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Upstream DNA Sequences Determine Different Autoregulatory Responses of the Adenovirus Types 5 and 3 E1A Promoters

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Adenovirus types 5 and 3 (Ad5 and Ad3), two human adenovirus serotypes of evolutionarily divergent subgroups, show very different levels of E1A gene expression early after infection of permissive cells. Since adenovirus E1A gene expression is known to be transcriptionally autoregulated, we have investigated the difference between Ad3 and Ad5 by monitoring transient expression of a reporter gene under transcriptional control of the E1A promoter of Ad5 or Ad3. There was only a modest difference between the basal levels of transcription driven by these two E1A promoters. This difference was amplified from 10 to 100 times by the different net responses of the E1A promoters to concomitantly expressed E1A genes. Each promoter had a characteristic net response to positive and negative regulation by E1A gene products. The Ad5 E1A promoter was more strongly repressed, whereas the Ad3 E1A promoter was more strongly activated by E1A gene products. Experiments with a chimeric Ad5/3 E1A promoter indicated that these different autoregulatory responses are determined by DNA sequences which are more than 50 base pairs upstream from the E1A transcriptional start site. A plausible target DNA sequence for positive and negative autoregulation by E1A gene products is discussed.

The genetic program of the adenovirus is initiated soon after the arrival of viral genomes in the nuclei of infected cells. E1A gene products at this early stage of infection home to the nucleus to regulate, either positively or negatively, the transcription of various viral and cellular genes (7, 9–11, 31, 32, 34, 35). Enhancer elements are thought to be targets for E1A-mediated repression (1, 37, 38), but the role of specific DNA target sequences in transactivation of transcription is not as well defined. Transactivation has been reported to be independent of DNA sequences in the proximity of some E1A-activated promoters (5, 13, 21). More recent evidence suggests that E1A gene products activate transcription by increasing site-specific binding of cellular transcription factors to the affected promoters (9, 15–17, 20, 29, 40). Thus, the positive and negative regulatory effects of adenovirus E1A gene products may have in common mechanisms which involve specific DNA targets that interact with cellular transcription factors.

Expression of the E1A gene occurs immediately after infection in the relatively unperturbed environment of ongoing cellular gene transcription. This is facilitated by multiple transcriptional enhancer elements associated with the E1A promoter (4, 6, 8). The presence of one or more of these enhancer elements renders the E1A promoter sensitive to autorepression (18, 19, 30). The E1A promoter can also respond to positive regulation by E1A products (5, 23, 28, 34). Autoregulation of the adenovirus E1A gene therefore involves a eucaryotic promoter which can function in uninfected cells and which is also responsive to both the activating and the repressing actions of E1A gene products.

Divergent evolution of adenoviruses has led to many known human adenovirus serotypes. These represent a resource of natural variation in properties such as E1A-mediated regulation of gene expression. We have studied adenovirus serotypes 3 and 5 (Ad3 and Ad5), which represent subgroups B and C human adenoviruses, respectively. Ad3 and Ad5 have been found to express their E1A genes with remarkably different intensities and kinetics early after infection (34). This is seen by Northern (RNA) blot analysis of Ad5 and Ad3 E1A-specific mRNA from infected A549 cells, a permissive human lung tumor cell line (34). Within 3 h after infection, very high levels of Ad3 E1A mRNA can be detected. The E1A mRNA in Ad5-infected cells appears later after infection and in lower amounts than with Ad3. This comparison suggests that a very different balance of E1A gene autoregulation is maintained by Ad5 and Ad3 early after infection by these viruses. This difference between Ad5 and Ad3 may be attributable to the functions of their E1A gene products or to the responsiveness of their respective E1A promoters.

