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Autoregulation of Adenovirus E1A Gene Expression

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We examined E1A gene expression by two evolutionarily divergent human adenoviruses, type 5 (subgroup C) and type 3 (subgroup B). Adenovirus type 3 (Ad3)-infected A549 cells contained much larger amounts of E1A-specific RNA than adenovirus type 5 (Ad5)-infected cells, from very early (3 h) through the late stages (20 h) after infection. The appearance of such abundant Ad3 E1A transcripts was delayed after infection of Ad5 E1A-expressing 293 cells, suggesting a down regulation of the Ad3 E1A gene by Ad5 E1A gene products. In a reciprocal manner, coinfection of A549 cells led to typically early and intense Ad3 E1A transcription and strongly inhibited transcription of the Ad5 E1A gene. Transient expression assays were developed so that the autoregulation of the E1A gene could be studied apart from the more complex background of infected cells. The DNA sequence surrounding the transcription start site of the Ad3 E1A gene was placed 5′ to the sequence which encodes the bacterial chloramphenicol acetyltransferase gene. Cotransfection of HeLa cells with Ad3 or Ad5 E1A-expression plasmids increased the expression of the Ad3 E1A promoter-driven chloramphenicol acetyltransferase gene. Taken together, these results suggest dual autoregulatory features of adenovirus E1A gene expression. The positive and negative effects appear to be temporally distinguished under different conditions, both in viral infection and in transient assays with plasmid-cloned genes.

The adenovirus E1A gene is widely recognized as an important model system for understanding regulation of gene expression at the level of transcription in animal cells (30, 35). The roles played by the E1A gene in the process of adenovirus-induced oncogenesis provoke further interest in this system. Recent work in this field has focused on the profound effects which E1A gene products bear upon transcription from heterologous viral and cellular promoters. E1A-dependent transactivation and repression of transcription have both been documented with selected promoters (2, 4, 12, 20, 38, 40, 41).

Early expression of the subgroup C human adenovirus type 2 or 5 (Ad2 or Ad5) E1A gene yields two mRNA species, 12S and 13S, which encode very similar translation products, differing only by an internal sequence of 46 amino acids. Specific association of the positive and negative regulatory activities with the individual 289 or 243 amino acid E1A products is subject to ongoing debate in the current literature. Similarly unresolved is the role, if any, of *cis*-acting DNA sequences in the proximity of E1A-induced or E1A-repressed promoters.

Several reports have described transcriptional enhancer activity associated with DNA sequences upstream from and within the E1A coding region. At least four nonoverlapping enhancer elements have been described by laboratories that used different experimental approaches (16–18, 29). These sequences have been suggested to facilitate transcription of the E1A gene itself. Once expressed, the E1A gene product(s) can then activate in *trans* the expression of other early viral genes from the limited number of DNA templates available before DNA replication.

Relatively little attention has been given to the role of E1A gene products in the control of E1A gene expression, i.e., autoregulation. Stringent inhibition of protein synthesis with anisomycin decreases levels of Ad2 E1A mRNA by 20- to 50-fold (25). Such conditions decrease even further, 50- to 200-fold, the levels of other E1A-dependent early Ad2 mRNAs. Mutant Ad2 15606 (29) bears a single base deletion (nucleotide 897) which effects a frameshift in both the 12S and 13S early E1A mRNAs. Transcription of the E1A gene of this mutant appears reduced to less than 5% of wild-type levels at early times after infection. These results suggest that expression of E1A is strongly amplified in the presence of functional E1A gene products.

Two in-frame mutations of Ad2 have been studied which further suggest a positive autoregulatory aspect of E1A expression. Ad2 *pm*975 incapacitates the E1A 12S splicing option and expresses its 13S mRNA, as well as E1B, E2, E3, and E4 species, at or above wild-type levels (27). Ad2 *dl*1500 incapacitates the E1A 13S splicing option and expresses E1A as 12S mRNA at only 20% of wild-type levels (28). Similar effects were observed earlier with Ad2 *hrl* (2), a frameshift mutant which specifically impairs the 13S gene product and expresses three to four times lower levels of E1A mRNA. Less efficient transactivation of Ad5 early genes by the 12S product alone was recently reported by Winberg and Shenk (44) with Ad5 mutants having 12S- and 13S-specific cDNA substitutions.

In summary of these observations, constitutive transcription of Ad2 or Ad5 in the absence of E1A products generates levels of E1A mRNA which are only 2 to 5% of the levels which accumulate in the presence of the functional 13S mRNA-encoded E1A gene product.

We recently described a group of human adenovirus type 3 (Ad3; subgroup B) variants with deletions, substitutions, and tandem reiterations in the region 5′ to the E1A transcription start site (23, 33). In the prelude to studies of how these variants expressed the Ad3 E1A gene, control experiments revealed two unexpected observations. (i) Ad3 expresses far greater levels of E1A RNA early after infection than does Ad5. (ii) Expression of region E1 by one serotype (Ad3 or Ad5) strongly delays or represses the expression of E1A by the other serotype.

These results, and the background of literature on E1A gene expression, suggested to us that Ad3 more effectively amplifies the early expression of the E1A gene. Furthermore, E1A products appear to be involved in both positive and negative autoregulatory functions. Plasmid cotransfec-

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tion, transient expression assays lend further support to this dual autoregulatory model of adenovirus E1A gene expression. Expression of Ad3 E1A or Ad5 E1A can either increase or decrease expression of a probe gene placed under control of the Ad3 E1A promoter, depending on whether the transfections are concomitant or sequential.

MATERIALS AND METHODS

Cells and viruses. HeLa, A549, and 293 cells were maintained as monolayer cultures in Dulbecco modified minimal essential medium supplemented with 10% calf serum–100 U of penicillin per ml–100 μg of streptomycin per ml–1 μM *p*-hydroxybenzoic acid butyl ester (antimycotic). Ad3 (strain G.B.) and Ad5 (strain adenoid 75) were obtained from the American Type Culture Collection and were propagated and purified by procedures used in this laboratory (11, 33, 39).

