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Expression of an Antigenic Adenovirus Epitope in a Group B Coxsackievirus

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Group B coxsackieviruses (CVB) cause human myocarditis, while human adenovirus type 2 (Ad2) is implicated as an agent of this disease. The L1 loop of the Ad2 hexon protein has been demonstrated to be antigenic in rabbits. To evaluate the feasibility of a multivalent vaccine strain against the CVB and Ad2, we cloned the sequence encoding the Ad2 hexon L1 loop, flanked by dissimilar sequences encoding the protease 2A (2Apro) recognition sites, into the genome of an attenuated strain of CVB type 3 (CVB3/0) at the junction of 2Apro and the capsid protein 1D. Progeny virus (CVB3-PL2-Ad2L1) was obtained following transfection of the construct into HeLa cells. Replication of CVB3-PL2-Ad2L1 in diverse cell cultures demonstrated that the yield of the chimeric virus was between 0.5 to 2 log units less than the parental strain. Western blot analyses of the CVB3 capsid protein 1D in CVB3-PL2-Ad2L1-infected HeLa cells demonstrated production of the expected capsid protein. Viral proteins were detected earlier and in approximately fourfold greater amounts in CVB3-PL2-Ad2L1-infected HeLa cells than in CVB3/0-infected cells. Cleavage of the CVB3-PL2-Ad2L1 polyprotein by 2Apro was slowed, accompanied by an accumulation of the fusion 1D-L1 loop protein. Reverse transcription-PCR sequence analysis of CVB3-PL2-Ad2L1 RNA demonstrated that the Ad2 hexon polypeptide coding sequence was maintained in the chimeric viral genome through at least 10 passages in HeLa cells. Mice inoculated with CVB3-PL2-Ad2L1 demonstrated a brief viremia with no replication detectable in the heart but prolonged replication of virus in the pancreas in the absence of pathologic changes in either organ. CVB3-PL2-Ad2L1 induced binding and neutralizing anti-Ad2 antibodies, in addition to antibodies against CVB3 in mice. CVB3-PL2-Ad2L1 was used to challenge mice previously inoculated with CVB3/0 and with preexisting anti-CVB3 neutralizing-antibody titers; anti-Ad2 neutralizing and binding antibodies were induced in these mice at higher levels than in mice without anti-CVB3 immunity. The data demonstrate that a CVB vector can stably express an antigenic polypeptide of Ad2 from within the CVB open reading frame that results in the induction of protective immune responses against both viruses.

The six serotypes of the group B coxsackieviruses (CVB1 to CVB6) are human enteroviruses (family *Picornaviridae* [26]). Coxsackievirus capsids are 29-nm-diameter icosahedral structures with the typical enterovirus canyon-like depressions surrounding the fivefold axes (46, 47), which, by analogy to polioviruses and rhinoviruses, are binding sites for the cell membrane receptor human coxsackievirus adenovirus (Ad) receptor (HCAR) (9, 12, 60). The CVB genome encodes four capsid and seven nonstructural proteins, including two proteases, within a single open reading frame (ORF) with a coding capacity of 2,185 amino acids (16, 37, 63). While diverse viruses can cause human myocarditis (11), the CVB serotypes are those most commonly implicated. CVB have been isolated from hearts of pediatric patients with myocarditis (20, 21, 35, 66), enteroviral RNA has been detected by reverse transcription-PCR (RT-PCR) and in situ hybridization in approximately 20 to 25% of heart samples from patients with myocarditis or dilated cardiomyopathy (reviewed in references 5 and 41), and murine models of experimental CVB-induced myocarditis exist that recapitulate many aspects of the human disease counterpart (reviewed in references 27, 33, 34, and 67). More recently, human Ad DNA has been detected in hearts of patients with myocarditis (40), with subsequent sequence analysis of the amplimers from diseased hearts shown to be consistent with infections by Ad2 (53). There are no commercially available vaccines against either CVB or Ad.

The small enterovirus genome is limited in terms of being able to add foreign sequences to it for successful expression. The crystallographic solution of poliovirus and rhinovirus capsid structures led to an early focus on expressing small peptides in external loops of capsid proteins (3, 18, 25, 29), but such inserts were limited to short peptides and chimeric viral genomes were generally unstable. Expression of foreign sequences within the enterovirus ORF, utilizing the ability of viral proteases to cleave both in *cis* and in *trans* to process the foreign polypeptide, were found to alleviate in part the size limitation of clonable inserts. Two sites within the enterovirus genome have been identified as useful, i.e., those immediately upstream of the start of translation and at the junction of the sequences encoding the capsid protein P-1D (1D) and the protease P-2A (2Apro). Although success was originally reported at the site upstream of the enteroviral ORF (68), insertions at this site tend to delete readily; expression of simian immunodeficiency virus (SIV) polypeptides suggested that this insertion site was less preferable, on the basis of stability and chimeric virus replication rate, than that between the 1D and the 2Apro (59). Once deletion of the inserted coding sequence from the enterovirus genome has occurred, the selection of the more efficiently replicating deleted genome (parental or near parental) quasispecies to become the dominant population can be rapid (48). Expression vectors that utilize the 1D/2Apro cloning site rely on 2Apro to cleave autocatalytically between

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its amino terminus and the carboxyl terminus of the inserted polypeptide and then to cleave the enterovirus capsid protein 1D from the foreign polypeptide sequence in *trans*. This is made possible by engineering short oligonucleotide tracts that encode the 2Apro recognition sites on either side of the inserted fragment in the chimeric viral genome. Different SIV antigenic sequences were expressed using this site in a poliovirus vector; replication of the viruses in mice transgenic for the poliovirus receptor demonstrated that the SIV peptides can be presented appropriately to stimulate host immune responses (19, 59).

Here we describe an attenuated chimeric CVB3 strain that stably expresses the antigenic L1 loop of the Ad2 hexon protein (61) at the junction of the CVB3 capsid protein 1D and the 2Apro. The progeny chimeric virus overexpresses viral protein, replicates, and induces neutralizing and binding anti-Ad2 antibodies, as well as anti-CVB3 neutralizing antibodies, in mice. Anti-Ad2 immunity can be induced in the presence of preexisting murine anti-CVB3 immunity.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of HeLa cells as well as cultures of murine fetal heart fibroblasts (MFHF) (65) and COS-1 cells were propagated in minimal essential medium containing 10% fetal bovine serum and 50 μg of gentamicin per ml. Human cardiac artery endothelial cell (HCAEC) cultures were obtained from Clonetics (Walkersville, Md.) and were propagated as monolayers, as suggested by the supplier, in proprietary medium purchased from Clonetics. The cells were grown at 37°C in a humidified 5% CO_2–air mixture. The infectious cDNA copy of the CVB3/0 genome (15, 16, 28) (GenBank accession no. M88483) was used as the background for the construction of the Ad2 hexon L1 loop polypeptide-expressing chimeric strain, CVB3-PL2-Ad2L1. Human Ad2 (American Type Culture Collection [ATCC], Manassas, Va.) and CVB3 strains were passaged in HeLa cell monolayers to produce virus stocks.

