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Functional Diversity of E1A Gene Autoregulation among Human Adenoviruses

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Autoregulation of the adenovirus E1A gene involves its constitutive expression and positively and negatively regulated transcription. Dissection of this process will identify basal-level *cis* elements and autoregulatory targets of the E1A promoter and functional domains within the *trans*-acting E1A gene products. In this report, the DNA sequence of the human subgroup B adenovirus type 3 (Ad3) E1A gene is presented and compared with that of the E1A genes of similar and distantly related human adenoviruses. The cDNA forms of the Ad3 E1A gene, corresponding to two major early mRNA species, are cloned, sequenced, and subcloned into plasmid expression vectors. Cotransfections of cell cultures are performed with Ad5 or Ad3 E1A gene expression plasmids and a reporter gene under control of the Ad5 or Ad3 E1A promoter. The Ad5 and Ad3 E1A promoters are similarly repressed by either serotype's 12S cDNA gene products. The Ad3 E1A promoter responds much more strongly than the Ad5 E1A promoter to transactivation by 13S cDNA gene products. In contrast, the 13S cDNA gene of Ad5 has greater transactivation activity than that of Ad3. Experiments with missense mutations of the Ad5 E1A gene indicate that transactivation of the Ad5 E1A promoter is weak, just reversing or balancing negative autorepression. Single amino acid substitutions in the conserved, repressive functional domain 2 of the E1A gene modulate transactivating activity that is usually associated with the separate and distal conserved functional domain 3. These results suggest a strong structure-function relationship influenced by the variable sequences separating these conserved domains.

Natural variation among similar viruses or strains of viruses provides many examples of evolutionary constraints, based upon the structure or function of essential gene products. In a complementary fashion, the immune responses of infected host organisms impose divergent pressures on certain viral genes. Late viral genes are often cited as examples of these counterpoised forces in viral gene evolution. Essential early viral genes may also be subject to complementary constraints and divergent pressures.

The adenovirus E1A early gene is the first component of viral genome expression in the nucleus early after infection. The intimate associations of E1A gene products with the cellular mechanics of transcription regulation (3, 4, 8, 36, 41, 55) create a set of functional constraints on this essential early gene. A role for E1A antigens as targets for cell-mediated immunosurveillance (1, 2, 32) may be analogous to the immunological basis for antigenic drift of late-gene capsid antigens.

The levels and the kinetics of E1A gene expression in cells infected by adenovirus type 5 (Ad5) or adenovirus type 3 (Ad3) are remarkably different (51). We attribute this to evolved differences of positive and negative autoregulation of this essential viral gene's expression (18, 51). Adenovirus E1A gene expression involves autoregulatory repression and transactivation of transcription (13, 18, 23, 25, 28, 37, 46, 51). These opposed levels of control are mediated by two early E1A gene products, distinguished by their 5′ donor sites for the RNA splicing of a common E1A gene transcript. Two functional domains (14, 30, 33) are associated with enhancer-targeted repression, and these are present in the translation products of both E1A mRNA species. A third functional domain (14, 30, 33) is associated with transactivation of transcription, and this domain is found in the translation products of the longer 13S mRNA. The E1A promoter contains elements that represent targets for the regulatory activities of the E1A gene products (18), although such targets may be accessed indirectly, through intermediate cellular factors associated with developing or active transcription complexes.

The E1A gene is also noted for the variety of cellular genes which respond to E1A-mediated positive and negative regulation of transcription (14, 16, 17, 34, 35, 44, 45, 47, 48, 53). Evolutionary balances of functional constraints and antigenic pressures on the E1A gene may have determined the variation of more complex properties of adenoviruses, such as tissue tropism and oncogenicity. The variety of such virus-host cell-host animal interactions and the involvements of the E1A gene are seminal features of the large family of (human) adenovirus serotypes (42, 57).

The different intensity and kinetics of Ad5 and Ad3 E1A gene expression early after productive infection are recapitulated in transfections of the same host cells with plasmid expression vectors. Our earlier studies (18, 51) were done with vectors expressing cDNA forms of the Ad5 E1A gene or genomic forms of the Ad5 and Ad3 E1A genes. These E1A gene constructs are cotransfected into cells with a reporter gene (chloramphenicol acetyltransferase [CAT]) expressed under control of either the Ad5 or Ad3 E1A promoter. The E1A promoter of Ad3 responds more to transactivation by the 13S cDNA of Ad5 than does the

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homologous Ad5 E1A promoter. The Ad5 E1A promoter responds more strongly than the Ad3 E1A promoter to autorepression mediated by the 12S cDNA form of the Ad5 E1A gene.

In this report, we present the DNA sequence of the Ad3 E1A gene and describe the successful cloning and expression of its 12S and 13S cDNAs. New plasmid expression vectors enable cotransfections with the E1A cDNA genes of both Ad3 and Ad5 and the CAT reporter gene, expressed under control of the homologous or heterologous Ad3 and Ad5 E1A promoters. The repressive activities of the 12S cDNA gene products of Ad3 and Ad5 are very similar. There is a significant difference between the activities of Ad3 and Ad5 E1A 13S cDNA gene products in transactivation of the Ad3 E1A promoter. Experiments with missense mutations in functional domains 2 and 3 of the Ad5 E1A gene address the remarkably different responses of the Ad5 and Ad3 E1A promoters to transactivation by E1A products. The variable region separating conserved functional domains 2 and 3 appears to be important in the role of both domains in E1A *trans* autoactivation.

MATERIALS AND METHODS

Cells and viruses. HeLa cell cultures were maintained in 110-mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml), and an antimycotic agent (1 μM *p*-hydroxybenzoic acid butyl ester). Adenovirus type 3 (strain G.B.) was originally obtained from the American Type Culture Collection and was propagated and purified by procedures used routinely in this laboratory (50, 51).

Enzymes. Restriction endonucleases, T4 DNA ligase, and the Klenow and T4 DNA polymerases were purchased from New England Biolabs. Chemically modified T7 DNA polymerase, Sequenase, was purchased from United States Biochemical Co. Enzymes were used with the buffers and incubation conditions specified by the vendors.

Plasmid expression vectors. The construction and use of plasmids expressing the CAT gene under the control of adenovirus E1A promoters were described in earlier publications (18, 51). In the present study, we used an Ad3 E1A promoter-CAT plasmid (51) and the Ad5/3 chimeric promoter-CAT plasmid described by Jones and Tibbetts (18). The latter CAT expression vector has upstream E1A promoter sequences derived from Ad5, joined at a common *Pvu*II restriction site (−50 bp) to the identical transcription initiation region of the Ad3 E1A CAT plasmid. Construction of the plasmids expressing the genomic form and the 12S and 13S cDNA forms of the Ad5 E1A gene was described earlier (18).

We modified our original plasmid vector, which expresses the genomic form of the Ad3 E1A gene (51). This plasmid DNA was first restricted at the unique sites for *Cla*I and *Sph*I, followed by blunting and polishing of the noncomplementary sticky ends with T4 DNA polymerase and blunt-end ligation with T4 DNA ligase. The modified plasmid was then cloned by transformation of *Escherichia coli* competent cells, screening for ampicillin-resistant colonies with plasmids about 540 bp smaller than the original Ad3 E1A gene expression vector. The sequences deleted in this procedure remove a remaining portion of the tetracycline resistance gene of the original pBR322 vector without affecting the expression of the cloned genomic form of the Ad3 E1A gene in transfected human cells. Deletion of these plasmid sequences removes the pBR322-derived *Bam*HI and *Hin*dIII restriction sites from the Ad3 E1A expression vector, promoting the remaining, Ad3-specific *Bam*HI and *Hin*dIII sites to unique status. This facilitates our procedure, which substitutes cDNA sequences for genomic DNA sequences between these two restriction sites, as described in a following section.

Genomic expression vectors, corresponding to missense mutations *hr*3 and *hr*4 of the Ad5 E1A gene, as described in an earlier publication (9), were obtained from Gary Glenn and Robert Ricciardi of the Wistar Institute. The missense mutations of these mutants debilitate the transactivation activity of the Ad5 E1A (13S) products, consistent with the loci of the mutations in functional domain 3 of the E1A gene.