The average DNA sequence homology of the Ad3 and Ad5 E1A genes is about 50%, with different regions that are strongly conserved or significantly divergent (12, 36). In contrast, the physical organization and structure of the Ad3 and Ad5 E1A genes are very similar (Fig. 1). The E1A promoters and their upstream regulatory elements are located from about 100 to 500 base pairs (bp) from the left end of the 36-kbp linear double-stranded DNA genome. Two species of E1A mRNA are generated early after infection, distinguished only by choice of 5′ splice donor sites. The translation products of the longer 13S mRNA and the shorter 12S mRNA differ only by internal sequences of 31 or 46 amino acids for Ad3 or Ad5, respectively. Comparisons of aligned E1A gene DNA and amino acid sequences of different adenoviruses reveal conserved regions that suggest three highly conserved functional domains within the 5′ exon or amino-terminal moiety (12, 36). Functional tests support this suggestion (2, 24, 25, 27, 39, 41). Domains 1 and 2 are common to the proteins translated from both the 12S and 13S mRNAs. Most of domain 3 appears only in the 13S mRNA translation products. Missense mutations in domain 1 or domain 2 of Ad5 E1A affect enhancer-targeted repression

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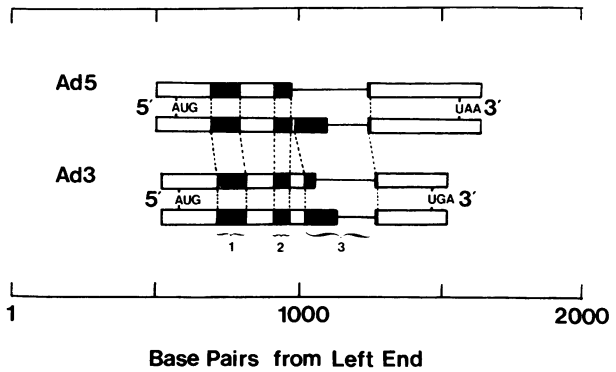

FIG. 1. The E1A genes of Ad5 and Ad3. The physical maps of the mRNA species transcribed from the E1A genes are shown in position near the left ends of the viral genome. The alignment is based on the DNA sequences of the Ad5 (36) and Ad3 E1A genes. The Ad3 *Bgl*II K fragment spans the promoter and coding sequences of the E1A gene, and its DNA sequence has been determined in our laboratory by Robert Hall. This Ad3 DNA sequence has 98% identical homology with that of Ad7 (36). Early E1A mRNA species 12S and 13S (upper and lower species, respectively, for Ad5 and Ad3) are distinguished by the choice of splicing donor site. Introns are shown as single lines joining the 5′ and 3′ exons of each mRNA. Translational start and stop codons are indicated. The regions shown in black represent the three domains of highly conserved E1A coding sequences (2, 12, 24, 25, 27, 39, 41). Domains 1 and 2 are associated with enhancer-targeted repression by E1A gene products. The 13S mRNA-specific domain 3 functions in E1A-mediated transactivation of transcription. The gap between domains 2 and 3 corresponds to a region of variable length among aligned adenovirus E1A DNA or protein sequences, from one codon in Ad5 subgroup C, 17 codons in Ad3 subgroup B, and 21 codons in Ad12 subgroup A (12, 19, 36).

function; missense mutations in domain 3 affect E1A transactivation of transcription. Thus, the 12S mRNA encodes E1A proteins which are negative regulators of transcription. The 13S mRNA-encoded proteins have potential for both positive and negative regulatory functions.

In this study, we have constructed and analyzed plasmids which express the chloramphenicol acetyltransferase (CAT) reporter gene under control of E1A promoters derived from Ad3 and Ad5. Our results indicated that upstream DNA sequences of these promoters determine very different responses to E1A gene products in cotransfected cells. The dominant autoregulatory response of the Ad3 E1A promoter reflected transactivation, whereas autorepression was more dominant for the Ad5 E1A promoter. This difference is consistent with the large difference in the onset and levels of E1A gene expression seen in cells early after infection with Ad3 or Ad5.