Construction and transfection of CVB3-PL2-Ad2L1. The construction of the infectious CVB3-PL2-Ad2L1 cDNA is outlined in Fig. 1a. For insertion of the Ad2 polypeptide encoded sequence, a CVB3/0-based subclone, pBSPL2, with a polylinker containing *Bam*HI, *Avr*II, *Eco*RV, and *Pst*I sites and flanked by a short sequence encoding the cleavage site of 2Apro was generated. Rather than flank an inserted sequence with perfect nucleotide repeats encoding the 2Apro recognition sites, changes were incorporated in the nucleotide sequence encoding the 2Apro cleavage site downstream of the insert that resulted in a sequence 49 nucleotides (nt) long with 69% similarity to the upstream wild-type site (Fig. 1b). This modified cDNA was first generated by PCR amplification using the pCVB3-0 cDNA (16) as template with primers containing the altered nucleotide sequence with restriction sites and then inserted in a subcloned fragment of the pCVB3-0 cDNA (defined by the *Avr*II-*Sca*I fragment between nt 2034 and 5137 [numbering from GenBank accession no. M33854] cloned in the *Xba*I and *Eco*RV sites of pBluescript II SK$^+$ (Stratagene, La Jolla, Calif.).

Two primers, HexA and HexD, (5′-TCCGGATGAAAAAGGGGTGCCTCT TCCAAAG and 5′-GCCTCTGCAGTCAGACAGATGTGTGTCTGG, respectively), were used to amplify the L1 loop region from Ad2 DNA (Genbank locus ADRCG, nt 19624 to 19776); this fragment added a *Bam*HI restriction site upstream and a *Pst*I site downstream in frame with the CVB3 ORF. Cleavage at these two sites generated a fragment that was subsequently ligated into sublone pBSPL2 using the polylinker sites. The Ad2 insert-containing subclone was ligated into the pCVB3/0 cDNA (16) genome using the unique *Bgl*II (nt 2042) and *Xba*I (nt 4947) sites. Sequence analysis of the resulting chimeric cDNA, pCVB3-PL2-Ad2L1, verified the existence of the Ad2-L1 loop coding sequence in frame with the CVB3 ORF (data not shown).

To generate progeny virus, 3.5 μg of pCVB3-PL2-Ad2L1 were transfected into 3×10^5 HeLa cells in a six-well plate using an Effectene transfection reagent kit (Qiagen, Valencia, Calif.) as suggested by the supplier. Three days posttransfection, cultures were frozen and thawed three times and centrifugally cleared of cell debris. One-third of the cleared supernatant was used to inoculate HeLa cells to obtain a CVB3-PL2-Ad2L1 virus stock (passage 2). Progeny virus was subjected to titer determination on HeLa cell monolayers and stored aliquoted at −74°C.

RT-PCR and sequence analysis. Total RNA was extracted from virus-infected cells (RNAzol; Life Technologies, Gaithersburg, Md.) and cDNA was synthesized using a one-step RT-PCR system as directed by the supplier (Superscript One-Step RT-PCR system; Life Technologies). The RNA sequence of pCVB3-PL2-Ad2L1 RNA across the cloning site was deduced by cycle sequencing of the resulting amplimers (ThermoSequenase; Amersham Life Science, Cleveland, Ohio). Enzymatic amplifications were performed for 40 cycles at an annealing temperature of 57°C using primers ID9 and DI4 (5′-CTAGACTCTGCCAATA CGAG [nt 3201 to 3220] and 5′-GTGCTCACTAAGAGGTCTCTG [nt 3406 to 3426], respectively). Nucleotide numbering is based upon the CVB3 sequence (accession no. M88483).

Single-step growth curves. Replication of the chimeric strain was compared to that of the parental strain using single-step growth curves as described elsewhere (65). Briefly, HeLa cells were inoculated at a multiplicity of infection of 20. After washing and refeeding of the cell monolayers, cultures were frozen at various times, thawed, and subjected to titer determination on HeLa cell monolayers for infectious virus.

Western blot analysis of viral proteins in infected cells. Translation of CVB3 proteins was studied by Western blot analysis of whole-cell virus-inoculated lysates basically as described elsewhere (14). HeLa or MFHF cultures were inoculated with virus at a multiplicity of infection of 20 or 50, respectively, and the monolayer cultures were lysed at various times postinoculation with Laemmli buffer containing 2-mercaptoethanol (36). Proteins were electrophoresed in 14% acrylamide gels containing sodium dodecyl sulfate (SDS) (Novex, San Diego, Calif.) and electroblotted to Immobilon-P nylon membranes (Millipore, Bedford, Mass.). The blots were probed with a 1/1,000 dilution of the primary antibody, a polyclonal horse anti-CVB3 neutralizing antibody (ATCC) that detects CVB3 capsid protein 1D. The primary antibody was detected using peroxidase-conjugated rabbit anti-horse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, Pa.) at a dilution of 1/125,000. The second antibody was detected using an ECL$^+$ kit and Hyperfilm (Amersham, Arlington Heights, Ill.) as directed by the supplier. A NucleoVision gel documentation system and software (Nucleo Tech, San Mateo, Calif.) were used to capture and analyze the X-ray film images.

Inoculation of mice with virus. Male BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were obtained at 3 to 4 weeks of age. The mice were inoculated intraperitoneally with 5×10^5 50% tissue culture infective doses $TCID_{50}$ of either CVB3-PL2-Ad2L1 or the control CVB3/0 strain in 0.1 ml of unsupplemented medium. To determine whether the chimeric virus replicated in mice, mice were sacrificed 1, 2, 4, 6, or 8 days postinoculation and murine sera, pancreata, and hearts were obtained to measure virus titers in tissue as described elsewhere (64). To determine whether the chimeric virus induced antiviral antibodies, mice were inoculated once, twice (14 days apart), or three times (each 14 days apart). Mice in each series were sacrificed 14 days following the final inoculation, and blood, hearts, and pancreata were removed for analysis. Longer times postinoculation were not investigated. Sera were isolated from clotted blood samples and stored frozen until use. For histopathologic analysis, hearts and pancreata were fixed in 10% buffered formalin, embedded, sectioned, and stained with eosin and hemotoxylin (65). For studies of immunity in preimmune mice, CVB3/0 was inoculated using 5×10^5 $TCID_{50}$/0.1 ml intraperitoneally once or twice (14 days apart). Fourteen days later, one group of five randomly chosen mice were sacrificed and the pooled serum was tested for the presence of anti-CVB3 neutralizing antibodies. Two other groups of mice were challenged twice (14 days apart) with 5×10^5 $TCID_{50}$ of CVB3-PL2-Ad2L1 (CVB3/0-1x, Ad2-CVB3/0-2x or (CVB3/0-2x, Ad2-CVB3/0-2x) (Table 1). The mice were sacrificed 14 days after the final inoculation to obtain murine serum.