Expression vectors corresponding to the 13S cDNA of Ad5 E1A mutants *pm*936 and *pm*953, as described in an earlier publication (29), were obtained from Jim Lillie and Michael Green of Harvard University. The missense mutations of these mutants debilitate enhancer-targeted repression of the Ad5 E1A (12S and 13S) products, consistent with the loci of the mutations in functional domain 2 of the E1A gene.

DNA sequencing of the Ad3 E1A gene. The DNA sequence of the left-end, 1,565-bp *Bgl*II K fragment of the Ad3 genome was determined from viral DNA (21, 23, 24, 52) and from viral DNA fragments derived from a functional, plasmid-cloned Ad3 E1A gene (51). A variety of partial and complete *Msp*I and *Mbo*I restriction fragments of viral DNA were cloned into the *Acc*I or *Bam*HI site of phage M13mp18 DNA and then subjected to routine dideoxynucleotide sequence analysis (43). Sequences from the left-end region of Ad3, corresponding to the inverted terminal repetition and E1A promoter of Ad3, were obtained by Maxam-Gilbert chemical DNA sequencing protocols (31), as reported earlier (21, 24, 52). The exon-intron junctions, corresponding to the 12S and 13S E1A mRNA species of Ad3, have been confirmed by fluorescence automated sequencing (38) of Ad3 E1A-specific cDNA clones, described in a following section.

Infection of cells and early viral RNA preparation. HeLa cell cultures were infected with Ad3 at 1.5×10^{10} virion particles per 5×10^6 cells per plate (cells were about 80% confluent). Medium was removed from the cells before virus was added in a volume of 0.5 ml per plate, using phosphate-buffered saline with 2% calf serum. After 30 min at 37°C, the virus inoculum fluid was removed and replaced with 10 ml per plate of DMEM culture medium containing 5% calf serum, antibiotics, and antimycotic.

Six hours after infection, the cultures were washed with phosphate-buffered saline prior to extraction of RNA by the method of Chirgwin et al. (5). The infected cells were lysed by addition of 0.8 ml of 4 M guanidine isothiocyanate–25 mM sodium acetate (pH 6)–120 mM β-mercaptoethanol per plate. Lysates were forced through 18-gauge syringe needles to reduce viscosity, then layered over a 1.2-ml cushion of 5.7 M CsCl, and centrifuged at 37,000 rpm for 16 h at 20°C with a Du Pont AH50 rotor and OTD75 ultracentrifuge to pellet the RNA. The supernatant was removed, and the total RNA pellet was resuspended in 300 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. Polyadenylated RNA was isolated from total RNA by oligo(dT)-cellulose (Collaborative Research) column chromatography. Samples were applied to columns in 0.5 M NaCl–10 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.5% sodium dodecyl sulfate. Columns were washed with the application buffer and then with 0.1 M NaCl–10 mM Tris-HCl (pH 7.5)–1 mM EDTA. The polyadenylated RNA fraction was recovered by elution with 10 mM Tris-HCl (pH

7.5)–1 mM EDTA, and the concentration was estimated from the A_{260} measured with a microcell with a Beckman DU70 spectrophotometer. The eluted polyadenylated RNA fractions were ethanol precipitated and resuspended in diethylpyrocarbonate-treated water at a concentration of 0.5 μg/μl.

Synthetic DNA oligomers. The DNA sequencing primer (−40, M13 universal forward primer) was obtained from New England Biolabs or United States Biochemical Co. We are now preparing oligonucleotide primers for DNA sequencing in this laboratory with a Du Pont Generator single-column DNA synthesizer with standard phosphoramidite reagents (NEN-Du Pont). The two oligomers used in our Ad3 E1A cDNA cloning protocol were synthesized with a SAM-1 instrument, phosphoramidite reagents, and solvents from BioSearch: oligomer 365, 5′-TGATTTAGAGATAGAC GGGCCGGAG-3′; and oligomer 366, 5′-TACTAAGGTCCA AAGGTCCATCCCC-3′. The DNA sequences of these two oligomers flank, on opposite strands, the unique *Bam*HI and *Hin*dIII restriction sites in the 5′ and 3′ exons of the Ad3 E1A gene.

Ad3 E1A-specific cDNA. First-strand cDNA synthesis was performed with the polyadenylated fraction of RNA isolated early after infection of HeLa cells by Ad3 and the components of a cDNA synthesis kit from Promega. Priming of the first-strand cDNA was done with 1 μg of oligomer 366 (above) and 2 μg of polyadenylated RNA; it was annealed by heating and then cooled in a total volume of 10 μl. DNA synthesis proceeded for 60 min at 42°C in a 25-μl reaction mix containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 4 mM sodium PP_i, 1 mM each dATP, dCTP, dGTP, and dTTP, 25 U of RNasin, and 30 U of avian myeloblastosis virus reverse transcriptase. The reaction mixtures (and products) were frozen and stored at −20°C until the next step of cDNA amplification by polymerase chain reaction (PCR). These first-strand cDNA products represent the noncoding strand of the Ad3 E1A gene from the 3′ exon position flanking the *Hin*dIII site (oligomer 366 sequence) towards the 5′ exon and site of E1A transcription initiation.

Amplification and cloning of Ad3 E1A cDNA sequences. The components of a Perkin Elmer-Cetus GeneAmp PCR kit and the vendor's procedures were used, with some modifications, to synthesize a second-strand cDNA from the first-strand cDNA of the Ad3 E1A gene (above) and then to amplify the cDNA segments prior to cloning.

First-strand cDNA products (2 μl of the reaction mix described above) were combined with 10 μl of 10× PCR buffer (0.1 M Tris-HCl [pH 8.3], 0.5 M KCl, 15 mM $MgCl_2$, 0.1% gelatin), 18 nmol each of dATP, dCTP, dGTP, and dTTP, 100 pmol of oligomer 365, 90 pmol of oligomer 366, 64 μl of H_2O, and 2.5 U of *Taq* DNA polymerase in a final volume of 100 μl. Samples were covered with a layer of light mineral oil and amplified in a Perkin Elmer-Cetus thermal cycler. The initial denaturation step was 94°C for 150 s, followed by 45 amplification cycles of three steps: 60 s at 94°C, 120 s at 47°C, and 180 s at 72°C. The final extension step was 10 min at 72°C. The sample was removed from beneath the oil overlay and ethanol precipitated. DNA amplification products were analyzed by agarose gel electrophoresis.

The PCR amplification products, generated as described above with Ad3 early mRNA and cDNA primers 366 and 365, were digested with *Bam*HI and *Hin*dIII at the restriction sites just inside the primer DNA sequences. The DNA was ligated to *Bam*HI- and *Hin*dIII-restricted pEMBL18 (6) vector DNA and transformed into *E. coli* UT481 (F′ bearing) competent cells, using the ampicillin resistance marker for plasmid selection and white colonies to indicate an interrupted *lacZ* gene.

Recombinant phagemid DNA was isolated for DNA sequencing to identify the intron junctions associated with the 12S and 13S cDNA species and to screen for possible adventitious mutations resulting from the cDNA synthesis or PCR amplification steps prior to cloning. The f1 phage strain IR1 was used as the helper for propagation and packaging of the recombinant phagemid DNA. The universal M13 sequencing oligomer was used for sequencing of the single-stranded phagemid DNA, with a fluorescence automated Du Pont Genesis 2000 DNA sequencer (38). The sequences of the cDNA clones were compared with established sequences of the wild-type (wt) Ad3 E1A gene. Eight candidate clones of the 12S and the 13S cDNAs were analyzed, with only one apparent (missense) mutation found in one 13S cDNA clone. Later subcloning of this mutant's cDNA in an expression vector revealed modestly diminished transactivation of the Ad3 E1A-CAT reporter plasmid. The four 12S and four 13S clones each have intron junctions which agree precisely with the junctions reported for the closely related E1A gene of adenovirus type 7 (7).