Transient expression of the CAT gene, using the Ad3 and Ad5 E1A promoters. We have described the properties of a plasmid (Ad3)pE1ACAT (Fig. 2A) which expresses the bacterial CAT gene in human cells under control of the Ad3 E1A promoter (34). Levels of the CAT gene product are strongly elevated in cells cotransfected with (Ad3)pE1ACAT and in plasmids expressing the E1A gene of either Ad3 or Ad5 (34).

A similar plasmid, (Ad5)pE1ACAT, was constructed, joining the E1A promoter of Ad5 to the CAT gene (Fig. 2B). The Ad5 E1A promoter region (from nucleotides 1 to 612, including part of the 5′ exon) was recovered from a genomic Ad5 E1A plasmid (34) by restriction with *Eco*RI and *Bst*X-I; this fragment was joined to segments of vector pBR322 and plasmid pSV0CAT (obtained from Peter Howley of the National Cancer Institute) (3). Transformed colonies were screened by colony hybridization with a ^{32}P-labeled probe representing the CAT-simian virus 40 DNA insert. Orientation of the Ad5 E1A promoter, the CAT coding sequences, and the 3′ processing signals of simian virus 40 were confirmed by restriction mapping. Levels of CAT in HeLa cells transfected with the (Ad5)pE1ACAT plasmid were about 20 times lower than those in cells transfected with equivalent amounts of (Ad3)pE1ACAT; cotransfection of HeLa cells with the (Ad5)pE1ACAT plasmid and E1A gene-expressing plasmids (of Ad3 or Ad5) decreased the levels of CAT gene expression (results not shown). This suggests major differences between the autoregulatory properties of the Ad3 and Ad5 E1A promoters, but stringent interpretation of these results must take into account other details distinguishing the two pE1ACAT constructs. The 5′ regions of the mRNAs transcribed from the plasmids are different, as are their respective translation product CAT enzymes.

To simplify matters, we constructed a third plasmid, (Ad5/3)pE1ACAT (Fig. 2C), by homologous substitution of a *Pvu*II restriction fragment from (Ad3)pE1ACAT (Fig. 2A) into (Ad5)pE1ACAT (Fig. 2B). Plasmids (Ad3)pE1ACAT and (Ad5/3)pE1ACAT should express the CAT gene with identical mRNA and translation products, although transcriptional control of the plasmids should reflect DNA sequence differences upstream from the start site for transaction. The two plasmids differ functionally by the Ad3- or Ad5-derived DNA sequences upstream from the viral *Pvu*II restriction site. The DNA sequences of Ad3 and Ad5 in this region near the E1A gene TATA motif are very similar. DNA sequences from this region of the three pE1ACAT plasmids are shown below for comparison.

```
                 −60          −50          −40          −30      (bp from 5′ mRNA)
                  |            |            |            |
Ad5  5′ TTTTATTATTATAGTCAGCTGACGTGTAGTGTATTTATACC 3′
Ad5/3 5′ TTTTATTATTATAGTCAGCTGATCGCTAGGGTATTTAAACC 3′
Ad3  5′ TTTTTACGTAGGTGTCAGCTGATCGCTAGGGTATTTAAACC 3′
                          Pvu II                  TATA
```

HeLa cells were transfected at approximately 80% confluence. Cells from the desired number of 100-mm-diameter tissue culture dishes were removed by trypsinization and pooled for transfection with calcium phosphate-precipitated DNA in suspension (34). After 3 h of gentle rotation with the transfecting DNA, the cells were glycerol shocked (mixed with equal volume of 20% glycerol in Dulbecco modified Eagle medium and then centrifuged for 2 min at 2,000 rpm), resuspended with 10 ml of fresh medium, and returned to fresh tissue culture dishes. Cells were harvested at 40 to 48