Virus binding antibody enzyme linked immunosorbent assay. To determine the titer of anti-Ad2 binding antibody in murine sera, an enzyme-linked immunosorbent assay was constructed by coating 96-well flat-bottom plates (Dynex Technologies, Chantilly, Va.) with Ad2 that had been prepared from HeLa cell monolayers. The enzyme-linked immunosorbent assay was performed using a peroxidase detection system (mouse-hybridoma subtyping kit; Boehringer Mannheim, Indianapolis, Ind.). Briefly, 96-well plates were coated for 1 h at room temperature with 7×10^3 to 8×10^3 $TCID_{50}$ of Ad2 per well. After washing and postcoating as specified by the manufacturer the plates were incubated with different dilutions of the CVB3-PL2-Ad2L1 immune murine serum (1/2, 1/20, 1/100, 1/500, 1/1,000, 1/5,000, and 1/10,000) for 30 min at 37°C. After the plates were washed, peroxidase-conjugated goat anti-mouse IgG was applied, and bound secondary antibodies were subsequently visualized using the peroxidase substrate 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) provided by the kit. Color intensity was evaluated in a microplate reader (Skatron, Sterling, Va.) at a wavelength of 405 nm.

Anti-Ad2 and anti-CVB3 neutralizing-antibody assays. Neutralizing-antibody titers in murine sera were determined as described elsewhere (7, 8). Aliquots of murine sera were heated at 56°C for 45 min prior to use. Stocks of CVB3/0 or Ad2 with known titers were diluted so that 100 to 200 infectious particles were dispensed per well of 96-well titer plates seeded the previous day with HeLa cells. Prior to dispensing, CVB3 or Ad2 was mixed with serially diluted murine sera and incubated at 37°C for 1 h. Titers from triplicate wells were read at 48 to 96 h, when the control wells containing only virus demonstrated complete cytopathic effects (detached and rounded up cells for CVB3; rounded up and refractile cells for Ad2).

RESULTS

Construction of the chimeric CVB3 genome and generation of progeny virus. The infectious cDNA copy of the chimeric CVB3 genome, pCVB3-PL2-Ad2L1, containing the sequence encoding the L1 loop of Ad2, was constructed as described in Materials and Methods (outlined in Fig. 1). Progeny virus