Plasmids. The plasmids constructed for expression of Ad3 E1A, Ad5 E1A, and the chloramphenicol acetyltransferase (CAT) gene under control of the Ad3 E1A promoter are shown (Fig. 1, 2, and 3). Plasmid pCT142 (23) corresponds to the left *Hin*dIII-I fragment of Ad3 DNA and was used to probe Ad3 E1A-specific RNA sequences. Another plasmid, bearing the left *Xba*I fragment of Ad5, was obtained from Thomas Shenk (Princeton University) and used to probe Ad5 E1A-specific RNA.

Plasmid pCT13 (22) contained the left end *Sal*I-C fragment of a variant of Ad3 in part of the pBR322 vector. The region between the Ad3 *Hpa*I and left proximal *Bam*HI sites was replaced with the corresponding sequences derived from the parental Ad3 sequences of pCT132 (22). The resulting plasmid was then restricted with *Sal*I and *Bgl*II, and protruding 5′ ends were blunted with Klenow fragment of DNA polymerase I. Blunt-end ligation with T4 DNA ligase provided the plasmid shown in Fig. 1. The Ad3 E1A region from nucleotide 14 in the inverted terminal repetition (−498 relative to the E1A cap site) to the *Bgl*II site (57 base pairs (bp) beyond the E1A poly(A) site) is present in this plasmid which is designated (Ad3)pE1A. The plasmid was constructed by Molly McGrane of our laboratory. Landmarks associated with the 12S and 13S E1A mRNAs are shown (Fig. 1).

A plasmid obtained from Thomas Shenk contained the left end *Xho*I-C fragment (5,790 bp) of Ad5 DNA cloned into the

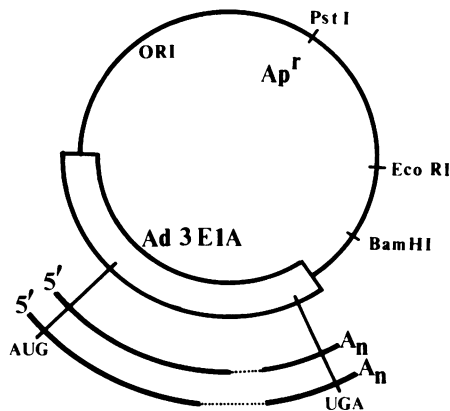

FIG. 1. A plasmid with Ad3 *Bgl*II-K DNA sequences for expression of the Ad3 E1A gene. Dotted lines represent E1A gene introns. A_n, 3′ poly(A) on the E1A mRNA.

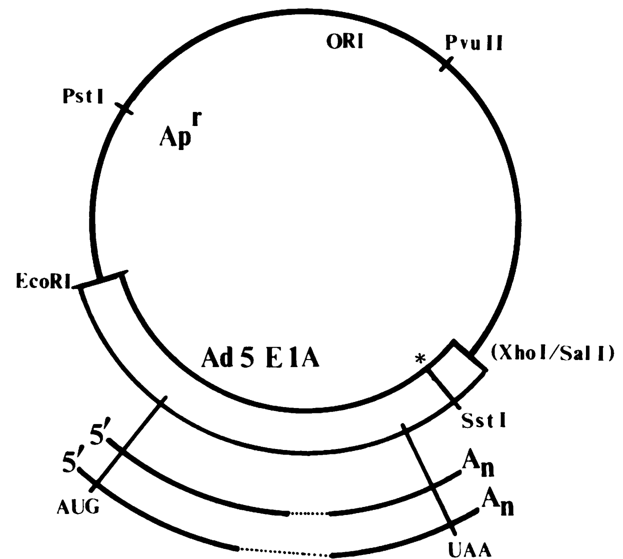

FIG. 2. A plasmid with Ad5 *Sst*I-E DNA sequences for expression of the Ad5 E1A gene. Dotted lines represent E1A gene introns. A_n, 3′ poly(A) on the E1A mRNA.

tetracycline resistance gene of pBR322. This plasmid was restricted with *Sst*I and ligated to provide a smaller DNA which still spanned the Ad5 E1A gene. The Ad5 DNA proceeds from nucleotide 1 at the *Eco*RI site through the E1A gene to the left proximal *Sst*I site (Fig. 2, asterisk, nucleotide 1772). This joins with a short segment of Ad5 DNA (nucleotides 5646 through 5790) which is part of the IVa2 gene intron. Landmarks associated with the 12S and 13S E1A mRNAs are shown over the corresponding E1A insert region of the plasmid (Fig. 2).

Plasmid pCT132 (22) was restricted with *Bam*HI, treated with the processive nuclease BAL 31, subjected to *Eco*RI linker (GGAATTCC) addition, restricted by *Eco*RI, ligated, and transformed into *Escherichia coli* HB101 (substrain N38) (Fig. 3A). The resulting family of ampicillin-resistant plasmids placed an *Eco*RI cloning site near the amino terminus of the Ad3 E1A gene; the specific plasmid used in this study has the *Eco*RI linker site after nucleotide 645 of the Ad3 DNA sequence (22).

The CAT gene was excised from pBR325 (3, 31) as the largest *Hha*I restriction fragment, and from this a 900-bp *Sau*96 I to *Ban*I fragment was obtained. Restricting the shortened 5′ Ad3 E1A plasmid (Fig. 3A) with *Eco*RI, blunting the protruding ends with Klenow fragment and deoxynucleotides, and blunt-end ligating the E1A and CAT fragments were followed by transformation of *E. coli* HB101 (substrain N38), with selection for chloramphenicol and ampicillin resistance. Restriction mapping verified the correct orientation of the CAT gene with respect to the viral E1A promoter (Fig. 3B). Resistance to chloramphenicol verified function of the procaryote promoter and the gene; expression of the CAT gene in transfected human cells (data not shown) verified function of the viral promoter fused upstream of the CAT gene. No consensus sites for polyadenylation of mRNA (AATAAA) occur in the region of CAT, pBR322, or Ad3 sequences 3′ to the CAT gene, although there are three locations of AATAA in pBR322 within 200 bp of the *Eco*RI junction. It is not known if these function as polyadenylation signals for expression of the CAT gene from this plasmid.