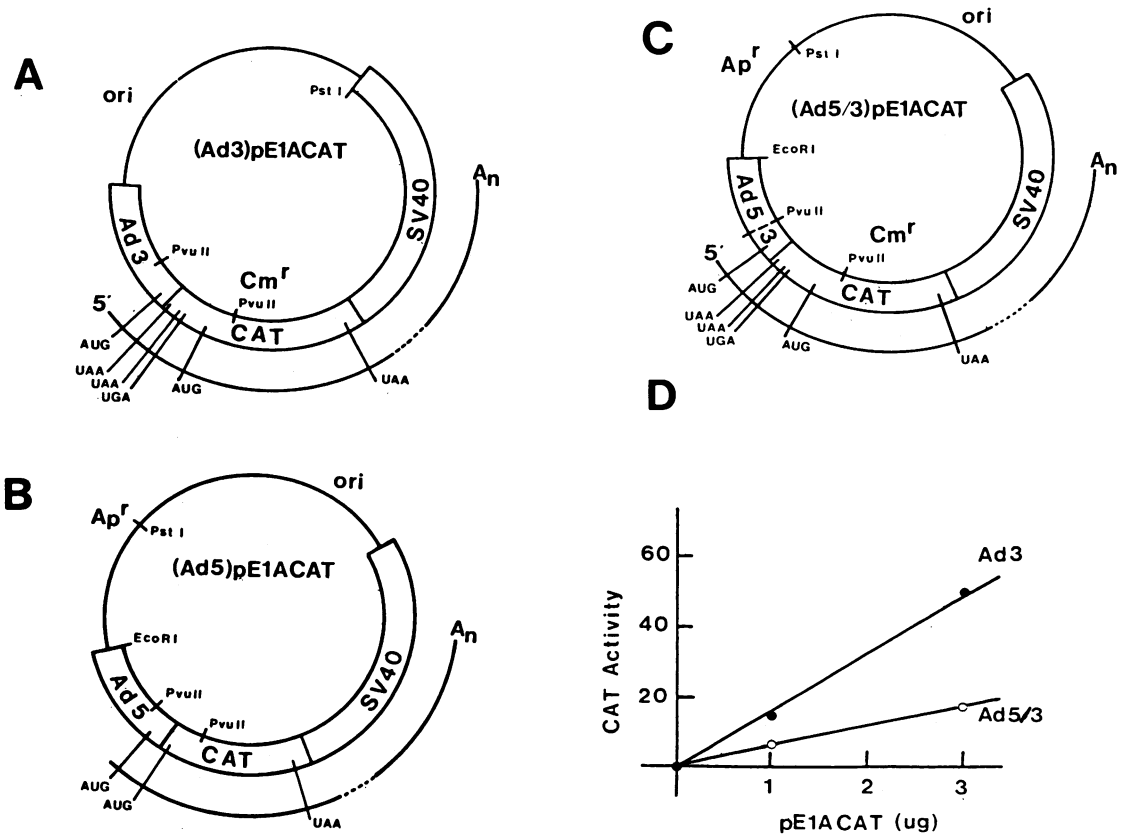

FIG. 2. Plasmids which express the CAT gene under control of adenovirus E1A promoters. The (Ad3)pE1ACAT (A) plasmid was described earlier by Tibbetts et al. (34). The (Ad5/3)pE1ACAT (C) plasmid was constructed from (Ad5)pE1ACAT (B) by substitution of the *Pvu*II restriction fragment which spans the junction of the Ad5 E1A promoter and the CAT gene, with the corresponding fragment from (Ad3)pE1ACAT. The CAT genes of the two plasmids (panels A and C) differ by DNA sequences which lie upstream from the *Pvu*II sites of the E1A promoters, 50 bp upstream from the transcriptional start site for the Ad3 E1A gene. (D) Level of expression of CAT in HeLa cells transfected with equivalent amounts of (Ad3)pE1ACAT or (Ad5/3)pE1ACAT.

h after transfection for extraction and assay of CAT as described by Gorman et al. (3). Spots revealed on plastic-backed thin-layer chromatography plates by autoradiography were cut out, and radioactivity was determined by scintillation spectroscopy. In this report, one unit of CAT activity corresponds to acetylation of 20 pmol of [^{14}C]chloramphenicol (1% of total substrate in a 100-μl reaction) in 15 min at 37°C. In this and other similar experiments, we have observed only three times greater levels of CAT in cells transfected with (Ad3)pE1ACAT compared with those for (Ad5/3)pE1CAT (Fig. 2D).

