. These hybridization and PCR data are consistent with other investigators' results which have demonstrated that EV22 RNA lacks highly conserved genomic sequences found in many enteroviral RNAs (9, 20, 58; C.-F. Yang and M. A. Pallansch, unpublished data).

Like the cardioviruses, which do not directly shut off host cell protein synthesis but outcompete host cell message for translational machinery (27), EV22 does not shut off host cell protein synthesis in infected-cell cultures. This is in contrast to the rapid shutoff exhibited by the prototypic enteroviruses PV1 (18, 56) and CVB3 (Fig. 4A). As it is established that cardioviral RNA translates efficiently in RRL, whereas the prototypic enteroviral genome is a poor in vitro (RRL) mRNA (6, 26, 49, 66–68), we compared the in vitro translational efficiency of EV22 RNA with those of CVB3 and EMCV RNAs in RRL (Fig. 5). Translation of EV22 RNA

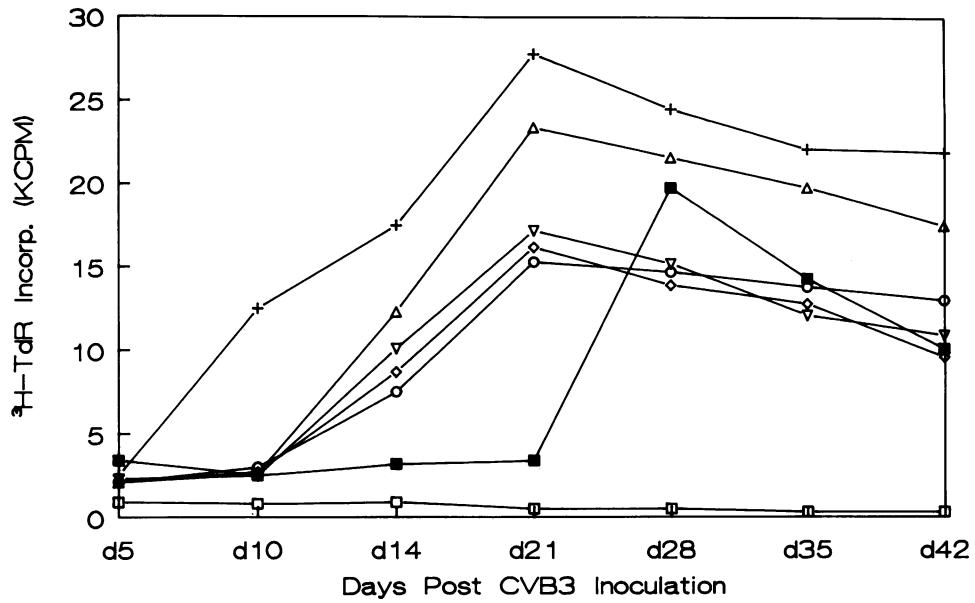

FIG. 7. Proliferative responses of splenocytes obtained from mice inoculated intraperitoneally with 10^5 PFU of CVB3. Mice were sacrificed on days 5, 10, 14, 21, 28, 35, or 42 days postinoculation. Spleen cells (2×10^5 per well) were exposed for 5 days in culture to 1.0 μg of antigen derived from HeLa cells infected with CVB3 (Δ), CVB2 (▽), CVB6 (○), CVA16 (◇), PV1 (+), EMCV (□), or EV22 (Omaha) (■) per well. Cells were assayed for proliferative ability on day 5 in culture. Standard deviations for each datum point never exceeded 1,200 cpm. The background level of incorporation in cells exposed to the control uninfected HeLa cell membrane antigen preparation never exceeded 1,721 ± 621 cpm (data not shown) and has been subtracted from each point. KCPM, Thousands of counts per minute.

was quantitatively more efficient (threefold) than that of CVB3 and on a par with that of EMCV. Interestingly, we noted that translation of EV22 RNA continues even at 90 min in vitro, while the translation of EMCV RNA is unchanged after about 45 to 60 min (Fig. 5A). Further studies are needed to examine the possibility that EV22 RNA may be a more stable message than EMCV RNA under these conditions. Although it is clear that translation in RRL is not adequate for efficient translation of prototypic enterovirus RNA without supplementation with mammalian cell extracts (6, 13, 52), and therefore the translational results presented here may reflect suboptimal conditions with artifactual translational products (13, 52), it is nevertheless of interest that an enteroviral RNA should be as efficient an in vitro message as a cardioviral RNA under identical conditions.