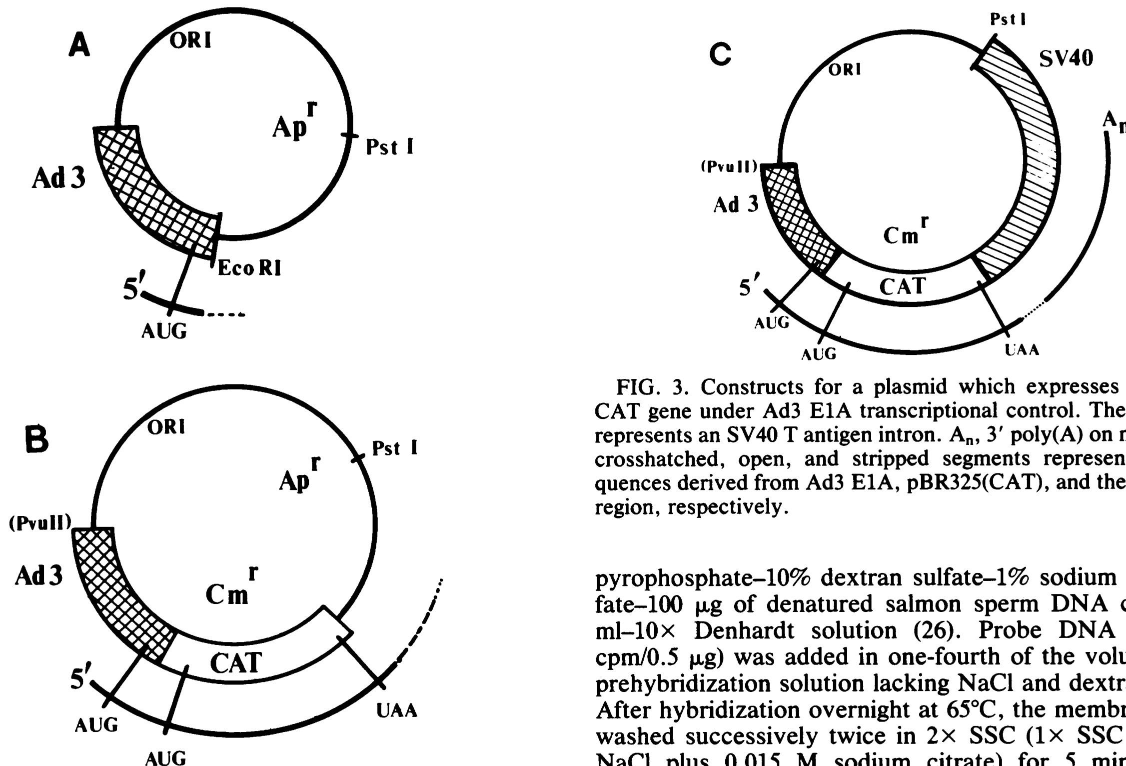

FIG. 3. Constructs for a plasmid which expresses the *E. coli* CAT gene under Ad3 E1A transcriptional control. The dotted line represents an SV40 T antigen intron. A_n, 3' poly(A) on mRNA. The crosshatched, open, and stripped segments represent DNA sequences derived from Ad3 E1A, pBR325(CAT), and the SV40 early region, respectively.

The 3' processing signals of simian virus 40 (SV40) from the CAT expression vector pSV2CAT were placed into our Ad3 E1A-promoted CAT expression vector, resulting in 5 to 10 times higher levels of CAT expression (data not shown). Each plasmid showed the typically increased level of CAT expression in 293 cells compared with that in HeLa or A549 cells. The plasmid shown in Fig. 3B was restricted at its single *Eco*RI site (within the CAT gene) and at the *Pst*I site within the pBR322 β-lactamase gene. Plasmid pSV2CAT (9) was similarly restricted, and the *Eco*RI (in CAT) to the *Pst*I site in SV40 (0.04 map units) was ligated with the Ad3 E1A-CAT fragment. Transformation of *E. coli* HB101 (substrain N38) yielded a plasmid as shown above (Fig. 3C) with a chloramphenicol-resistant, ampicillin-sensitive phenotype. This plasmid is designated (Ad3)pE1ACAT in this report.

Isolation and analysis of RNA from infected cells. A549 or 293 cells were infected with freshly prepared, three times CsCl-banded Ad5 or Ad3 virions at a multiplicity of 2,000 particles per cell. RNA was extracted from uninfected cells or at 3, 6, 9, and 20 h after infection by using guanidinium thiocyanate and pelleting through a CsCl cushion as described in detail by Chirgwin et al. (5).

Samples of total RNA (10 μg) were electrophoresed through horizontal 1.5% agarose-formaldehyde gel columns (15 by 15 by 5 mm, 20-slot combination) with 20 mm morpholinepropanesulfonic acid (pH 7.0)–5 mM sodium acetate–1 mm EDTA, essentially as described by Maniatis et al. (26). After 8 h of electrophoresis at 60 V at room temperature, the RNA was transferred from the gel to GeneScreen membrane (New England Nuclear Corp., Boston, Mass.) and hybridized with nick-translated DNA probes (see below) under conditions recommended by the suppliers. The membranes were prehybridized for 8 to 10 h at 65°C in 50 mM Tris hydrochloride (pH 7.5)–1 M NaCl–0.1% sodium pyrophosphate–10% dextran sulfate–1% sodium lauryl sulfate–100 μg of denatured salmon sperm DNA carrier per ml–10× Denhardt solution (26). Probe DNA (2×10^7 cpm/0.5 μg) was added in one-fourth of the volume of the prehybridization solution lacking NaCl and dextran sulfate. After hybridization overnight at 65°C, the membranes were washed successively twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature, twice in 2× SSC–1% sodium dodecyl sulfate for 30 min at 65°C, and twice in 0.1× SSC for 30 min at room temperature. After drying, the membranes were autoradiographed overnight with intensifying screens.