Thus, when idiosyncrasies of different mRNA and translation products from the plasmids were eliminated, there appeared to be only a modest difference in the basal levels of transcription determined by the upstream DNA sequences of the Ad3 and the Ad5 E1A promoters. This difference alone does not seem sufficient to explain the large differences in the levels of early E1A gene expression seen in viral infections with Ad3 and Ad5 (34).

Cotransfections and E1A autoregulation of (Ad3)pE1ACAT or (Ad5/3)pE1ACAT. We proceeded to compare the expression of CAT with the (Ad3)pE1ACAT and (Ad5/3)pE1ACAT plasmid in HeLa cells cotransfected with different amounts of plasmids that express the genomic, 12S cDNA or 13S cDNA forms of the adenovirus E1A gene (Fig. 3). Cotransfections with the 12S cDNA form of the Ad5 E1A gene repressed both the Ad3 E1A promoter and the chimeric Ad5/3 E1A promoter, but the Ad5 regulatory DNA sequences appeared to be more sensitive to this repression. The 13S cDNA form of the Ad5 E1A gene led to both positive and negative effects on the transcription of the E1A CAT genes, depending on the amount of the cotransfecting E1A-expressing plasmids. Bifunctionality of the 13S cDNA of Ad5 E1A was most evident in the responses of the Ad5/3 E1A promoter (Fig. 3B). It was weakly stimulated in cells cotransfected with low molar ratios of the 13S cDNA and E1A CAT plasmids (pE1A/pE1ACAT, 0.10) and showed weak repression at an equimolar ratio of the cotransfecting plasmids. The (Ad3)pE1ACAT plasmid was maximally stimulated at the lower ratio of cotransfecting 13S cDNA and was less well stimulated at higher levels of the E1A gene input (Fig. 3A). The level of CAT expressed with the Ad3 E1A promoter was increased 60-fold by the 13S Ad5 E1A cDNA. The Ad5/3 E1A promoter was transactivated only 2.5-fold under equivalent conditions.

Transcripts from the genomic Ad5 E1A can be spliced to generate a mixture of both 12S and 13S mRNA products. (Ad5/3)pE1ACAT responded to cotransfecting genomic Ad5