The cDNA sequences of the eight cDNA candidates were excised from the pEMBL18 vectors by restriction with *Bam*HI and *Hin*dIII for substitution into the corresponding *Bam*HI-*Hin*dIII region of the modified genomic Ad3 E1A expression plasmid (described above). The restricted plasmid and cDNA fragments were mixed, ligated with T4 DNA ligase, and used to transform *E. coli*, with selection based on the plasmid ampicillin resistance gene. The desired plasmids were selected by agarose gel electrophoresis on the basis of reduced sizes compared with the genomic Ad3 E1A expression vector. The expression and activities of the cloned 12S and 13S Ad3 E1A genes were evaluated in cotransfections of HeLa cell cultures with E1A promoter-CAT gene expression plasmids. The four 12S candidate clones were indistinguishable in cotransfection functional assays (18, 51). Three of the four 13S candidate clones were also indistinguishable from one another in such assays.

Transfections of HeLa cells and analysis of E1A gene expression. HeLa cells were prepared for DNA transfections from cultures at approximately 50 to 70% confluence on plates. Transfections generally followed the procedures of Graham and Van der Eb (11), Gorman et al. (10), and Tibbetts et al. (18, 51) with modifications. Cells were removed from plates by trypsinization, pooled, and dispensed as equal aliquots into separate tubes for individual transfections in suspension. Each tube of cells (4 ml per transfection in DMEM–5% calf serum) received 200 μl of a transfection mixture containing 20 μg of plasmid DNA (carrier plus expression vector) and 5 μg of sheared, denatured salmon sperm DNA in HEPES-buffered phosphate (125 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 5 mM dextrose, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.05]). $CaCl_2$ was added to the transfection mixtures at 125 mM for precipitation with DNA, 30 min prior to transfection.

Following 3 h of gentle rotation at 37°C in the CO_2 tissue culture incubator, the transfected cells were mixed with equal volumes of cold 20% glycerol in DMEM and immediately centrifuged for 5 min at 2,500 rpm. The cell pellets were resuspended in 10 ml of DMEM–10% serum–antibiotics–antimycotic and returned to new culture plates for posttransfection incubation at 37°C.

Transfected cells were harvested at various times after

FIG. 1. DNA sequence of the Ad3 *Bgl*II K fragment, spanning the E1A gene. This sequence has been reported in U.S. patent 4,920,211. Coding regions are indicated in boldface. Numbers indicate base pairs along the E1A gene-encoding r strand, proceeding from the left end of the viral genome. Lowercase italic letters above the DNA sequence highlight landmarks, which are identified in Table 1. Sequences corresponding to oligomers 365 and 366, used in cDNA cloning procedures (see Materials and Methods), are underlined.

TABLE 1. Landmarks in the DNA sequence of the Ad3 E1A gene[a]

Landmark	Position (bp)	Description
a	95	*Hpa*I restriction site
b	135	End of left-end inverted terminal repeat (47)*
c	210	−300 E1A (distal) enhancer element (49)*
d	286	E2F binding motif, TTTCGCG (58)*
e	295	E2F binding motif, CGCGAAA (58)*
f	310	−200 E1A (proximal) enhancer element (49)*
g	327	E2F binding motif, TTTCGCG (58)*
h	479	E1A gene TATA element, TATTTA (39, 42)*
i	511	5′ end of E1A transcripts (39, 42)*
j	575	E1A translation start codon (39, 42)*
k	721	Sequence of oligomer 365 (underlined)
l	745	*Bam*HI restriction site
m	1061	12S E1A mRNA 5′ splice donor site
n	1155	13S E1A mRNA 5′ splice donor site
o	1250	Common E1A mRNA 3′ splice acceptor site
p	1385	*Hin*dIII restriction site
q	1408	Complement of oligomer 366 (underlined)
r	1453	E1A translation stop codon (39, 42)*
s	1461	*Pst*I restriction site
t	1495	Polyadenylation signal, AATAAA (39, 42)*
u	1514	E1A mRNA 3′ polyadenylation site (39, 42)*
v	1551	E1B gene TATA element, TATAA (39, 42)*
w	1565	*Bgl*II restriction site

transfection by scraping the cells from each plate into 1.2 ml of Versene (0.5 mM EDTA in phosphate-buffered saline). The suspensions were centrifuged in 1.5-ml microcentrifuge tubes for approximately 2 min at 12,000 rpm, and the cells were then resuspended in 150 μl of 0.25 M Tris-HCl (pH 7.5). The cell suspensions were subjected to three cycles of freeze-thawing between −80 and 37°C and then centrifuged in the microcentrifuge for 10 min at about 12,000 rpm. Cellular extracts were assayed for CAT enzyme activity in 100-μl reaction mixes with 80 nmol of acetyl coenzyme A and 2 to 2.5 nmol of [^{14}C]chloramphenicol, as described previously (51). One unit of CAT activity corresponds to acetylation of 1% of substrate (20 nmol) in 15 min at 37°C under our standard assay conditions. Sample extracts (50 μl of the 100-μl reaction mix) are routinely diluted in assays to provide results in the range of 1 to 50% substrate acetylation. Higher levels of acetylation are not linear with the concentration of CAT in the extract. Activities of CAT in the

[a] Features of Ad3 E1A gene topography are identified as landmarks which correspond to lowercase italic letters above the DNA sequence in Fig. 1. A schematic map of the Ad3 E1A gene is presented in Fig. 2. Features inferred by comparisons with published results for human Ad7 or other work are indicated by asterisks and reference citations, respectively.

extract are calculated to take into account the extent of extract dilution (if any). Average levels of CAT activity recovered from transfected cell cultures are presented plus or minus the standard error of the mean.

RESULTS

DNA sequence of the Ad3 E1A gene. The DNA sequence of the 1,569-bp *Bgl*II K restriction fragment of human Ad3 is presented in Fig. 1. This figure represents new data and previously published data for the entire length of both complementary strands. Landmarks within the DNA sequence include the genomic inverted terminal repetition of Ad3 (52), some of the *cis* elements of the Ad3 E1A promoter (12, 18, 21), and sites associated with transcription and mRNA processing of the E1A gene (7, 54). These landmarks and others are described in Table 1. Figure 2 provides a schematic map of the Ad3 E1A gene and its two major early mRNA species.

The DNA sequence of the Ad3 E1A gene is very similar to that determined for the closely related subgroup B serotype Ad7 (7). The E1A gene-coding regions of Ad3 and Ad7 differ by only 10 of 786 bp. Their 13S mRNA translation products differ by only 3 of 262 amino acids, as I for V, V for I, and I for R (single-letter amino acid code). The 12S- and 13S-specific 5′ splicing donor sites and common 3′ splice acceptor sites of Ad3, determined as described later, are identical to those specified by Ad7. The 13S intron and the 5′ and 3′ noncoding DNA sequences of these two viruses differ by only 19 of 779 bp. The enhancer elements (12) and other identified *cis* elements of their E1A promoters are identical DNA sequences.

The close relationship of the subgroup B Ad3 and Ad7 E1A genes is reminiscent of that of the E1A genes of subgroup C serotypes Ad2 and Ad5 (54). Aligned DNA sequences of Ad3 and Ad7 or of Ad2 and Ad5 reveal 98 to 99% sequence identity. Aligned DNA sequences representing the two different subgroups, such as Ad5 and Ad7, show only 52% sequence identity through the E1A gene region. Conserved features in the E1A regions of subgroup B and subgroup C adenoviruses include an inverted terminal repeat region and left-end origin of DNA replication, transcriptional enhancer elements at −200 and −300 bp, a TATA element of the E1A promoter as TATTTA, 40 bp of nearly identical DNA sequence about the E1A 5′ RNA cap site, conserved functional domains 1, 2, and 3 of the E1A coding sequences, and a nuclear homing signal at the carboxy terminus of E1A proteins.

We and others (14, 18, 25, 54) have observed differences among functional components of subgroup B and subgroup C E1A genes. These distinguishing features include the number and arrangement of E2F binding sites in E1A promoters, different 12S E1A splice donor sites, and DNA sequence and separation of conserved functional domains 2 and 3. A schematic comparison of the Ad5 and Ad3 E1A gene structures, similar to the format of Fig. 2, was presented in our previous report (Fig. 1 of reference 18).