Probe DNA was prepared by nick translation of plasmids containing E1A-specific sequences of Ad5 or Ad3 (see above). The conditions for labeling the DNA with [α-^{32}P]dATP were those of Rigby et al. (32) as modified by Haase et al. (14). Specific activity of the probes was about 4×10^7 cpm/μg of DNA.

Transfections and CAT assays. Cells at 60 to 80% confluence in plates (diameter, 100 by 15 mm) were used for transfections. The transfection procedure used was that of Gorman et al. (9) or with the two modifications described here. Before the addition of the DNA, cells were trypsinized and suspended in 5 ml of medium per plate of cells. The calcium phosphate-DNA precipitate was then added and gently mixed with the cell suspension. After 3 h at 37°C, the cells were shocked by mixture with an equal volume of 20% glycerol in phosphate-buffered saline for 1 min. After centrifugation, the cells were suspended in fresh medium and plated onto the original number of plates. All plasmids were transfected at a concentration of 10 μg of DNA per plate (about 10^7 cells) unless otherwise noted. Sheared, denatured salmon sperm DNA (10 μg per plate) was added as carrier DNA for each transfection.

Assays of CAT activity were performed as reported by Gorman et al. (9). Each plate of washed, pelleted cells was sonicated in 0.1 ml of 0.25 M Tris hydrochloride (pH 7.8). The sonicated cells were spun for 15 min at 12,000 rpm (Microfuge; Beckman Instruments, Inc., Fullerton, Calif.) at 4°C; 50 μl of supernatant was removed and mixed with 40 μl of H_2O–2 μl of 40 mM acetyl coenzyme A–1 μl (0.1 μCi) of ^{14}C-labeled chloramphenicol (43.2 mCi/mmol; New England Nuclear). Reactions were carried out for 1 h at 37°C and then extracted with 1 ml of cold ethyl acetate. The organic layer was removed, dried, and the chloramphenicol was sus-

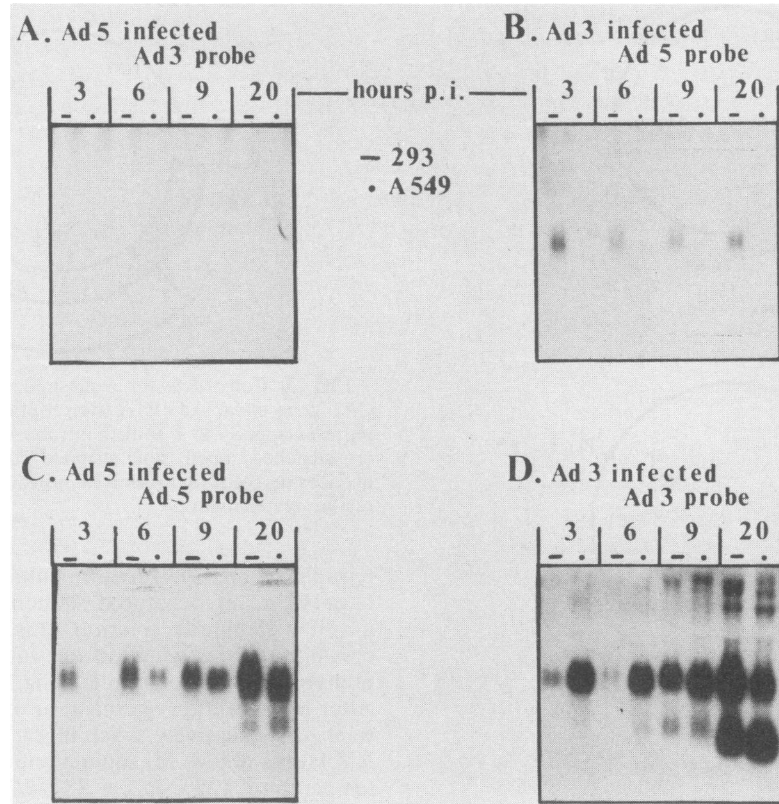

FIG. 4. Northern analysis of E1A-specific DNA from Ad5- and Ad3-infected cells. RNA was isolated from 293 and A549 cells at 3, 6, 9, and 20 h after infection with Ad5 or Ad3. In each case the multiplicity of infection was 2,000 particles per cell. (A) The Northern blot of RNA from Ad5-infected cells was probed with nick-translated Ad3 E1A-specific DNA, and no hybridization was detected. (B) The Northern blot of RNA from Ad3-infected cells was probed with nick-translated Ad5 E1A-specific DNA, and hybridization was observed for the 12S and 13S Ad5 E1A RNA of 293 cells. The Ad3 infection of these cells appears to have had little effect upon constitutive expression from the integrated Ad5 E1A genes. (C) The course of Ad5 E1A expression during infection of 293 and A549 cells, with accumulating 12S and 13S RNA and the appearance of 9S late-specific RNA at 9 to 20 h. Ad5 infection of 293 cells appears (by 6 h) to have generated higher levels of Ad5 E1A than corresponding infection of A549 cells, although expression from the integrated and viral E1A genes could not be distinguished in this experiment. (D) The course of Ad3 E1A expression during infection of 293 cells and A549 cells. Far greater levels of Ad3 E1A 12S and 13S RNA appear in A549 cells at early times and throughout infection than those seen for Ad5 (panel C). Furthermore, the A549 cells accumulated much larger levels of the late-specific smaller E1A RNA, appearing by 6 h and abundant by 20 h. The second striking result of this experiment is the difference seen in parallel infection of 293 cells by Ad3. In 293 cells the onset of intense Ad3 E1A expression was delayed until at least 9 h after infection.

pended in 20 μl of ethyl acetate, spotted on silica gel–thin-layer chromatography plates, and chromatographed with a chloroform-methanol (95:5) solvent. After autoradiography, the spots were cut from the thin-layer chromatography plate, and radioactivity was measured in a liquid scintillation counter to determine the percent acetylation of the chloramphenicol.