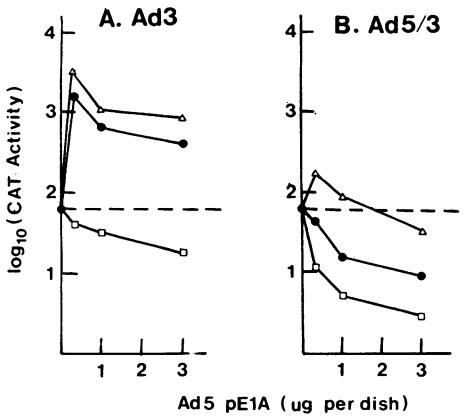

FIG. 3. Responses of (Ad3)pE1ACAT (A) and (Ad5/3)pE1ACAT (B) to expression of the Ad5 E1A gene in cotransfected HeLa cells. HeLa cells were transfected with 3 μg of the E1A CAT plasmids per plate and different amounts of Ad5 E1A genomic or cDNA expression plasmids. The Ad5 E1A plasmids were derived from the Ad5 E1A expression plasmid described previously (34). The plasmid was modified to delete a small downstream region of viral DNA left by the original construction strategy. The genomic Ad5 E1A DNA sequence was removed from this plasmid by restriction with *Eco*RI and *Sst*I and then was joined to the 2,298-bp DNA fragment of *Eco*RI-*Pvu*II-restricted pBR322 vector DNA. Procedures such as polishing 3′ overhangs with T4 DNA polymerase or blunt-end ligation with T4 DNA ligase and general cloning techniques followed those of Maniatis et al. (26). Plasmids containing segments of 12S or 13S cDNA of the Ad2 E1A gene were obtained from Jim Lillie and Michael Green (Harvard University). Ad2 and Ad5 are both subgroup C human adenoviruses, and they are nearly identical in this region (36). These plasmids were propagated in Dam$^-$ *Escherichia coli* to facilitate excision of the *Cla*I to *Xba*I restriction fragments which span the (deleted) introns of the E1A gene. These short fragments were cloned by substitution of the corresponding *Cla*I-to-*Xba*I region of the genomic (Ad5)pE1ACAT plasmid. Different forms of the cotransfecting Ad5 E1A gene were used as indicated, using genomic Ad5 E1A (●), 13S cDNA of Ad5 E1A (△), and 12S cDNA of Ad5 E1A (□). The 12S cDNA repressed CAT expression under E1A promoter control, especially with the Ad5/3 chimeric promoter. Activation of transcription by the bifunctional 13S cDNA form of Ad5 E1A was much more pronounced in the case of the (Ad3)pE1ACAT plasmid. The ordinates of each graph represent levels of CAT enzyme activity on a logarithmic scale to give equal weight to the positive and negative responses of the E1A CAT promoters to the E1A gene products.

E1A with levels of CAT that were intermediate between those obtained with the 12S and 13S cDNA plasmids. Autorepression apparently dominated positive autoregulatory responsiveness of (Ad5/3)pE1ACAT. In contrast, the same genomic Ad5 E1A-expressing plasmid stimulated (Ad3)pE1ACAT expression only slightly less than that seen in the cotransfections with Ad5 13S cDNA.

Similar cotransfection experiments have been performed with genomic Ad3 E1A expression plasmids (data not shown). Again, (Ad3)pE1ACAT was strongly stimulated in *trans* under the same conditions as those in which (Ad5/3) pE1ACAT was strongly repressed.

The limited difference in basal level gene expression of the Ad3 and Ad5/3 E1A CAT plasmids in transfected cells (Fig. 2D) was magnified by the very different responses of the plasmids to E1A gene products (Fig. 3). The combination of these two factors, especially the autoregulatory component, explains the very different levels of E1A gene expression attained early after infection of cells with Ad3 or Ad5 (34). The transfection experiments showed greatest levels of autostimulated gene expression at low ratios of E1A plasmid to E1A CAT reporter gene. This may mimic conditions early after viral infection when transcription from parental viral genomes is first affected by the E1A gene products that begin to accumulate in the infected cell nuclei.

The E1A promoters of the (Ad3)pE1ACAT and (Ad5/3)pE1ACAT expression plasmids differ only in the DNA sequences which lie more than 50 bp upstream from the transcriptional start site of the E1A gene. Functional differences in the autoregulatory responses of the Ad3 E1A and Ad5/3 E1A promoters are thus determined by differences in the DNA sequences of Ad3 and Ad5 in this upstream region. Basal level transcription of the E1A gene is supported by enhancer elements (4, 6, 8). The promoter-proximal enhancer element is approximately 200 bp upstream from the E1A transcription start site (4). The core DNA sequence of this enhancer is the same for Ad3 and Ad5, 5′-GAGGAAGTGAA-3′. DNA sequences which surround this enhancer element may contribute to the modest threefold difference in basal levels of Ad3 and Ad5/3 basal E1A CAT gene expression. These DNA sequences may also determine the different extents to which the Ad3 and Ad5 promoters are subject to repression by E1A gene products.