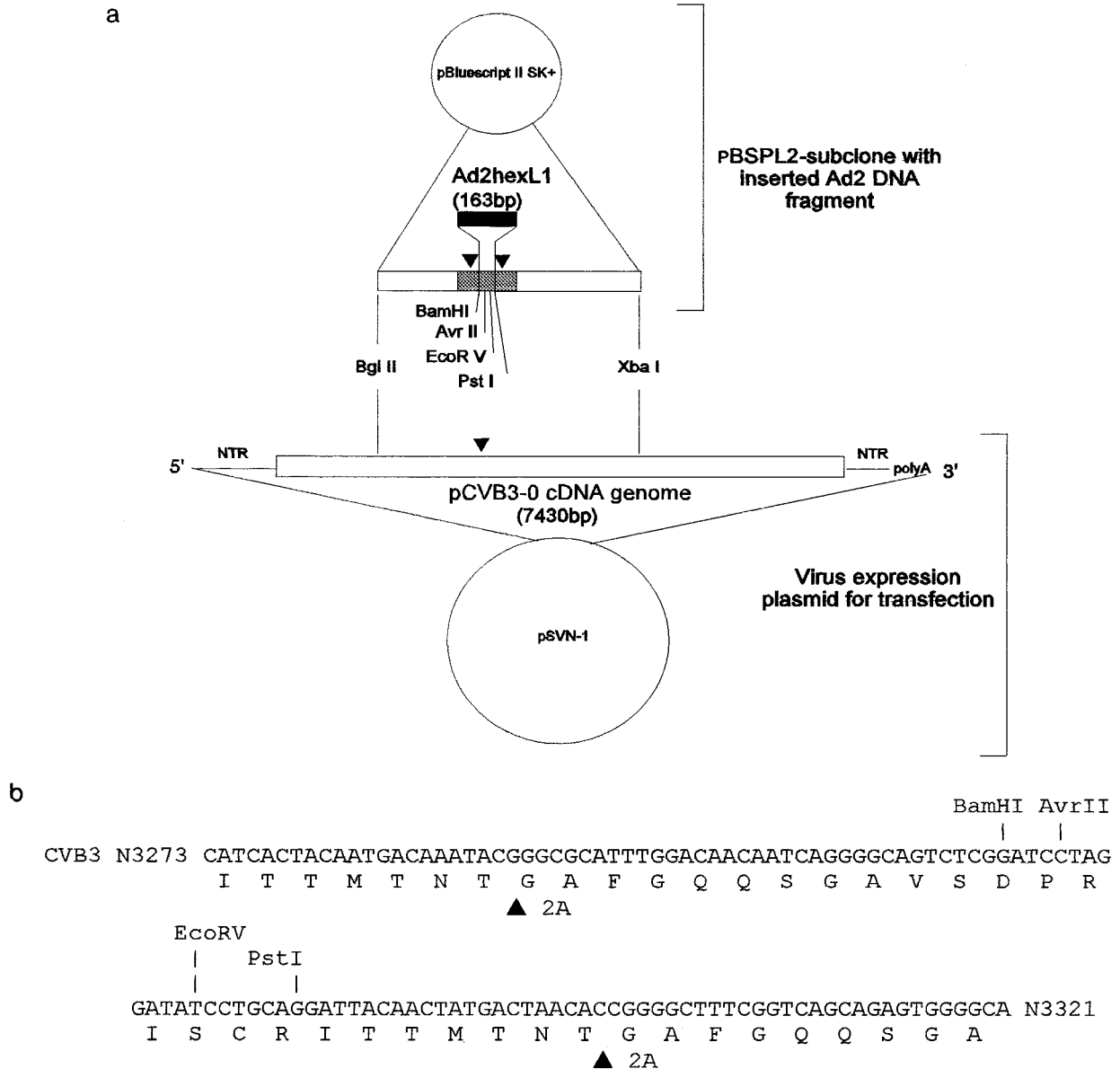

FIG. 1. Construction of pCVB3-PL2-Ad2L1. (a) The construction of the recombinant plasmid using a CVB3/0-derived subclone, pBSPL2, is described in Materials and Methods. The CVB3 ORF is indicated by open bars, the Ad2 hexon L1 loop insert is indicated by a solid box, and the polylinker is indicated by a shaded box. Regions encoding 2Apro recognition sites are indicated by black arrows. NTR, nontranslated region. PolyA, poly(A) tract located at the 3′ end of the CVB3 genome. (b) The nucleotide sequence of the polylinker with the flanking dissimilar duplicated region that encodes the modified 2Apro cleavage site (shaded bar) is given. Nucleotide numbering is based on the CVB3/0 genome found at GenBank accession no. M88483.

(CVB3-PL2-Ad2L1) was propagated on HeLa cell monolayers from supernatants of frozen and thawed, centrifugally cleared HeLa cell transfections. Cell culture supernatants containing progeny virus were cleared of cellular debris by centrifugation, subjected to titer determination on HeLa cell monolayers, and frozen in aliquots at −74°C. Viral RNA was isolated from CVB3-PL2-Ad2L1 stocks and reverse transcribed, and a fragment spanning the capsid protein 1D-2Apro junction was enzymatically amplified and sequenced to determine whether the progeny virus contained the L1 loop coding sequence. No differences were detected from the expected sequence (data not shown). All chimeric virus stocks used in these experiments were derived and sequence verified in this fashion.

Characterization of chimeric virus replication in cell cultures. To investigate chimeric virus CVB3-PL2-Ad2L1 replication in cell culture relative to its parental strain CVB3/0, we inoculated HeLa cells, COS-1 cells, primary HCAEC cultures, and primary MFHF cultures (Fig. 2) with both viruses. The chimeric virus CVB3-PL-Ad2L1 replicated at similar rates in HeLa cell monolayers (Fig. 2a) but yielded approximately 10-fold less infectious virus titer. Under these conditions, HeLa cell cultures demonstrate advanced cytopathic effects by 7 to 9 h postinoculation. The yields of both viruses were reduced in the other cell cultures tested relative to the titers achieved in HeLa cells (Fig. 2b), with titers of the chimeric strain 0.5 to 2 log units lower than those of CVB3/0. The data suggest that

TABLE 1. Antibody response to CVB3-PL2-Ad2L1 infection in mice[a]

Antiserum[b]	Virus-neutralizing titer[c]		Virus-binding titer[d] (Ad2)
	CVB3/0	Ad2	
CVB3-PL2-Ad2L1-1x	1/16	<1/2	1/20
CVB3-PL2-Ad2L1-2x	1/32	1/4	1/100
CVB3-PL2-Ad2L1-3x	1/64	1/8–1/16	1/1,000
CVB3/0-1x and CVB3-PL2-Ad2L1-2x	1/128	1/16–1/32	1/5,000–1/10,000
CVB3/0-2x and CVB3-PL2-Ad2L1-2x	1/128	1/32	1/10,000
Hyperimmune CVB3	>1/1,000		
Hyperimmune Ad2		>1/1,000	>1/10,000

[a] Mice were inoculated once (CVB3-PL2-Ad2L1-1x), twice (CVB3-PL2-Ad2L1-2x; 14 days apart), or three times (CVB3-PL2-Ad2L1-3x; each 14 days apart) with 5×10^5 $TCID_{50}$ of virus. For studies of anti-Ad2 responses in CVB3/0-immunized mice (inoculated once or twice, 14 days apart), mice were challenged twice with CVB3-PL2-Ad2L1.

[b] Pooled sera were measured for neutralizing as well as binding antibodies as described in the text. Hyperimmune CVB3/0, polyclonal horse anti-CVB3 serum (ATCC); Hyperimmune Ad2, polyclonal mouse serum obtained 2 weeks after the last of four inoculations (each 3 weeks apart) with Ad2.

[c] Neutralizing-antibody titers are expressed as the reciprocal of the highest antibody solution preventing virus-induced cytopathic effects in three of three wells.

[d] ELISA binding antibody titers were defined as the highest serum dilution giving an absorbance value of more than 0.2 optical density unit at 405 nm above control levels.

expression of the Ad2 L1 loop polypeptide partially attenuates virus replication relative to the parental strain.

Western blot analysis of viral protein translation in infected-cell cultures. For the chimeric virus CVB3-PL2-Ad2L1 to replicate successfully, the capsid protein 1D must be cleaved by 2Apro at its carboxyl terminus, where it forms a junction with the artificially inserted Ad2 hexon L1 loop polypeptide. To investigate the efficiency of this cleavage event, we studied the processing of capsid protein 1D in infected HeLa cells by Western blot analysis. Proteins from HeLa cells inoculated either with CVB3-PL2-Ad2L1 or with CVB3/0 were separated on SDS-containing 14% polyacrylamide gels, blotted, and probed with a polyclonal horse neutralizing anti-CVB3 antibody that detects the CVB3 capsid protein 1D. Since an antibody that recognizes the Ad2 hexon L1 loop sequence on Western blots was unavailable, detection of the Ad2 polypeptide was not performed. Using the anti-CVB3 antibody, the 34-kDa CVB3 capsid protein 1D was detected at 5 h postinoculation in cells inoculated with the chimeric virus, whereas the same band was detected later, at 7 h, in the CVB3/0-inoculated cultures (Fig. 3). A prominent band in cells infected with the chimeric virus that migrated with an apparent size of a fusion protein consisting of the capsid protein 1D and the hexon L1 loop polypeptide (41 kDa) was also detected. Densitometric comparison of the Western blot signals demonstrated that CVB3-PL2-Ad2L1 overproduced both capsid protein 1D and the uncleaved 1D/Ad2 L1 loop chimeric protein by approximately 3.8-fold relative to 1D translation in CVB3/0-infected cells. These results suggested that the lower yields of chimeric virus in cell cultures might be linked to the delayed processing at the capsid protein 1D/Ad2 L1 loop junction by 2Apro.