Cloning and expression of Ad3 E1A-specific cDNA. Different levels of Ad5 and Ad3 E1A gene expression early after infection with wt and mutant viruses, have been attributed to different responses of their E1A promoters and also to different activities of E1A gene products (18, 25, 51). Our recent work with cDNA expression clones of the Ad5 E1A gene localized determinants of these different autoregulatory responses in the upstream DNA sequences of the Ad5 and Ad3 E1A promoters. In order to analyze possible differences

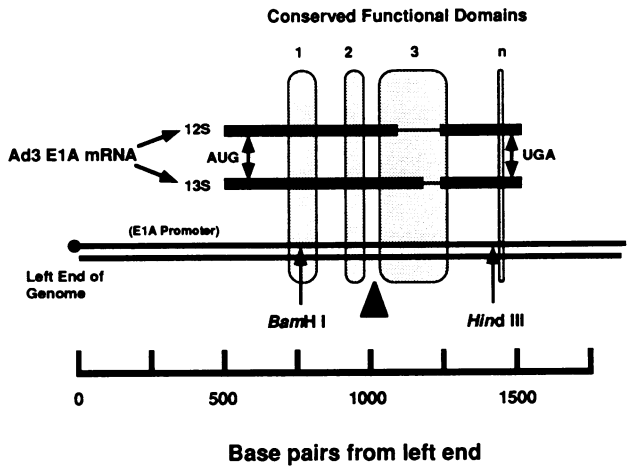

FIG. 2. Map of the left end of the Ad3 viral genome, including the E1A gene and its 12S and 13S mRNA species. The thick horizontal lines above the double-stranded DNA genome (left-end segment) represent the two major early mRNA species of the E1A gene. The thin lines in the middle of the mRNAs correspond to the introns removed by RNA splicing of the primary E1A transcript. The shaded areas surrounding the DNA and mRNA sequences correspond to the three highly conserved functional domains of adenovirus E1A genes (30–32). The very thin area near the 3′ end of the gene, labeled n, corresponds to the conserved pentapeptide region which has been associated with nuclear targeting of E1A proteins from the cytoplasmic site of mRNA translation (44). The unique sites for restriction endonucleases *Bam*HI and *Hin*dIII are shown in the 5′ and 3′ exons of the gene, respectively. The AUG start codon and UGA stop codon of the mRNA species are indicated by the vertical double arrows. The large black wedge highlights the nonconserved region of variable length and DNA sequence between conserved functional domains 2 and 3. This region may be of importance in modulating the negative and positive transcriptional regulation activities associated primarily with the conserved domains (25). An alignment of these landmarks of the Ad3 E1A gene with corresponding features of the more familiar Ad5 E1A gene was presented as Fig. 1 in our recent report (18).

in Ad3 and Ad5 E1A gene product activities, it was necessary to construct cDNA expression clones corresponding to the 12S and 13S mRNA species of the Ad3 E1A gene.

Several conventional protocols for synthesis and cloning of E1A cDNA expression clones were unsuccessful, in our laboratory and others. The successful procedure described in Materials and Methods involved synthesis of a segment of first-strand cDNA with mRNA purified from cells early after Ad3 infection, reverse transcriptase, and an oligonucleotide primer complementary to the 3′ exon sequence of Ad3 E1A mRNA. A second primer, representing the 5′ exon sequence of the Ad3 E1A mRNA, *Taq* DNA polymerase, and an additional quantity of the first primer were used in a PCR procedure to synthesize the second-strand cDNA and then amplify the corresponding cDNA sequence.

An analysis of the cDNA synthesis-amplification products by agarose gel electrophoresis is presented in Fig. 3. More than 90% of the total products represent species smaller than 500 bp, and these do not appear to represent the Ad3 E1A gene. In spite of the specific oligonucleotide primers used for cDNA synthesis and amplification, an identical pattern of these abundant, smaller products is obtained with mRNA isolated from uninfected HeLa cells. These spurious, if perhaps significant, products appear with a wide variety of reaction conditions, including different durations, tempera-

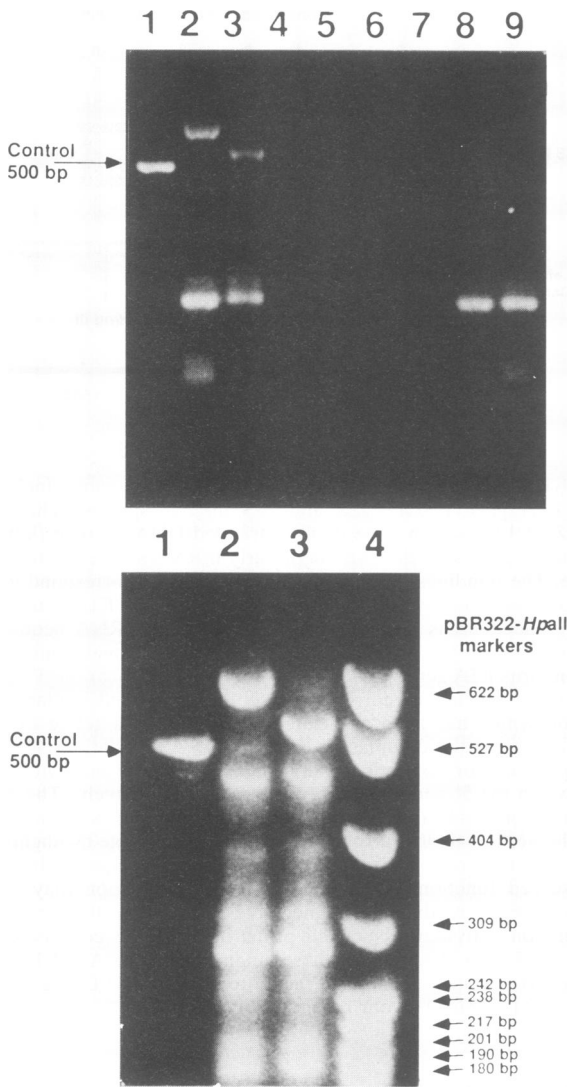

FIG. 3. Analysis of the products of cDNA synthesis and PCR amplification in 1.8% agarose gels (3 V/cm for 16 h in 0.1 M Tris-borate [pH 8.3]–1 mM Na_2H_2EDTA–0.5 μg of ethidium bromide per μl). The products of the cDNA PCR amplification reactions are visualized by ethidium fluorescence on longwave UV transillumination. (Top) Lane 1 corresponds to the 500-bp control product DNA provided with the PCR GeneAmp kit. Lanes 2, 3, 8, and 9 show PCR products amplified from HeLa cell mRNA preparations with oligomers 365 and 366, as described in Materials and Methods. Lanes 8 and 9 show products from mRNA isolated from uninfected HeLa cells. These extraneous products also appear in lanes 2 and 3, which were derived from Ad3-infected HeLa RNA. Lanes 3 and 9 show the same products as in lanes 2 and 8 following digestion with *Bam*HI and *Hin*dIII restriction enzymes. Ad3 E1A-specific products, susceptible to cleavage by *Bam*HI and *Hin*dIII, appear as the largest species in lanes 2 and 3 and correspond to the 13S cDNA product. A slightly smaller and less abundant species, the 12S cDNA product, is also present but difficult to see unless the gels are overloaded with products. The empty lanes 4 and 5 represent negative controls for mock reactions lacking only reverse transcriptase, and lanes 6 and 7 represent negative controls for mock reactions lacking only the oligonucleotide primers. (Bottom) In this overloaded gel, lanes 1, 2, and 3 correspond to lanes 1, 2, and 3 in the top panel, respectively. Lane 4 shows length markers derived from a pBR322 DNA *Hpa*II restriction digest. The two Ad3 E1A-specific cDNA products appear as 630- and 535-bp species in lane 2. The smaller species corresponds to 12S Ad3 E1A cDNA product and is typically recovered in amounts less than 10% of the larger, 13S-specific cDNA product. Each is reduced by about 75 bp following cleavage by *Bam*HI and *Hin*dIII (lane 3).

tures and repetitions of the thermal cycling, and concentrations of monovalent and divalent salts or *Taq* DNA polymerase.