Reagents and miscellaneous procedures. Enzymes were obtained from New England BioLabs, Inc., Beverly, Mass., and used as recommended by the supplier. Radioactive nucleotides were obtained from New England Nuclear Corp. Procedures such as digestions with BAL 31, linker addition, and general cloning technique followed Maniatis et al. (26).

RESULTS

E1A expression in cells infected by Ad3 or Ad5. RNA was isolated from A549 or 293 cells at different times after infection with Ad3 or Ad5 virions (2,000 particles per cell) and analyzed by Northern hybridization. RNA from cells infected with Ad5 was probed with Ad3 E1A-specific DNA, and no hybridization was observed (Fig. 4A). When RNA from Ad3-infected cells was probed with Ad5 E1A-specific DNA, hybridization was observed only for samples of Ad3-infected 293 cells (Fig. 4B). The level of Ad5 mRNA in the Ad3-infected 293 cells was similar to that in uninfected 293 cells (1, 10; data not shown). The DNA sequences through the E1A genes of subgroup C (Ad2 and Ad5) and subgroup B (Ad3 and Ad7) are only 50% homologous (42); thus, the high level of discrimination between Ad5 and Ad3 sequences was expected.

The time courses of Ad5 and Ad3 E1A expression in infected 293 and A549 cells are shown in Fig. 4C and D. The 12S and 13S mRNA of Ad5 E1A appeared from 6 to 9 h after infection of A549 cells (prolonged exposure of the autoradiograms revealed a weak signal at 3 h). The E1A mRNAs of Ad5-infected 293 cells could not be distinguished as to genomic or viral origin but there was a slightly increased level of these species in comparison with that of the Ad5-infected A549 cells.

At 3 h after infection of A549 cells, the levels of 12S and 13S mRNA of Ad3 E1A were much higher than early levels of Ad5 E1A expression under parallel conditions (Fig. 4C and D). This feature of intense Ad3 E1A expression contin-

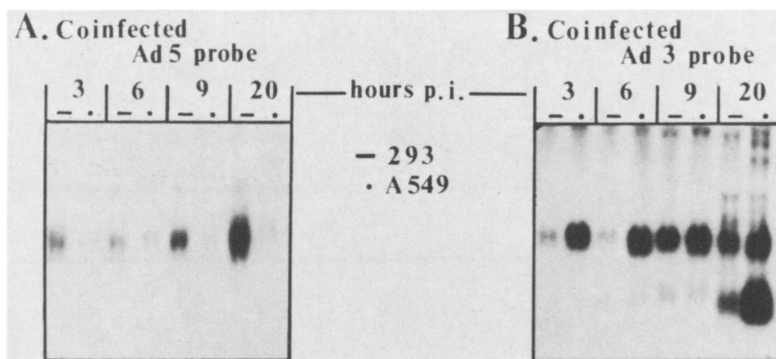

FIG. 5. Northern analysis of E1A-specific RNA from cells coinfected by Ad5 and Ad3. As described in the legend to Fig. 4, RNA was isolated from 293 and A549 cells at 3, 6, 9, and 20 h after infection, in this case coinfection by Ad3 and Ad5 virions at 1,00 particles each per cell. The total multiplicity of infection is thus 2,000, as in the experiment shown in Fig. 4. (A) The resulting Northern blot probed with Ad5 E1A-specific DNA. There is negligible effect of coinfection with Ad3 upon the levels of Ad5 E1A expression in the 293 cells. In A549 cells, however, the effect of coinfection with Ad3 is dramatic. Expression of Ad5 E1A was very inhibited by the coinfecting Ad3. (B) Northern blot of coinfected cell RNAs probed with Ad3 E1A-specific DNA. In this case the A549 cells show very little, if any, difference in the time course or intensity of Ad3 E1A expression compared with those cells infected by Ad3 alone (Fig. 4D). In 293 cells, however, the coinfection with Ad5 delayed the onset of intense Ad3 E1A expression and at 20 h a somewhat lower amount of E1A transcripts accumulated, compared with that in cells infected by Ad3 alone (Fig. 4D).

ued throughout the infection. Since the multiplicities of infection (particles per cell), numbers of cells, amounts of RNA, and specific activities of the E1A DNA probes were the same, the intensities of the RNA bands suggest at least a 20- to 50-fold difference in the levels of early and late E1A expression between Ad3 and Ad5.

There was a pronounced delay in the appearance of E1A mRNAs on Ad3 infection of 293 cells (Fig. 4D). At 3 and 6 h after infection there appeared to be at least 90% inhibition of Ad3 E1A expression. From 9 to 20 h after infection the E1A mRNAs approached levels comparable to those in Ad3 infection of A549 cells.

Is transcription of E1A from incoming Ad5 genomes similarly delayed in 293 cells? The answer will require an Ad5 mutant with E1A transcripts that can be distinguished from those of the 293 cells. Experiments similar to those performed by Spector et al. (37), but analyzing mutant Ad5 *dl*313 E1A-transcripts early after infection of 293 cells, would address this question of homologous early-negative control of E1A transcription.