Stability of the Ad2 hexon L1 loop coding sequence in the CVB3 vector genome. Western blot data suggested that the Ad2 L1 loop coding sequence was maintained and expressed in the CVB3-PL2-Ad2L1 genome. However, an alternative hypothesis was that we were investigating a mixed population of virus, such that viral RNAs with and without the Ad2 L1 loop fragment coding sequence were being translated in the infected cells. Viral RNA with the Ad2 insert deleted might be producing the capsid protein 1D, while insert-containing RNA would be producing both 1D and the chimeric 1D-Ad2L1 loop protein. Although sequence analysis strongly suggested that the virus stocks were uniformly chimeric and not deleted with respect to the Ad2 L1 loop insert coding sequence, we tested the hypothesis by examining the CVB3-PL2-Ad2L1 RNA populations in infected HeLa cells by RT-PCR and sequence analysis. To determine the stability of the inserted sequence in the CVB3 genome as a function of time in cell culture, we concurrently passaged CVB3-PL2-Ad2L1 10 times in HeLa cells. Viral RNA was isolated from virus stocks at each pass and used as template in RT-PCRs with primers located outside of and flanking the insertion site in the CVB3 genome. Analysis of the amplimers by agarose gel electrophoresis showed that the inserted Ad2 sequence remained stable in the CBV3 genome for at least 10 passages in HeLa cell monolayers, generating the expected size of 446 bp for the insert-containing amplimer (Fig. 4a). In passages 8 and 10, we detected a low level of an amplimer that would correspond in size to that generated from a CVB3 genome with the Ad2 insert deleted. These smaller amplimers, as well as representative insert-containing bands from passages 3, 5, and 10, were isolated from agarose gels and sequenced using as sequencing primers the same primers that had been used in the RT-PCR analysis. Sequence analysis revealed that the 446-bp amplimers contained the expected Ad2 hexon L1 loop coding sequence and the flanking sequences in frame with the CVB3 ORF (Fig. 4b). The smaller 225-bp fragments from pass 8 and 10 were indeed from viral genomes that had deleted the L1 loop sequence (Fig. 4c). The sequence of these deleted genomic fragments also demonstrated that the viral ORF had been maintained intact and therefore that these amplimers had not been derived from nonviable viral RNA. These results do not support the hypothesis that two different virus populations were present in the passaged CVB3-PL2-Ad2L1 or that significant deletions were generated de novo in each passage and transmitted as progeny virus. Evidence for a deleted virus population, potentially capable of replication, was obtained by amplification analysis only in two later passages. The passage data are consistent with a dominant L1 loop insert-containing viral quasispecies that remains stable through at least 10 passages in HeLa cell monolayers, suggesting that the chimeric 1D/Ad2L1 protein observed in the Western blot analysis is most probably due to a delay in 2Apro cleavage at the engineered site between 1D and the Ad2 hexon protein fragment.

Characterization of chimeric virus replication and pathogenicity in mice. To determine if the chimeric virus replicates in mice, mice were inoculated and sacrificed on days 1, 2, 4, 6, and 8 postinoculation. Virus titers in the murine sera, pancreata, and hearts were subsequently measured on HeLa cells (Fig. 5).

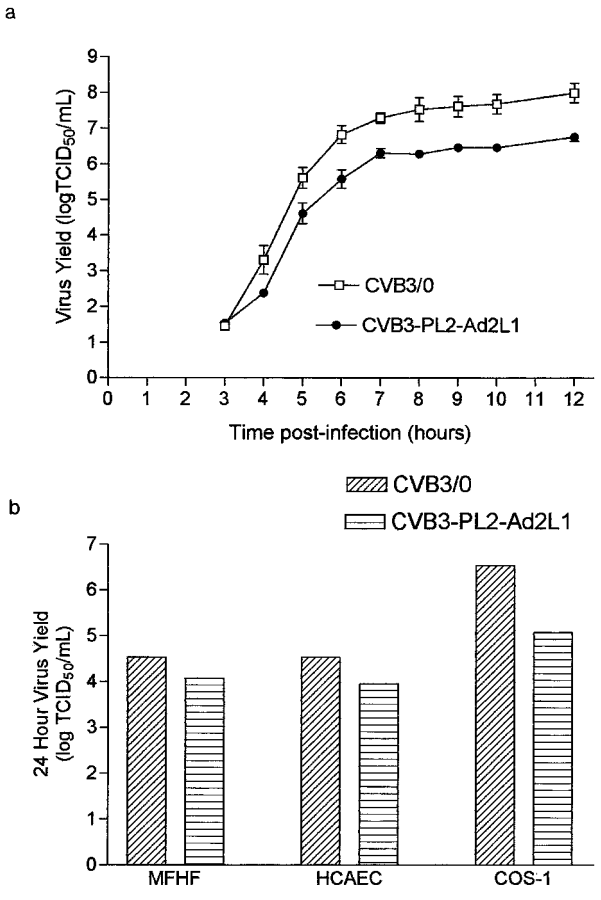

FIG. 2. Replication of CVB3-PL2-Ad2L1 in cell culture. (a) Single-step growth curves of CVB3-PL2-Ad2L1 and CVB3/0 in HeLa cells were obtained as described in the text. Cultures were harvested by freezing at the indicated times postinoculation. Virus titers were determined on HeLa cell monolayers and are expressed as the logarithm of $TCID_{50}$ per milliliter with each data point representing the mean titer and standard deviation of triplicate independent experiments. (b) Yields of infectious virus in MFHF, HCAEC, and COS-1 cultures 24 h after inoculation with CVB3-PL2-Ad2L1 or CVB3/0.

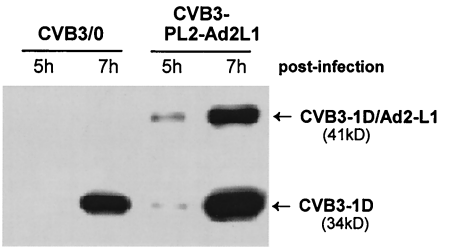

FIG. 3. Western blot analysis of viral proteins in CVB3-PL2-Ad2L1-inoculated HeLa cells. Proteins were harvested 5 and 7 h postinoculation from HeLa cell monolayers inoculated with either CVB3-PL2-Ad2L1 or CVB3/0, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an anti-CVB3 antibody. Anti-CVB3 primary antibody was detected with a horseradish peroxidase-linked second antibody, and the signal was developed using an enhanced chemiluminescence system (ECL^+; Amersham). Molecular masses at which the CVB3 capsid protein (CVB3-1D) and the chimeric protein (CVB3-1D/Ad2-L1) migrate are indicated.