The products of reactions initiated with mRNA from Ad3-infected cells contain two additional species, about 630 and 535 bp. The ratio of yields of the larger and the smaller products, estimated by ethidium fluorescence in the gel, is typically about 10:1. The lengths are those expected for the Ad3 E1A cDNA products, predicted from the Ad3 DNA sequence, the specific oligomer primers, and the intron junctions described for Ad7 E1A mRNA (7). Treatment with *Bam*HI and *Hin*dIII reduces the lengths of the two Ad3-specific products by about 75 bp each. The sizes of the nonspecific products do not appear to change following treatment with these restriction enzymes. Apparently, only the two Ad3 mRNA-specific products have the *Bam*HI and *Hin*dIII restriction sites associated with the amplified regions of the 5′ and 3′ exons of the Ad3 E1A gene.

The *Bam*HI- and *Hin*dIII-treated cDNA products from Ad3-infected mRNA preparations were ligated with *Bam*HI- and *Hin*dIII-restricted pEMBL18 (6) vector DNA for cloning of the tentatively identified Ad3 cDNA sequences. Several transformants with inserts of 550 or 457 bp were selected. DNA sequence analysis confirmed these to be cDNA fragments corresponding to the Ad3 E1A gene 13S and 12S mRNA species, respectively. Ad3 E1A 12S and 13S cDNA inserts were excised from the pEMBL18 vectors and substituted for corresponding genomic DNA sequence between the *Bam*HI and *Hin*dIII sites of the modified Ad3 E1A gene expression plasmid (51) (see also Materials and Methods). These new Ad3 E1A cDNA constructs were then evaluated for transcriptional regulation activities by cotransfection analysis of transient-gene expression with CAT reporter gene expression plasmids. Ad3 E1A-CAT reporter gene expression was analyzed in cells cotransfected with the 12S or 13S Ad3 E1A cDNA expression clone (Fig. 4).

Significantly lower levels of CAT gene product, 29% of the control values, were recovered from cells cotransfected with the 12S cDNA clone than from cells transfected with the Ad3 E1A-CAT plasmid alone. This is consistent with enhancer-targeted repression of transcription, expected for the 12S cDNA species and the Ad3 E1A promoter used for expression of the CAT gene. The activities of four different constructs of the Ad3 E1A 12S cDNA expression plasmid were indistinguishable in similar cotransfection assays with the repressible Ad3 E1A-CAT, Ad5 E1A-CAT, and pSV2-CAT (10) reporter plasmids (results of these comparisons not shown).

Transactivation of the Ad3 E1A-CAT plasmid is evident in cotransfections with the Ad3 E1A 13S cDNA expression clone (Fig. 4). Three different constructs of the Ad3 E1A 13S cDNA expression plasmid showed similar transactivation activities, 20-fold stimulation over controls, in cotransfection assays under these conditions. A fourth 13S cDNA clone had two- to threefold-lower transactivation activity in such assays, but it was later determined to have sustained a single base change (C_{1142}→T), a missense mutation (H→Y) near the right end of conserved functional domain 3 (results not shown). This mutation is likely to have been introduced early during the rounds of DNA synthesis and

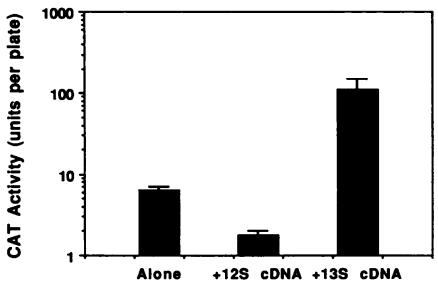

FIG. 4. CAT gene expression in cell cultures cotransfected with Ad3 E1A-CAT (10 μg per plate) and the 12S or 13S cDNA clone of the Ad3 E1A gene (3 μg per plate). Repressive activity of the 12S cDNA clone is evident from the lower level of CAT gene product harvested 42 h after cotransfection. The average amount of CAT product recovered from these cultures was only 25 to 30% of that recovered from cells transfected by Ad3 E1A-CAT alone. Transactivation activity of the 13S cDNA clone appears as levels of CAT product 20 times greater than in cells transfected by Ad3 E1A-CAT alone. Error bars indicate intervals 3 standard errors above the average levels of CAT (6 to 15 samples per average).

amplification with avian myeloblastosis virus reverse transcriptase and *Taq* DNA polymerase.

E1A gene autoregulation with 12S and 13S cDNA clones of Ad5 and Ad3. The availability of 12S and 13S cDNA clones of both the Ad5 and Ad3 E1A genes enables analysis of the positive and negative autoregulation of the transient expression of these genes in transfected cells. Parallel experiments permit comparisons of E1A promoter responses and effects of E1A gene products in homologous and heterologous cotransfections. The data summarized in Table 2 represent multiple transfection experiments performed with several different batches of HeLa cells. Each set of transfections was performed with either the Ad5 or Ad3 E1A promoter for CAT gene expression, at 10 μg of E1A-CAT plasmid per plate, alone or cotransfected with the 12S or 13S cDNA clone of the Ad5 or Ad3 E1A gene at 3 μg per plate. CAT gene product activity was assayed in extracts from the cultures 40 to 44 h after transfection.

The average levels of CAT from transfected cell cultures presented in Table 2 have larger variances than would have been the case if the experiments were performed as replicates with a single batch preparation of HeLa cells for transfection. Our other dose-response and time course experiments (Fig. 5 and 6) (reference 18 and unpublished results) indicate that this particular set of conditions—batch of cells, inputs of reporter plasmid (10 μg per dish) and E1A gene plasmid (3 μg per dish), glycerol shock treatment, and time after transfection—typically underestimates the potential levels for E1A autotransactivation. With 10 to 100 times less E1A (13S cDNA or genomic) plasmid input, transactivation often approaches 500-fold-higher expression of the Ad3 E1A-CAT gene. Nevertheless, the results presented in Table 2 indicate that the different responses of Ad3 and Ad5 E1A promoters are statistically significant ($P < 0.05$). Statistically significant differences are also observed for the transactivating activities of the Ad3 and Ad5 13S E1A cDNA genes.

Homologous cotransfection experiments, in which the E1A-CAT and the E1A-cDNA clones have the same promoters, generate CAT product which should reflect the levels of transcription from that E1A promoter in the transfected cells. With this consideration, the data in Table 2 support the following conclusions.

TABLE 2. E1A-CAT gene expression in cells transfected alone or with homologous and heterologous E1A 12S and 13S cDNA clones[a]

Transfecting plasmid	Cotransfecting plasmid	No. of independent transfections	CAT activity (U/plate)
Single transfections			
Ad3 E1A-CAT		15	6.3 ± 0.3
Ad5 E1A-CAT		15	6.7 ± 1.2
Homologous cotransfections			
Ad3 E1A-CAT	Ad3 12S cDNA	6	1.8 ± 0.2
Ad5 E1A-CAT	Ad5 12S cDNA	6	0.6 ± 0.1
Ad3 E1A-CAT	Ad3 13S cDNA	9	113.7 ± 13.0
Ad5 E1A-CAT	Ad5 13S cDNA	9	16.2 ± 1.9
Heterologous cotransfections			
Ad3 E1A-CAT	Ad5 12S cDNA	3	1.6 ± 0.1
Ad5 E1A-CAT	Ad3 12S cDNA	6	1.2 ± 0.5
Ad3 E1A-CAT	Ad5 13S cDNA	9	221.7 ± 37.7
Ad5 E1A-CAT	Ad3 13S cDNA	9	2.9 ± 0.6

[a] HeLa cell cultures were transfected by the procedure described in Materials and Methods. Each culture dish of HeLa cells received 10 μg of the indicated E1A-CAT reporter plasmid and, if cotransfected, 3 μg of the indicated E1A cDNA expression plasmid. The number of independent transfections is specified with the mean and standard error for CAT activity per plate, recovered 40 to 44 h after transfection.

(i) Similar levels of E1A-CAT transcription are supported by the Ad3 and Ad5 E1A promoters in the absence of E1A cDNA expression clones.

(ii) Lower levels of transcription from the Ad3 and Ad5 E1A promoters occur in cells homologously cotransfected with 12S E1A cDNA expression vectors. This autorepression assay led to levels of CAT product which were 9 and 29%, respectively, of those expressed by the Ad5 and Ad3 E1A-CAT vectors alone. These data suggest, as reported earlier (18), that the Ad5 E1A promoter is more sensitive to autorepression than the Ad3 E1A promoter.