E1A expression in cells coinfected by Ad3 and Ad5. RNA was isolated from 293 and A549 cells at different times after coinfection by Ad3 and Ad5 virions (1,000 particles per cell each) and analyzed by Northern hybridization. The results shown in Fig. 5A reveal little effect of Ad3 upon expression of the Ad5 E1A genes in coinfected 293 cells compared with 293 cells infected by Ad5 alone (Fig. 4C). In A549 cells, however, the presence of coinfecting Ad3 strongly inhibited expression of the Ad5 E1A gene. The course of Ad3 E1A expression in coinfected A549 cells (Fig. 5B) was indistinguishable from that of cells infected by Ad3 alone. In coinfected 293 cells, however, the Ad3 E1A mRNA was not only delayed in its appearance but was also present at diminished levels compared with 293 cells infected by Ad3 alone (Fig. 4D).

Taken together, results of viral infections and coinfections showed a markedly elevated capacity of Ad3 to express its E1A gene compared with that of Ad5. When Ad3 E1A products appeared very early after coinfection of A549 cells, the expression of the coinfecting Ad5 E1A gene was strongly repressed. When Ad5 E1A products were already present (293 cells) at the time of infection, however, the appearance of Ad3 E1A mRNA was delayed and might have been at reduced levels. The consistent aspect of these results is that the negative effects of ambient E1A products upon E1A transcription were reciprocal for Ad3 and Ad5. The more pronounced effect of Ad3 upon Ad5 in these experiments may reflect a general E1A-dose dependence of the inhibition of E1A transcription or a greater sensitivity of the Ad5 promoter to repression by E1A products.

Positive control of E1A products on E1A expression. A plasmid was constructed which placed the bacterial CAT gene under control of the Ad3 E1A transcription unit (see Materials and Methods). Sequences from nucleotide 14 to 645 of Ad3 pCT132 (22) were linked 5' to a 900-bp fragment from pBR325 which contained the CAT gene. The SV40 small T-antigen splice sites and early polyadenylation sites were excised from pSV2CAT (9) and placed 3' to the CAT gene. The resultant plasmid is designated (Ad3)pE1ACAT and is capable of expressing the CAT gene in both procaryotic and eucaryotic cells.

HeLa cells and 293 cells were transfected with (Ad3)pE1ACAT or pSV2CAT by using a modified calcium phosphate procedure and were assayed for the CAT gene 48 h later as described by Gorman et al. (9) (Fig. 6). When the CAT gene was under control of the Ad3 E1A promoter, greater levels of CAT expression were routinely observed in 293 cells than in HeLa cells. The pSV2CAT plasmid, however, gave lower levels of CAT expression in 293 cells than in HeLa cells, consistent with the observations of others (4, 41).

It seemed likely that Ad5 E1A or E1B products or both in 293 cells were related to the greater levels of (Ad3)pE1ACAT expression in 293 cells than to those in HeLa cells. We therefore performed cotransfection experiments in HeLa cells with (Ad3)pE1ACAT and plasmids expressing the E1A gene of Ad3 or Ad5 (Fig. 7). These results show clearly that products expressed from the E1A gene of Ad3 (*Bgl*II-K fragment clone) or from the E1A gene of Ad5 (*Sst*I-E fragment clone) each strongly stimulate Ad3 E1A promoter-driven CAT expression. Up to 50 times more acetylated chloramphenicol products have been formed in assays of cotransfected cell extracts than those formed in extracts from cells infected by (Ad3)pE1ACAT alone. A

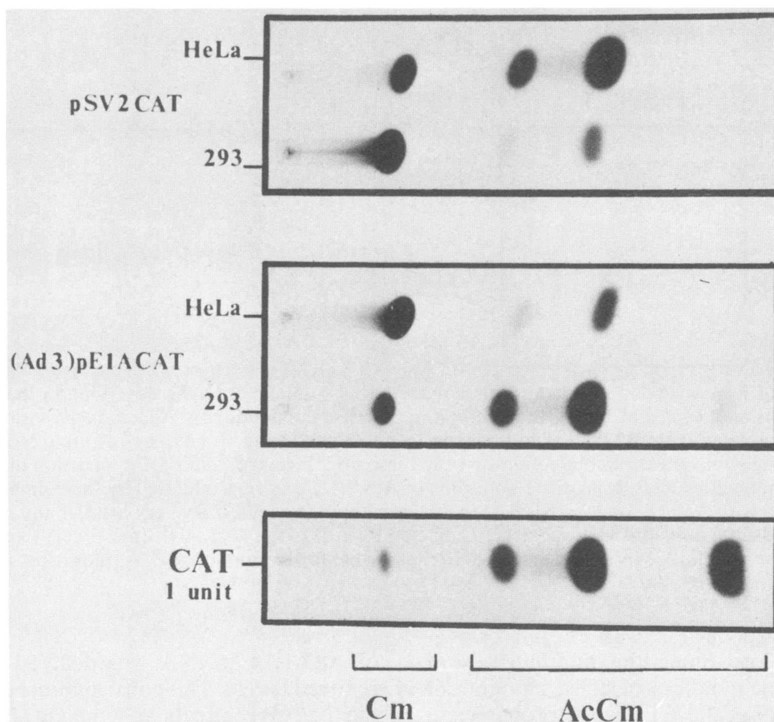

FIG. 6. Expression of the CAT gene by pSV2CAT and (Ad3)pE1ACAT in HeLa and 293 cells. Cells were transfected with plasmids by using a calcium phosphate procedure and were subsequently harvested for analysis of CAT enzyme activity as described by Gorman et al. (9). An indication of relative amounts of CAT gene expression is provided by the relative amounts of ^{14}C-labeled chloramphenicol (Cm) which was singly or doubly acetylated (AcCm) during parallel incubations with different cell culture extracts. Plasmid pSV2CAT with SV40 enhancer-associated early promoter shows less CAT expression in 293 cells than in HeLa cells, consistent with reports of repressive E1A activity (4, 40). In contrast, the (Ad3)pE1ACAT shows a much higher level of CAT activity in transfected 293 cells than in HeLa cells. This is consistent with positive regulatory action of E1A products (in this case heterologous Ad5 E1A products) upon the Ad3 E1A promoter. The bottom track shows acetylation from a reaction with 1 U of CAT (P-L Biochemicals, Inc., Milwaukee, Wis.). The fastest component (right) is 1,3-diacetylated chloramphenicol. We observed negligible amounts of this component in reactions with less than 80% acetylation. This region is not shown in subsequent assays.