CVB3-PL2-Ad2L1-infected mice experienced a brief viremia with prolonged viral replication in the pancreas but without detectable virus titers in hearts. Histopathological examinations of mice inoculated with the chimeric virus revealed healthy pancreas and heart tissues with no evidence of virus-induced lesions, in contrast to pancreatic inflammation and damage observed in CVB3/0-infected mice (data not shown). These experiments demonstrate that the chimeric virus CVB3-PL2-Ad2L1 is capable of replicating in mice and is attenuated for inducing disease in murine pancreatic tissues.

Antibody responses in mice to infection by CVB3-PL2-Ad2L1. A synthetic peptide containing the 13 amino acids of the Ad2 hexon L1 loop was shown by Toogood et al. to be antigenic in rabbits (61), promoting the generation of serotype-specific, anti-Ad2 neutralizing antibodies. To determine whether mice would mount an immune response against the Ad2 L1 loop polypeptide that was expressed during replication of the chimeric virus, CVB3-PL2-Ad2L1 was inoculated into BALB/c mice once, twice, or three times. Mice were sacrificed 14 days after the final inoculation. Five mice were in each group, and sera were pooled to assay for the presence of anti-CVB3 and anti-Ad2 neutralizing and binding antibodies. Antibodies in the murine sera bound immobilized Ad2 in an ELISA-based assay, ranging from 1/20 after one inoculation to 1/1,000 after three inoculations (Table 1). While anti-Ad2 neutralizing antibodies were negligible after a single inoculation, titers between 1/8 and 1/16 were obtained after three inoculations (Table 1). We also performed this experiment in C3H/HeJ mice ($H\text{-}2^k$ haplotype) with similar results, suggesting that the results were not due to a specific murine host (data not shown). Ad2-binding antibodies in the sera were subtyped using an ELISA. The primary component was IgG1 at a titer of 1/1,000, with detectable IgG2a at titers between 1/20 and 1/100. No IgG2b, IgG3, or IgA were detected in the murine sera (data not shown). Anti-CVB3 neutralizing antibodies were readily detected at titers ranging from 1/16 after one inoculation of CVB3-PL2-Ad2L1 to 1/64 after three exposures (Table 1). The results demonstrate that the CVB3-PL2-Ad2L1 chimeric virus induces both anti-CVB3 neutralizing antibodies and anti-Ad2 neutralizing and binding antibodies in experimentally inoculated mice and that the Ad2 hexon L1 loop is antigenic in mice as well as in rabbits.

Induction of anti-Ad2 immunity in mice with preexisting anti-CVB3 immunity. CVB are common causes of human infection (52). Although preexisting immunity to a viral agent can protect from disease caused by the specific virus, it does not necessarily preclude reinfection by that agent as has been shown by both poliovirus (PV) vaccines (43, 58) and more recently developed Ad vectors (10, 13, 44). To determine whether CVB3-PL2-Ad2L1 could induce anti-Ad2 immunity in mice with preexisting immunity against the CVB3 vector, mice were inoculated once or twice (14 days apart) with CVB3/0. We have shown previously that infectious CVB3/0 is cleared from mice by day 7 to 10 postinoculation (16). Mice were subsequently challenged with CVB3-PL2-Ad2L1 14 days after the last CVB3/0 inoculation and again 2 weeks later. Sera were isolated after sacrifice 2 weeks after the final challenge. Two weeks later, after the initial CVB3/0 inoculation, a group of five randomly chosen control mice were sacrificed. Sera from these mice were assayed for the presence of anti-CVB3 neutralizing activity; all sera expressed neutralizing anti-CVB3 antibody titers ranging between 1/8 and 1/32 (data not shown). Antibodies in pooled serum from mice inoculated once with CVB3/0 and then twice with CVB3-PL2-Ad2L1 were assayed by ELISA for the presence of binding antibodies. Anti-Ad2 binding antibodies from mice inoculated once with the chimeric virus were detected at titers between 1/5,000 and 1/10,000 (Table 1). Neutralizing anti-Ad2 antibodies were detected at serum dilutions between 1/16 and 1/32. These titers

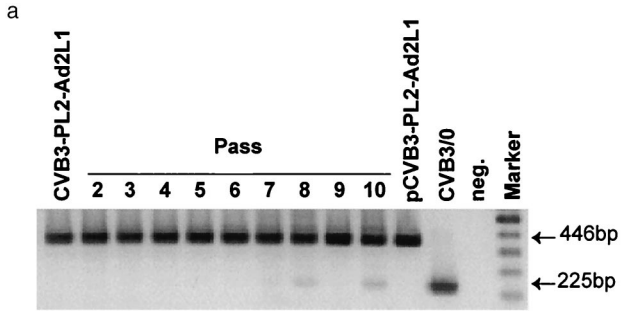

FIG. 4. PCR and sequence analysis of CVB3-PL2-Ad2L1. pCVB3-PL2-Ad2L1 was transfected into HeLa cells, and the resultant progeny virus (CVB3-PL2-Ad2L1, pass 1) was subsequently serially passaged in HeLa cell cultures (passes 2 to 10). Viral RNA was isolated from virus stocks at each passage, and the presence of the inserted Ad2 sequence was analyzed by PCR using primers flanking the insertion site in the CVB3 genome. (a) Amplimers were separated by agarose gel electrophoresis. CVB3-PL2-Ad2L1, RT-PCR amplimer using chimeric viral RNA as the template; pCVB3-PL2-Ad2L1, PCR amplimer using the chimeric plasmid DNA as the template; CVB3/0, RT-PCR amplimer using the parental CVB3/0 RNA as template; neg., RT-PCR using RNA as template from uninfected HeLa cells; Marker, 100-bp DNA ladder. (b and c) The sequence of the Ad2 insert-containing 446-bp amplimer (CVB3-PL2-Ad2L1) (b) and the sequence of the 225-bp Ad2 fragment-deleted amplimer (CVB3-PL2-Ad2L1del) (c) were obtained after isolation of the DNA fragments from agarose gels. Sequence analysis was performed with the same primers as for the RT-PCR analysis. Numbering is based on the CVB3/0 genome (Genbank accession no. M88483).