(iii) The autoactivated expression of CAT from the Ad3 E1A promoter with the homologous 13S cDNA of Ad3 E1A is at least seven times greater than the homologous transactivation of the Ad5 E1A promoter with the 13S cDNA of Ad5 E1A.

Comparisons of the results of homologous and heterologous cotransfection experiments lead to additional observations, including the following.

(iv) Transcription of Ad3 and Ad5 E1A promoters is repressed by expression of either the Ad3 or Ad5 12S cDNA gene. The levels of repression effected by the Ad3 and Ad5 12S cDNA clones are not statistically distinguishable.

(v) The Ad3 E1A promoter responds much more strongly to transactivation by the Ad3 or Ad5 13S E1A cDNA than does the Ad5 E1A promoter, as reported earlier (18). The Ad5 13S cDNA gene is at least two times more effective than the Ad3 13S cDNA clone as a transactivator of the Ad3 E1A promoter.

The responses of the Ad3 and Ad5 E1A promoters to homologous or heterologous autorepression are similar, although the Ad5 E1A promoter appears to be slightly more sensitive to repression. The autorepressive activities of the Ad3 and Ad5 E1A 12S cDNA genes appear to be very similar.

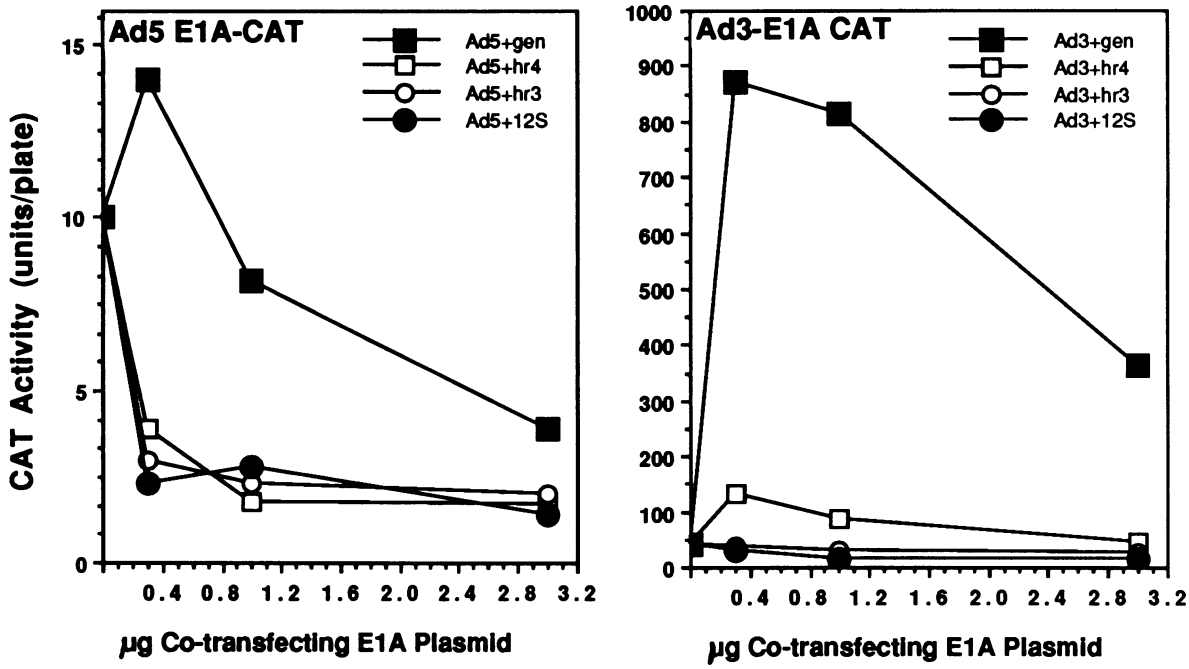

FIG. 5. Effects of Ad5 *hr*4 and *hr*3 mutants (domain 3, transactivation) on the cotransfection CAT gene expression from Ad3 and Ad5 E1A-CAT vectors. (Left) Dose-response analysis of cotransfecting Ad5 E1A plasmids for CAT gene expression under control of the Ad5 E1A promoter. The genomic (gen) form of the wt Ad5 E1A gene is weakly stimulating to CAT gene expression at very low input and repressive at higher inputs compared with expression in cells transfected with the Ad5 E1A-CAT plasmid alone. The repressive 12S cDNA of wt Ad5 strongly decreases the level of expression from the CAT reporter vector, as is also the case in cells transfected with genomic clones representing the domain 3 mutants *hr*4 and *hr*3. The mutants appear to abrogate what little transactivating activity is revealed by this assay with the poorly activated Ad5 E1A promoter. (Right) Corresponding experiments performed with the strongly activated Ad3 E1A-CAT vector. The change in ordinate scale reflects the very high levels of transactivation obtained in cells cotransfected with the genomic Ad5 E1A gene. Again, the *hr*4 and *hr*3 mutants are severely debilitated in their capacity to transactivate the Ad3 E1A promoter, although the data indicate that the *hr*4 lesion is somewhat less deleterious.

In distinct contrast, the Ad5 13S E1A cDNA gene product appears to have about 15-fold-higher transactivation activity, with the transactivation-responsive Ad3 E1A promoter target, than does the homologous Ad3 13S cDNA gene product. We draw this conclusion from a comparison of the results from different cotransfections. Lower levels of Ad5 than Ad3 E1A 13S cDNA gene expression take place in cotransfected cells, as judged from the relative levels of homologous E1A-CAT expression. Nevertheless, two-times-higher levels of Ad3 E1A-CAT transactivation are observed in cells cotransfected with the Ad5 than with the Ad3 13S cDNA gene. The results from different heterologous and homologous cotransfection experiments, using single batches of HeLa cells and lower inputs of the 13S cDNA expression plasmids, suggest that this difference in Ad5 and Ad3 13S E1A transactivation activities may be significantly greater than may be inferred from the data in Table 2 alone.

We acknowledge that alternative explanations of these results can be framed on the basis of variations in the E1A protein levels (stability, turnover, translatability, compartmentalization) in cells transfected with the various E1A expression mutants. Lacking reagents and data pertaining to the differential stability of variant E1A proteins, we prefer the plausible assumption that levels of transcription, reflected by the relative accumulations of the CAT reporter gene product, are the primary determinant of variation in E1A protein levels. A second concern arises in our use of E1A genes under the control of different E1A-responsive E1A promoters. To date, however, no eukaryotic promoter which is quantitatively refractile to the positive and/or negative transcriptional regulation effected by E1A gene products has been described.

Positive E1A gene autoregulation and conserved functional domain 3. It is plausible that the conserved functional domains of the adenovirus E1A gene, associated with positive and negative regulation of the transcription of other early viral and cellular genes (14, 30, 33), act in similar fashion in the transcriptional autoregulation of the E1A gene. Well-characterized plasmids bearing missense mutations in functional domains 2 and 3 of the Ad5 E1A gene were generously made available to us to test this assumption. In addition, these new cotransfection experiments provided further insight into the different balances of positive and negative autoregulation which characterize the E1A genes of Ad5 and Ad3.