The DNA sequences of Ad5 and Ad3 in the region of the −200 E1A enhancer core are compared below (asterisks emphasize DNA sequence identity, nucleotides are numbered from left end of the genome).

```
     bp 271  ←E2F motif→          −200 enhancer                                  bp 330
             |                                                                   |
Ad5: 5′  CCATTTTCGCGGGAAAACTGAATAAGAGGAAGTGAAA-TCTGAATAATTTTGT-GTTACTCA  3′
         ********** ******* *** ********** ***** * **** * ****
Ad3: 5′  CCATTTTCGCGCGAAAACTAAAT--GAGGAAGTGAATTTCTGAGTCATTTCGCGGTTATGCC  3′
             |                                                                   |
     bp 281  ←E2F motif→          −200 enhancer          E2F motif               bp 340
```

Immediately upstream from the −200 enhancer core of each E1A promoter there is a DNA sequence which is an inverted repetition of the binding-site motif (5′-TTTTCGCG-3′) for cellular transcription factor E2F described by Nevins and co-workers (15–17). A single base change (G↔C) distinguishes these elements in Ad3 and Ad5. A third E2F motif (5′-TTTCGCG-3′) lies just 3′ to the −200 enhancer core of Ad3, but this is not found at the corresponding location in Ad5 DNA. In Ad5 DNA, but not in Ad3 DNA, a single E2F motif occurs just 3′ to the −300 duplicate enhancer core (4, 14). This comparison of the distributions of E2F sites sug-

gests an evolutionary process of tandem reiteration and subsequent divergence of short DNA sequences in the E1A transcriptional control region. E1A-stimulated expression of the adenovirus E2 gene has been associated with increased binding of the E2F factor at that promoter (15, 16). Furthermore, the E1A promoter binds the E2F factor and is a strong competitor for the binding of E2F to the E2 promoter (17).

There is a significant difference in phasing between the inverted, repeated E2F sites and the −200 enhancer core DNA sequences. Two bases, -AA-, are present in Ad5 but not Ad3. These would place the sites for two different DNA-protein interactions in different proximity and spatial orientation along the axis of the DNA helix.

We suggest that the Ad3 E1A promoter can interact more effectively than the Ad5 E1A promoter with the cellular E2F transcription factor, leading to the more positive response of the Ad3 promoter to autoactivation by E1A gene products. Binding of E2F so closely to the DNA-protein interactions at the enhancer may further interfere with E1A-mediated repression associated with the enhancer element. Site-directed mutagenesis and our functional assays of E1A gene autoregulation provide an appealing approach to further analyze the roles of numbers and arrangements of the E2F-binding sites associated with the E1A promoters of different adenoviruses.

An additional line of evidence supports the hypothesis that the −200 E2F-enhancer region determines the autoregulatory responses of the E1A promoter. The defective mutant Ad3hr15 duplicates in tandem the 60-bp DNA sequence of Ad3 compared with that of Ad5 (18). The mutant encodes wild-type Ad3 E1A proteins, yet its E1A gene is transcriptionally silent early after infection due to extreme autorepression of its aberrant E1A promoter. Revertants of Ad3hr15 delete codons adjacent to the conserved functional domain 2 of the Ad3 E1A proteins, a region which influences enhancer-targeted repression (19). The revertant Ad3hr15-*dl*7 has the duplicated enhancer region in its E1A promoter, yet it overproduces E1A transcripts compared with those produced in wild-type Ad3. Thus, this duplicated upstream DNA sequence, bp 281 to 340 of Ad3, shows increased responses to both positive and negative autoregulation.

Recent studies of other promoters which respond to E1A-mediated transactivation have implicated cellular transcription factor TFII-D, which interacts with TATA-box elements having the sequence 5′-TATAA-3′ (22, 29, 40). The TATA elements of the E1A promoters from Ad3 and Ad5 do not correspond to the motif required for this interaction with TFII-D. This is consistent with our conclusion that sequences further upstream from the TATA element are the focus for positive E1A autoregulation.

Evolutionary variation in the E1A promoters of Ad3 and Ad5 has provided a unique opportunity to resolve the standing problem of DNA sequence-specific or DNA sequence-independent regulation of transcription by the adenovirus E1A gene. We appreciate at this stage that the modes of action of the E1A gene are likely to be several, that its effects are pleiotropic, and that different promoters may be idiosyncratically responsive to E1A gene products. We also expect that further analysis of the E1A autoregulatory system will provide useful insights as a model for the regulatory functions of the E1A gene.

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