b

```
CVB3-PL2-Ad2L1 N3273 CATCACTACAATGACAAATACGGGCGCATTTGGACAACAATCAGGGGC
                       I  T  T  M  T  N  T  G  A  F  G  Q  Q  S  G  A
                                  ⇐ 1D |▲ 2Apro

     BamHI
      |
AGTCTCGGATCCGGATGAAAAAGGGGTGCCTCTTCCAAAGGTTGACTTGCAATTCTTCTCAAATACTAC
 V  S  D  P  D  E  K  G  V  P  L  P  K  V  D  L  Q  F  F  S  N  T  T
           |-----------------------------------------------------------

CTCTTTGAACGACCGGCAAGGCAATGCTACTAAACCAAAAGTGGTTTTGTACAGTGAAGATGTAAATAT
 S  L  N  D  R  Q  G  N  A  T  K  P  K  V  V  L  Y  S  E  D  V  N  M
----------------------------Ad2 hexon L1 loop-------------------------

                                PSTI
                                 |
GGAAACCCCAGACACACATCTGTCTGACTGCAGGATTACAACTATGACTAACACCGGGGCTTTCGGTCA
 E  T  P  D  T  H  L  S  D  C  R  I  T  T  M  T  N  T  G  A  F  G  Q
--------------------------| 1D                    |▲ 2Apro ⇒

GCAGAGTGGGGCA N3321
 Q  S  G  A
```

c

```
CVB3-PL2-Ad2L1del CATCACAACTATGACTAACACCGGGGCTTTCGGTCAGCAGAGTGGGGCA
                   I  T  T  M  T  N  T  G  A  F  G  Q  Q  S  G  A
                              ⇐ 1D |▲ 2Apro
```

were between two- and fourfold higher than those observed in mice that had received only three successive inoculations of CVB3-PL2-Ad2L1. Mice that had been inoculated twice with CVB3/0 and then twice with CVB3-PL2-Ad2L1 showed binding and neutralizing antibodies detected at serum dilutions 1/10,000 and 1/32, respectively (Table 1). These data demonstrated that CVB3-PL2-Ad2L1 can induce anti-Ad2 immunity in mice with preexisting protective immunity against the CVB3 vector and that the immunity obtained was higher than that observed in mice inoculated only with the chimeric virus.

DISCUSSION

Ample evidence demonstrates that PV can be engineered as multivalent chimeric vaccines against non-PV infectious agents such as primate and human retroviruses (2, 17, 19, 54, 59), rotaviruses (42), hepatitis B virus (68), and potentially against cancer (38, 45) as well. However, it has been questioned whether intact enteroviruses can package sufficiently large proteins while simultaneously maintaining the foreign insert for sufficient time to serve as useful chimeric vaccines (48). Morrow and colleagues have demonstrated one potential approach to this problem through the use of a capsid protein packaging system that supplies PV capsid proteins in *trans*, permitting the insertion and expression of significantly larger proteins from within the viral ORF (6, 45, 50). Not in question is that naturally occuring infections by human enteroviruses or through vaccination by Sabin PV strains have demonstrated that enteroviruses are exceptional vaccines against themselves (43). The widespread application of the PV Sabin strain vaccines has led to nearly complete worldwide eradication of PV as causes of disease. Although continued use of PV as research tools in the laboratory following the expected worldwide eradication of PV is foreseen, using constructs in which the capsid proteins

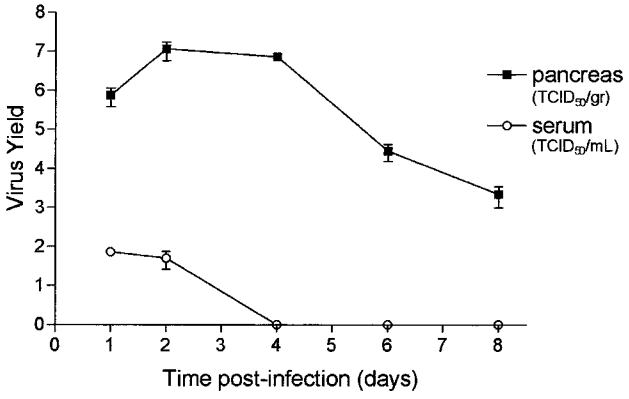

FIG. 5. Kinetics of CVB3-PL2-Ad2L1 replication in murine sera and pancreata. BALB/c mice were inoculated as described in the text with 5×10^5 $TCID_{50}$ of CVB3-PL2-Ad2L1 per mouse and then sacrificed on days 1, 2, 4, 6, and 8 postinoculation. For each mouse, virus titers were measured in serum ($TCID_{50}$/milliliter), pancreas ($TCID_{50}$/gram), and heart ($TCID_{50}$/gram). The data are shown as mean and standard deviation for three animals per time point. No titer was measurable in any heart tissue.

may be supplied in *trans*, the future of PV as intact chimeric viral vectors is less certain (22–24). The closely related CVB, as the best-studied non-PV enterovirus group, represents an attractive enterovirus system with which to complement or replace chimeric PV vaccine development.

Because of the high etiologic association of the CVB and Ad2 with human viral myocarditis (4, 21, 30, 40, 53, 56, 57, 62, 66, 67), we tested whether an artificially attenuated strain of CVB3 could express an antigenic sequence of Ad2 and then whether the progeny virus could induce protective immunity against both viruses in an experimental mouse system. The data presented in this report demonstrate that a CVB can be engineered to express an antigenic polypeptide from within the CVB3 ORF and that the resulting virus induces both neutralizing and binding antibodies against Ad2 and CVB3. While we did not investigate the long-term duration of the anti-Ad2 or anti-CVB3 response in mice, it will be of interest to determine the relative longevity of the immune responses against both viruses in vaccinated mice as a function of time. Murine Ad (Mav-1) replicates in and can kill mice (32), unlike human Ad. If the same region of the Mav-1 hexon loop L1 is similarly antigenic, it would also be possible in principle to test the protective effect of a multivalent vaccine against both CVB and Mav-1 in mice.

The use of common viruses as chimeric vaccines faces the potential obstacle of preexisting antivirus immunity in the proposed patient that may decrease or ablate the utility of such chimeric vectors. However, inoculation of inactivated (Salk-type) PV vaccines generate protective immunity in humans which can be complemented by subsequent exposure to the attenuated Sabin PV vaccines (1, 43). An emphasis on the use of Ad-based vectors in clinical settings has also raised this issue. Bramson et al. (10) have nonetheless demonstrated that an interleukin-12-expressing Ad vector still promotes tumor regression in mice with preexisting anti-Ad vector immunity. The results presented in this report demonstrate that mice with preexisting circulating anti-CVB3 antibodies did not prevent the generation of an anti-Ad2 response by the chimeric CVB3-PL2-Ad2L1 upon challenge; indeed, the levels of anti-Ad2 neutralizing immunity in the pooled serum of mice with preexisting anti-CVB3 immunity were higher than those in mice that received three inoculations of the chimeric CVB3 vector. Recent work in this laboratory has demonstrated that a single inoculation of an attenuated CVB3 strain induces anti-CVB3 protective immunity 28 days postinoculation at a 1/4 to 1/8 dilution of serum; this level completely protects these mice from heart and pancreatic disease following a challenge with a virulent CVB3 strain (14). Although these results are highly suggestive that preexisting immunity against a CVB vector does not pose a significant problem in administering a CVB-based chimeric vaccine, unanswered questions remain. Studies need to address preexisting anti-vector immunity on an individual-mouse basis rather than using pooled sera, as we did in this study. Since human sera can demonstrate high serotype-specific anti-CVB immunity, studies are necessary to probe at what levels, if any, of preexisting immunity the impact of a chimeric vaccine becomes minimal. Since inbred mice demonstrate different responses to infections by the CVB (56, 57), the influence of the murine genetic background on the efficacy of a chimeric CVB-based vaccine in the presence of preexisting serotype immunity will also need to be examined.