Figure 5 presents the results of cotransfections of HeLa cells with the Ad3 and Ad5 E1A-CAT expression vectors and E1A genes of the Ad5 mutants *hr*3 and *hr*4. These missense mutations, characterized by the work of Glenn and Ricciardi (9), represent single-base changes in functional domain 3 of the Ad5 E1A gene. They compromise transactivation of E1A-dependent early promoters, such as those of the adenovirus E2, E3, and E4 genes. The left panel of Fig. 5 shows the dose-response curves of Ad5 E1A-CAT reporter gene expression with increasing amounts of cotransfecting E1A expression plasmids. Genomic wt Ad5 E1A provides limited transactivation of the Ad5 E1A promoter, but CAT

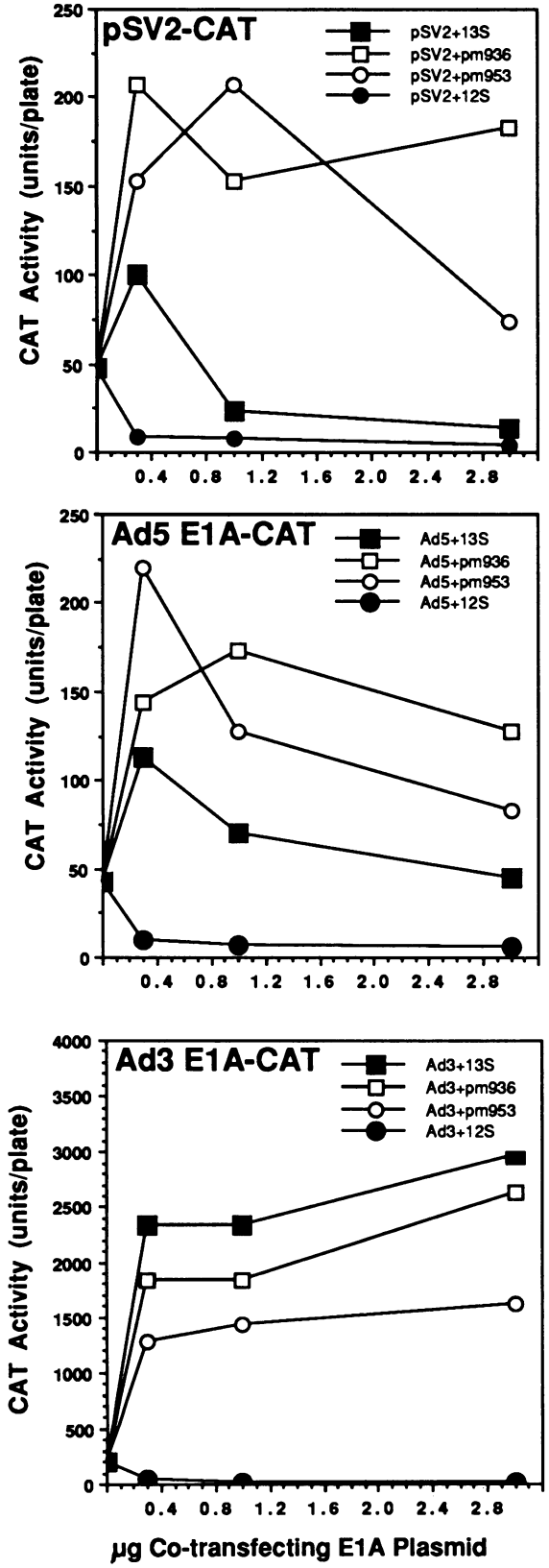

FIG. 6. Effects of the Ad5 *pm*936 and *pm*953 mutations (domain 2, repression), as cotransfecting 13S cDNA, on CAT gene expression from the pSV2-CAT and E1A-CAT vectors. (Top) The 12S cDNA, and to a lesser extent the 13S cDNA, of the wt Ad5 E1A gene products do appear at higher levels than in the cotransfections with the repressing wt Ad5 12S cDNA clone. The genomic clones expressing the mutant *hr*4 and *hr*3 E1A genes lead to repression levels similar to that of the wt 12S cDNA clone. This is consistent with the loss of autotransactivation activity due to the mutations in functional domain 3 of the Ad5 E1A gene.

The right panel of Fig. 5 shows the corresponding analysis performed as cotransfections with the Ad3 E1A-CAT reporter plasmid. The very different ordinate scales of the two panels reflect the much greater response of the Ad3 E1A promoter to transactivation by the wt Ad5 E1A gene. The level of Ad3 E1A-CAT expression is much lower in cells cotransfected with the mutant *hr*4 and *hr*3 E1A genes. In the case of the *hr*4 mutant, this experiment suggests diminished but not entirely abrogated transactivation activity.

Negative E1A gene autoregulation and conserved functional domain 2. Point mutations in functional domain 2 of the Ad5 E1A gene should compromise the enhancer-targeted repression activity of both 12S and 13S cDNA clones of the Ad5 E1A gene. Such mutations are represented by the Ad5 missense mutants *pm*936 and *pm*953, characterized by Lillie and Green (29). Figure 6 presents an analysis of CAT gene expression from three different promoters in cotransfections with wt and mutant forms of the Ad5 E1A gene.

The top panel of Fig. 6 shows the results obtained with the SV2-CAT (10) reporter gene. The early simian virus 40 (SV40) promoter has an enhancer element (72-bp repeat) which is a target for repression mediated by Ad5 E1A gene products. This promoter is not generally recognized as being responsive to transactivation by adenovirus E1A products. Except at the lowest inputs of cotransfecting 13S wt Ad5 cDNA, both cDNA forms of the wt Ad5 E1A gene repress transcription from the SV2 promoter. In cotransfections with 13S cDNA clones representing mutants *pm*936 and *pm*953, there appears to be no repression of the SV2 promoter but rather a modestly elevated level of CAT expression, only twice that recovered from cells transfected with SV2-CAT alone.

Expression of the Ad5 E1A-CAT reporter gene was evaluated with the same set of cotransfecting Ad5 E1A genes (Fig. 6, middle panel). The results are remarkably similar to

represses the enhancer-dependent transcription from the early SV40 promoter in the plasmid pSV2-CAT (41). The dose-response curves reveal no repression by the 13S cDNA clones corresponding to the two missense mutations of the Ad5 E1A gene. (Center) Remarkably similar results obtained when Ad5 E1A-CAT is used as the reporter. Again, the 12S cDNA of the wt Ad5 E1A gene represses the expression controlled by the Ad5 E1A promoter. This repression is compromised by the missense mutations. The transactivation expected in cotransfection with the wt Ad5 13S cDNA gene is weak. The overall patterns of results with the SV40 early promoter and the Ad5 E1A promoter are remarkably similar, including the ordinate scales of CAT activity. (Bottom) Effects of the Ad5 E1A domain 2 lesions on transactivation of transcription from the Ad3 E1A promoter. In this case, the 13S cDNA clones of the wt Ad5 and the mutants are all transactivating the reporter gene through its Ad3 E1A promoter. The Ad5 12S cDNA control represses the Ad3 E1A promoter. The plasmids with either mutation of domain 2, however, have less transactivation activity than the wt Ad5 13S cDNA gene. This suggests modulating effects of mutations in one domain on complementary activities associated with another domain in the same protein. Notice again the change in ordinate scale in this panel to reflect the significantly higher levels of CAT gene expression from the transactivation-responsive Ad3 E1A promoter.

those obtained with SV2-CAT described above. Cotransfection of Ad5 E1A CAT with the wt Ad5 13S cDNA shows only slightly greater activation than cotransfections with the Ad5 12S cDNA. The 13S cDNA clones of point mutants *pm*936 and *pm*953 do not appear to be capable of repressing the Ad5 E1A promoter, but the levels of transactivation effected by these mutants' E1A genes (as 13S cDNA) are not significantly greater than observed in the SV2-CAT experiment. These results were also confirmed in replicate cotransfections performed under the conditions described in Table 2, footnote *a* (results not shown). The Ad5 E1A promoter thus appears to be intrinsically weak in its response to 13S E1A-mediated transactivation. Autorepression does not appear to be a functional block to an otherwise stronger response to transactivation by the 13S cDNA genes of Ad5 wt, *pm*936, or *pm*953.

The last experiment of this series was done with the Ad3 E1A-CAT reporter gene (Fig. 6, bottom). The ordinate scale in this panel again reflects the intrinsically high response of the Ad3 E1A promoter to activation by the 13S cDNA products of the Ad5 E1A gene. wt Ad5 and the mutants *pm*936 and *pm*953 all lead to strong transactivation of the Ad3 E1A promoter when cotransfected as 13S cDNAs. A surprising result in this experiment is the lower level of transactivation activity associated with both mutant forms of the Ad5 13S cDNA. The locus of each mutation is within functional domain 2, associated with enhancer-targeted repression activity. Apparently even a subtle change, such as a missense mutation in one functional domain of the E1A protein, can modulate other functions associated with adjacent or distal domains.

The apparently lower transactivation of Ad3 E1A CAT by the Ad5 *pm*936 and *pm*953 E1A genes, shown in the bottom panel of Fig. 6, may reflect differences associated with the heterologous Ad3 E1A promoter as a target for transactivation. This is not particularly surprising, since the Ad5 E1A promoter, used to express the Ad5 wt and mutant 13S E1A cDNA genes in these experiments, does not respond well to transactivation.