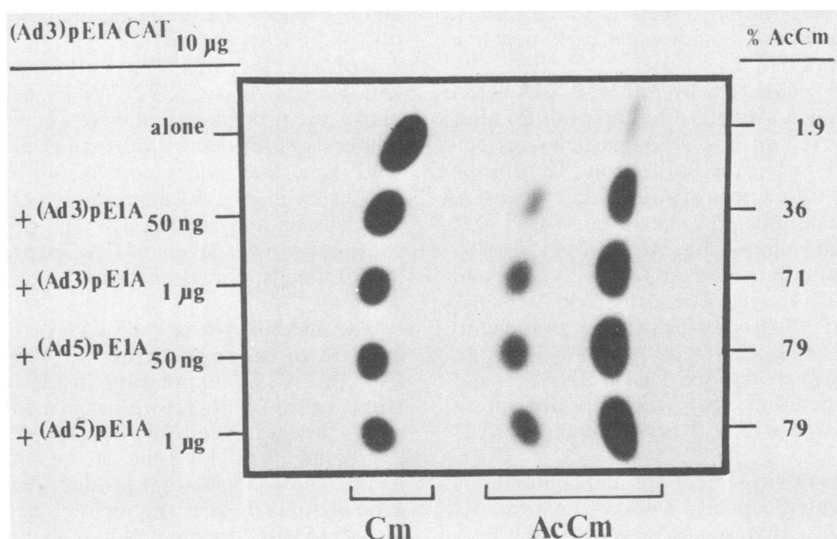

FIG. 7. Stimulation of CAT expression from (Ad3)pE1ACAT in HeLa cells by cotransfection with Ad3 or Ad5 E1A-expressing plasmids. HeLa cells were transfected with 10 µg per plate of (Ad3)pE1ACAT DNA either alone or together with 0.05 µg or 1.0 µg of Ad3 or Ad5 E1A-expressing plasmids. As suggested by the transfection of 293 cells described in the legend to Fig. 6, this experiment confirms that E1A products (Ad3 or Ad5) can exert a strong stimulation of expression from the Ad3 E1A promoter-driven CAT plasmid.

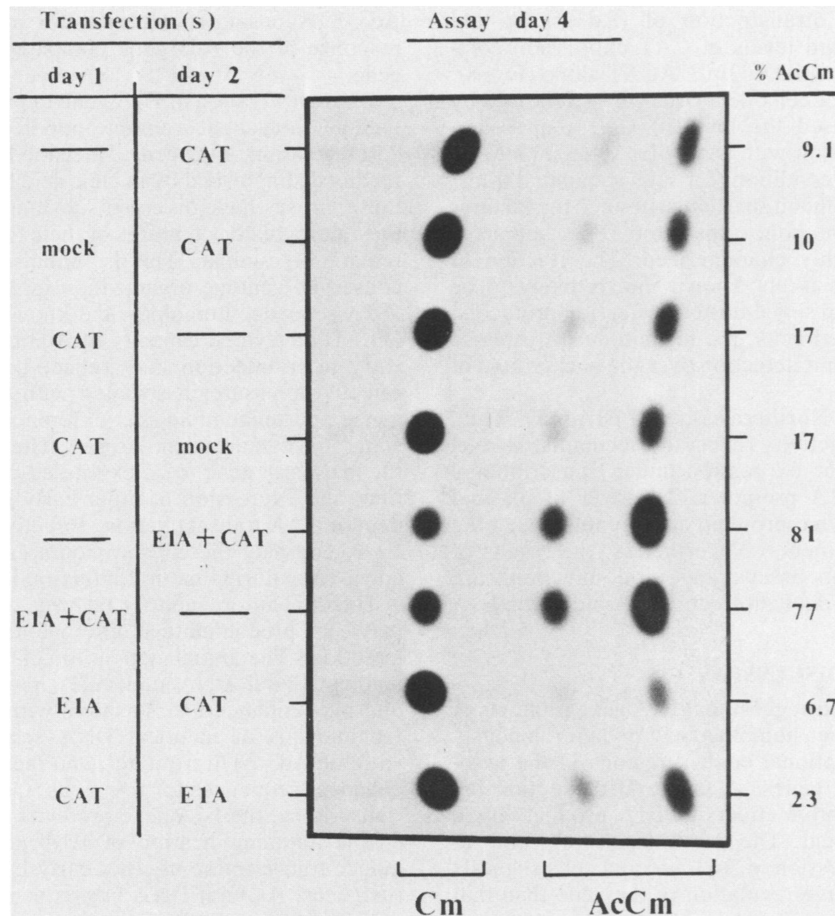

FIG. 8. Homologous stimulation or repression of CAT expression by (Ad3)pE1ACAT depends upon selection of a cotransfection or sequential transfection with (Ad3)pE1A. On day 1 HeLa cells were transfected (or not) with 10 μg of indicated plasmids (Ad3)pE1A or (Ad3)pE1ACAT or were mock transfected without DNA. At 24 h later on day 2, a second transfection was performed (or not) as indicated. At 36 h after the second step (mid-day 4), cells were harvested and assayed for CAT activity. The top four control lanes indicate no significant effect on CAT expression from (Ad3)pE1ACAT when cells were transfected once on day 1 or 2, or on both days, the first or second transfection without DNA (mock). Cotransfection of (Ad3)pE1ACAT with (Ad3)pE1A on either day 1 or day 2 showed equivalent stimulation of CAT expression compared with that of the cells transfected by (Ad3)pE1ACAT alone. Transfection with (Ad3)pE1A on day 1 followed by (Ad3)pE1ACAT on day 2 shows lower CAT expression compared with that of cells transfected by (Ad3)pE1ACAT alone. Thus, the products of Ad3 E1A expression can both stimulate and repress transcription expression of CAT under control of the homologous Ad3 E1A promoter. The bottom lane shows reversal of the order of transfection in the repressive case above. Here the (Ad3)pE1ACAT was stimulated by the subsequent expression of (Ad3)pE1A. The stimulation is less than that observed in the cotransfections, the transcribing complexes expressing CAT are thus less susceptible to stimulation or less abundant by the time the (Ad3)pE1A is introduced.

cotransfection titration assay revealed a plateau of maximum CAT expression in the range of 0.05 to 10 μg of the Ad3 E1A-expressing plasmid together with 10 μg of the (Ad3)pE1ACAT plasmid (results not shown).