TABLE 1. Incorporation of [^{3}H]thymidine by EV22-immune murine spleen cells upon exposure to viral antigens for 5 days in vitro

Viral or mock antigen	Mean (±SD) [^{3}H]thymidine incorporation by splenocytes from 5 animals	Stimulation index[a]
EV22	15,029 ± 2,199	9.9
CVB3	13,756 ± 2,184	9.1
CVB2	14,091 ± 1,391	9.3
CVB6	11,212 ± 2,734	7.4
CVA16	12,123 ± 1,421	8.0
PV1	10,928 ± 1,292	7.2
EMCV	2,012 ± 977	1.3
Mengo virus	1,392 ± 774	0.9
TMEV	3,129 ± 789	2.1
Influenza A virus	1,312 ± 791	1.4
HeLa	1,512 ± 198	1.0
Allantoic fluid	945 ± 79	1.0
Medium only	724 ± 89	

[a] Average counts per minute (cpm) incorporated in the presence of antigen/average cpm incorporated in the presence of mock antigen. Uninfected HeLa cells served as mock antigen for all picornavirus antigens. Uninfected allantoic fluid served as mock antigen for influenza virus antigen. A stimulation index of ≥3 is significant.

Enteroviruses share at least one group-specific antigenic epitope which is recognized by enterovirus-immune murine T cells both in vivo and in vitro (2; M. Beck and S. Tracy, Am. J. Pathol., in press). However, no cardiovirus (EMCV, TMEV, or Mengo virus) will serve as an in vitro antigen to stimulate proliferation of enterovirus-immune murine T cells, from which we infer that viruses in the cardiovirus genus lack the same epitope(s). We therefore tested whether EV22 possessed such an epitope. Coxsackievirus B3-immune T cells proliferated against EV22 in vitro (Fig. 7), and T cells from EV22-inoculated mice recognized and proliferated to enteroviruses as an antigen in vitro (Table 1). In neither case did cardioviruses function antigenically in the in vitro assay, and in other work (M. Beck and S. Tracy, unpublished data), we have been unable to demonstrate the common enteroviral epitope in hepatitis A virus. These data demonstrate that EV22 contains an epitope(s) conserved among enteroviruses but not present in other picornaviruses.

The lack of identity between the primary structures of EV22 RNA and other enteroviral RNAs suggests that EV22 RNA may exhibit significant secondary structure differences in important regulatory regions of the EV22 genome as well as changes at the coding level. Translation of enteroviral RNA in vitro is dependent upon specific primary and secondary structures in the 5′ nontranslated region of the genome (4, 50, 72), alterations of which can result in viruses with different biologic characteristics (14, 22, 34, 64, 80). Interestingly, Seal and Jamison (62) reported evidence for a significant secondary structure located 5′ in the EV22 RNA which is ribonuclease resistant. The prolonged in vitro translation of EV22 RNA which we observed (Fig. 5) might

then be related to a significant secondary structure which confers stability to the viral RNA during translation (71). The fact that EV22 does not shut off host cell protein synthesis in cell culture (Fig. 4A) lends credence to the possibility that EV22 RNA may act as a superior message in vivo, thereby alleviating a requirement to shut down host cell protein synthesis for a productive infection. Protein 2A, which is implicated in the cleavage of cellular protein p220 accompanying host cell shutoff of capped-message translation in poliovirus (32, 33, 39, 56, 68), may differ in EV22. The lack of nucleotide sequence identity may reflect a divergent amino acid sequence giving rise to a functionally different protein. A divergent amino acid sequence may also result in a divergent enterovirus-specific antigen, which although conserved in EV22 (Fig. 7, Table 1), may be altered, resulting in delayed recognition by CVB3-immune T cells.

In summary, EV22 is significantly divergent from prototypic enteroviruses in terms of genomic nucleotide identity. This difference may be reflected in the efficiency with which the viral genome acts as a message in in vitro translation. The inability to shut off host cell protein synthesis and the possession of a diverged enterovirus group antigen which is recognized by enterovirus-immune T cells suggests that the EV22 proteins vary significantly with respect to prototypic enteroviral proteins. The determination of whether EV22 is a divergent enterovirus or perhaps a human cardiovirus must await the elucidation of the complete nucleotide sequence of the EV22 genome.

ACKNOWLEDGMENTS

We thank Bert Semler and Raymond Roos for the gifts of the molecular clones of poliovirus type 1 Mahoney and TMEV, respectively, Howard Lipton for the gift of purified TMEV, Gail Woods for confirming the serotype of the EV22 (Omaha) isolate, and Ann Palmenberg for the gift of purified Mengo virus and for helpful discussions during preparation of the manuscript.

This research was supported in part by a Public Health Service grant (5-RO1-HL40303-02) from the National Institutes of Health and by a grant from the Edna Ittner Foundation (to S.M.T.), a University of Nebraska Medical Center Seed Grant (to N.M.C.), and the American Heart Association, Nebraska Affiliate (M.A.B.) and Texas Affiliate (C.J.G).

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