DISCUSSION

DNA sequencing of similar genes from closely related organisms is often the first point of entry into problem areas associated with prior and continuing evolution of gene structures and functions. Alignments of similar DNA or RNA sequences readily reveal a molecular topography of conserved and nonconserved domains in the gene of interest, reflecting a cross section of variation of that gene within a contemporary population. Establishing consensus sequences may prompt entertaining speculation about the derivation of contemporary variants from a putative common precursor. The conclusions drawn from such analysis may even be correct, if the contemporary pool of variants is derived from a relatively homogenous founder population. In general, however, this type of analysis is suspect, to the extent that one discounts the likely heterogeneity of the gene's precursors and temporal recombining intermediates.

A more immediate and productive application of comparisons of DNA sequences is correlation of conserved or nonconserved regions of the gene with specific functions of the gene products. Such mapping of functional domains develops by assay of activities associated with the gene as derived from wild-type strains, prevalent allelic variants, or spontaneous and induced mutants. There are risks associated in studies preoccupied with singular models of a particular gene's structure-function relationships. There are also risks to be managed with the notion that the most highly conserved of variant DNA sequences must be associated with domains of essential function.

Structural and functional dissection of the adenovirus E1A gene. Consideration of the adenovirus E1A gene and its autoregulation, as revealed from studies of the Ad3 and Ad5 models, leads to several ironies. The E1A promoters of subgroup B and subgroup C adenoviruses have identical duplicate E1A enhancer elements (12, 21, 40, 54). Spontaneous or induced deletions of the enhancer region cripple Ad5 E1A gene expression and Ad5 infections, but similar deletions in the Ad3 genome impair neither E1A gene expression nor the course of productive infection. What distinguishes these highly conserved elements as either essential or dispensable for the expression of the Ad5 and Ad3 E1A genes?

Dissection of the adenovirus E1A promoter has revealed multiple arrays of other *cis* elements, in particular, known motifs for interactions with cellular transcription factors such as E2F, CREB/ATF, and enhancer-binding factors. Brute-force deletions of selected regions of the E1A promoter can promote ordinarily subordinate elements to more dominant roles in the basal-level or autoregulated expression of the E1A gene. The design of assays, the selection of a particular model adenovirus, and the nature of specific mutagenic lesions may confound interpretation of structure-function relationships. For example, separate reports have asserted both DNA sequence independence and specific DNA sequence determinants involved in positive and negative autoregulation of the E1A promoter (13, 18). Similarly, conflicting reports have appeared with respect to the role of promoter targets of other E1A-dependent transcription units (20, 22, 26, 27, 44).

Ad3 E1A gene DNA sequence. The DNA sequence of the Ad3 E1A gene, presented in Fig. 1 and Table 1, is very similar to that of the closely related Ad7 E1A gene. This anticipated result provides a limited set of sequence differences which may be predicted to have minimal impact on the autoregulated expression of the E1A genes of these similar viruses. Alignment of the Ad3 E1A gene sequence with that of Ad5 follows the precedents established in the literature (14, 30, 33, 54) and prompts generalizations with regard to the coincidence of functional domains (generally mapped in Ad2 or Ad5) with the highly conserved domains of DNA sequences. The DNA sequence of the Ad3 E1A gene supports our selection of this serotype as a representative of the B subgroup with our present emphasis on functional diversity of E1A gene autoregulation in subgroup B and subgroup C adenoviruses.

Diverged patterns of promoter and product roles in E1A gene autoregulation. The results of homologous and heterologous cotransfections presented in this report suggest that extensive functional diversity has developed in the autoregulation of the Ad3 and Ad5 E1A genes. At the simplest level, this diversity is reflected by lower expression levels of the Ad5 E1A gene than of the Ad3 E1A gene (51). Our recent report (18) suggested that a primary determinant of this difference in the autoregulated E1A gene expression of Ad3 and Ad5 lies in the upstream DNA sequences, which specify different responses of E1A promoters to 13S E1A-mediated transactivation. The results presented in Table 2 and Fig. 6 further support this conclusion. The Ad5 E1A promoter behaves more like the SV40 early promoter, which is generally thought to be repressed and not transactivated at all by the action of adenovirus E1A gene products. The Ad3 E1A

promoter can be repressed by 12S cDNA products, but it is remarkable for its strong response to transactivation by 13S E1A gene products.

These results suggest a complementary coevolution of the E1A promoters and their autoregulating E1A gene products. The poorly transactivated Ad5 E1A promoter supports expression of a 13S cDNA product with greater transactivating activity. The opposite situation is found in the Ad3 E1A gene. The strongly transactivated Ad3 E1A promoter supports expression of a 13S cDNA product with lower transactivating activity.

The perspective on E1A gene autoregulation and complementary patterns of promoter-product coevolution provokes interest in the regulated expression of other early genes of Ad3 and Ad5. Adenoviral early-gene expression is generally recognized to be E1A dependent. In the case of Ad5, early-gene expression takes place in a milieu of limited quantities of the apparently strong Ad5 13S E1A transactivator. Basal-level expression of Ad5 early genes appears to be sufficient to evade abortive infection (at high multiplicity) with Ad5 *dl*312, a mutant which lacks the Ad5 E1A gene altogether.

In the case of Ad3, the early genes are expressed in the presence of very large quantities of what appears to be a weaker 13S E1A transactivator product. The E1A autoregulation mutant Ad3 *hr*15 (23, 25) invites comparison with Ad5 *dl*312. Although Ad3 *hr*15 encodes wt Ad3 E1A products, the result is apparent shutdown of further E1A transcription. Limited expression of the E1A gene supported by the mutant promoter is lethal; infection is aborted, with minimal cytocidal effects even at very high multiplicity. This may imply that the expression of other Ad3 early genes has coevolved to a state of greater dependence on the E1A transactivation function.

Another provocative problem is presented by the differences in E1A product activities and responses of the coevolved E1A gene promoters. Many host genes of adenovirus-infected or -transformed cells respond to positive or negative regulation by adenovirus E1A gene products. The promoters of these cellular genes do not typically resemble the particular arrays of enhancer elements or E2F motifs which characterize E1A promoters. We should anticipate that functional divergence of adenovirus E1A genes, revealed in our studies of autoregulation, is likely to be manifested in highly variable regulation of cellular genes, which are not constrained to coevolve with the adenovirus E1A gene. As suggested in the introduction, such variation may play determinant roles in the diverse tissue tropisms and pathoepidemiology of human adenovirus serotypes and subgroups.

Interdependence of functional domains and activities of E1A proteins. A structural relationship of conserved domains 2 and 3 appears to mutually influence their complementary functions in transcriptional regulation. The results presented in Fig. 6 illuminate this point. The missense mutations in Ad5 *pm*936 and *hr*953 compromise enhancer-targeted repression activity, associated with their loci in conserved domain 2. However, the assays with the transactivation-responsive Ad3 E1A-CAT reporter gene show significantly lower transactivation with these mutant Ad5 E1A genes. The single amino acid changes corresponding to their missense mutations appear to change the overall structure of the mutant 13S cDNA products and thereby compromise (in quantitative terms) the function of the distal transactivation domain.

This leads us to consider that the DNA sequence between conserved domains 2 and 3 (black wedge in Fig. 2) is a region of the E1A gene which is balanced in coevolution with the upstream, transactivation-responsive elements of the E1A promoter. This is reminiscent of the structure-function relationship associated with mutant Ad3 *hr*15 and its second-site revertants during passage of viral stocks in this laboratory (23, 25). The level of E1A gene expression in Ad3 *hr*15 represents effective transcriptional shutdown of the gene, while the same promoter overexpresses the E1A genes which bear reverting deletions in the variable region between conserved functional domains 2 and 3. Perhaps this mutant and its revertants functionally recapitulate aspects of the evolution of E1A gene autoregulation among adenovirus serotypes in the field. These aspects are the apparent reiteration and divergence of *cis* elements in the E1A promoter and deletions (or insertions) in variable regions separating conserved, functional domains in the E1A gene-coding region.

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