Because of concern regarding the instability of foreign sequences even when inserted within the viral ORF (48), we studied the Ad2 hexon L1 loop coding sequence stability in CVB3-PL2-Ad2L1 RNA by both RT-PCR and direct sequence analysis over 10 generations in HeLa cell cultures. We found the expected size amplimer in each passage, and only in passages 8 and 10 did we detect a smaller than expected amplimer, a band that corresponded to the band which would be generated from a virus population in which the insert was deleted. Sequence analysis confirmed that the expected 446-bp amplimer contained the L1 loop coding sequence in frame and as cloned in the plasmid pCVB3-PL2-Ad2L1 that was used to transfect cells and generate progeny virus. Sequence analysis of the smaller bands showed deletion of the insert in these bands and showed that in these, too, the ORF had been maintained, suggesting that these amplimers originated from a viable viral quasispecies. Nonetheless, over 10 passages, the dominant quasispecies remained CVB3-PL2-Ad2L1. In preliminary work, a similar RT-PCR-mediated examination of viral RNA isolated from murine pancreas 6 days after inoculation with the chimeric virus has demonstrated a mixed population, consisting of intact insert as well as deleted genomes lacking the insert (data not shown). That this chimeric virus induced protective neutralizing-antibody responses in mice against the vector CVB3 as well as against Ad2 argues forcefully for the stability of the insert during replication in mice and for correct presentation of the Ad2 polypeptide to the murine immune system.

We observed overproduction of viral capsid protein 1D and a second protein on Western blots that migrated with an apparent molecular weight of the capsid protein 1D plus the Ad2 hexon L1 loop fragment in proteins from CVB3-PL2-Ad2L1 infected HeLa cells. We believe that these findings are related. The larger 1D fusion protein could only have resulted from a delay in cleavage by 2Apro at the recognition site located between the CVB3 capsid protein 1D and the Ad2 L1 loop polypeptide. Delayed cleavage at this site was not due to alteration of the amino acid sequence of the 2Apro cleavage site; this site was engineered to be identical to that normally found in CVB3, while the sequence in and around the duplicated downstream 2Apro recognition was altered at the nucleotide level to discourage homologous recombination during viral replication. Further, direct sequence analysis of amplimers from virus RNA containing these sites demonstrated that both sequences that encode the 2Apro cleavage recognition sites remained intact and had not been changed. The production of infectious virus at rates similar to the parental virus strain, data that were confirmed in a variety of different cell cultures and by

replication of this virus and induction of anti-Ad2 responses in mice, demonstrated that 2Apro was only delayed in cleavage and was not inhibited. Overproduction of the viral proteins is, to the best of our knowledge, a novel finding in chimeric enteroviruses. The chimeric CVB3-PL2-Ad2L1 strain demonstrated a fourfold increase in production of viral proteins as judged by the detection of the viral capsid protein 1D on Western blots, although it replicated to lower titers. How can more protein synthesis be reconciled with a lower yield of infectious virus in CVB3-PL2-Ad2L1-infected cells? We propose the following mechanism. The 2Apro cleaves the 1D/Ad2 polypeptide site in the chimeric virus in *trans*, not in *cis* as it does in the wild-type virus. A delay in cleaving the site would thus produce not only authentically cleaved 1D but also a backlog of uncleaved 1D fusion protein. The higher rate and higher yield of viral protein translation may in part be due to recycling of newly synthesized viral RNA away from virion assembly into translation. The 1D fusion protein may compete with native 1D protein in the assembly process; as the virion assembly process dithers, newly synthesized viral RNA that normally would be encapsidated (51) is shunted into translation, increasing protein synthesis. It might therefore be expected that with increasing amounts of viral protein being synthesized, so too should viral RNA levels be increasing as only translatable viral RNA is replicated (49). Preliminary data from experiments measuring the amounts of total polyadenylated RNA synthesized in cells infected with CVB3-PL2-Ad2L1 or CVB3-PL2 as a function of time postinfection are consistent with this prediction (K. Höfling and S. Tracy, unpublished data). Can the fusion 1D protein be encapsidated in mature virions? The distantly related picornavirus hepatitis A virus does not have a functional 2Apro activity; instead, the 2A protein forms a terminal extension on the hepatitis A virus capsid protein 1D that is presumably cleaved in mature virions by host proteases (31, 39, 55). To model this in enteroviruses would most simply require the insertion of a peptide upstream of the 2Apro cleavage site in the terminal sequence of capsid protein 1D; to the best of our knowledge, such work has not been published. Since the carboxyl termini of proteins 1D line the southwest walls of the receptor-binding canyons that surround the fivefold axis of symmetry in the intact mature infectious virion surface (46; M. Rossmann, personal communication), such an extension is conceivable but might well result in a noninfectious virion. Perhaps more to the point is whether the chimeric 1D can be detected in newly assembled protomers in the infected cell, protomers that also may serve to inhibit intact virion assembly. We are currently examining both of these possibilities. A different CVB3-PL2 construct that expresses biologically active murine interleukin-4 also overexpresses protein 1D and correlates with a lower virus yield (N. Chapman et al., submitted for publication). However, discrete effects upon enterovirus polyprotein processing may be accentuated by the closely linked translational, transcriptional, and encapsidative processes in the viral life cycle (49, 51). It is worth noting that an overproduction of viral proteins could beneficially impact the utility of a vaccine based on such a vector.

In summary, the Ad2 hexon L1 loop and flanking amino acids were expressed from within the ORF of an attenuated strain of CVB3. The inserted Ad2 coding sequence affected the yield of CVB3-PL2-Ad2L1 relative to the parental virus, but it was maintained stably in the vector RNA through at least 10 generations in HeLa cell cultures. The chimeric virus replicated in mice and presented the Ad2 polypeptide to the immune system as demonstrated by the induction of both anti-Ad2 neutralizing and binding antibodies. The chimeric CVB3-based virus induced anti-Ad2 immunity in mice with preexisting anti-CVB3 immunity.

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