The results of transient expression assays suggest strong, positive autoregulatory control of the adenovirus E1A gene. The efficacy of the E1A-expressing plasmids present at only 1/200 of the concentration of (Ad3)pE1ACAT may reflect both a potent effect of E1A products at low concentrations and the accumulation of E1A products by positive feedback acting upon the pE1A as well as upon the (Ad3)pE1ACAT.

Negative control of E1A products on pSV2CAT and (Ad3)pE1ACAT expression. Borrelli et al. (4) and Velcich and Ziff (41) reported that Ad2 or Ad5 E1A product(s) can repress heterologous transcription units which include functional enhancer elements such as in plasmid pSV2CAT. Having established positive autoregulatory effects of Ad3 and Ad5 E1A products upon the Ad3 E1A transcription unit above, we examined the negative control properties of our Ad5 and Ad3 E1A plasmids. Cotransfection of HeLa cells with pSV2CAT and either (Ad5)pE1A or (Ad3)pE1A decreased CAT expression. In various cotransfection experiments with 10 μg of pSV2CAT DNA per plate and 1 to 10 μg of (Ad3)pE1A or (Ad5)pE1A per plate we observed 50 to 80% lower signals (CAT) than those observed in cells transfected by pSV2CAT alone (results not shown).

The results of viral infections presented earlier (Fig. 4 and 5) demonstrated the potential negative effects of E1A products upon E1A transcription. This was seen in situations where E1A products of one adenovirus (Ad3 or Ad5) were present in the cells before the onset of E1A transcription from the other adenovirus template. To simulate these conditions, plasmid DNA expressing Ad3 E1A was transfected into HeLa cells 24 h before the introduction of the (Ad3)pE1ACAT plasmid. This permittted a test of the possible homologous negative control of the E1A gene over

its own expression. Cotransfection of (Ad3)pE1A and (Ad3)pE1ACAT increased levels of CAT expression compared with transfection with (Ad3)pE1ACAT alone (Fig. 8). Sequential transfection of cells with (Ad3)pE1A followed by (Ad3)pE1ACAT decreased the level of CAT expression, compared with transfection with (Ad3)pE1ACAT alone.

To establish optimum conditions for such sequential transfection experiments is difficult, particularly since the kinetics of transcription initiation from transfected DNA templates have not been thoroughly characterized. The fraction of doubly transfected cells is not known, but is likely to be smaller than that found in single-dose transfection protocols. In the doubly transfected cells, the magnitude of response must be sufficient to permit detection over the background of singly transfected cells.

We recognize that our Northern assays of E1A mRNA and enzyme assays of CAT activity reflect the accumulated level of these products of gene expression under transcriptional controls of the Ad3 E1A promoter. Turnover of plasmid DNA, mRNA, and enzyme protein certainly influenced the outcome of such experiments. Nevertheless, the results of our transient expression assays and viral infections are consistent and expose a dual autoregulatory role of the E1A gene products.

DISCUSSION

The results we obtained suggest that E1A gene products of Ad3 and Ad5 can interact homologously or heterologously with their E1A transcriptional control regions in the autoregulation of their E1A genes. A temporal distinction between positive and negative effects of E1A products upon E1A expression is evident. The surprisingly early and intense level of E1A expression in Ad3-infected cells suggests a stronger level of positive regulation of the gene than that observed with the more familiar Ad5 infection. Our transient expression cotransfection assays directly demonstrated *trans* autoactivation of the E1A promoter by E1A products. Negative autoregulation of the E1A gene is suggested by our experiments in which the E1A promoter of infecting viral or transfecting plasmid DNA must develop to a transcribing nucleoprotein complex in a milieu of ambient E1A gene products.

Evidence from the literature, cited above, is consistent with the notion of transautoactivation of the E1A gene. This conclusion is inferred from decreased levels of E1A transcripts in cells infected with E1A-defective virus mutants or in infected cells treated with drugs inhibiting protein synthesis. Transient assay systems have been used to demonstrate transactivation of other viral and cellular promoters by E1A products. This paper is the first report which directly demonstrates transautoactivation of the E1A promoter by E1A products.

Ad3, a subgroup B human adenovirus, was found to express its E1A gene to much higher levels of mRNA early after infection of cells than the more familiar subgroup C Ad5 (Fig. 4). Northern blot analyses do not address the mechanism by which such high steady-state levels of Ad3 mRNA accumulate. Perhaps the half-life of Ad3 mRNA species is significantly longer than that for mRNAs of Ad5. Our present view is that the differences between levels of Ad3 and Ad5 E1A mRNA reflect different balances of positive and negative effects at the level of E1A transcription. Since both Ad3 and Ad5 E1A-expressing plasmids increased expression of the (Ad3)pE1ACAT to similar extents (Fig. 7), we are drawn to the hypothesis that subtle differences of DNA sequence in the region of the Ad3 and Ad5 E1A promoters account for a greater autoactivating response of the Ad3 gene compared with that of the Ad5 gene.

Our investigation of E1A gene expression by Ad5 and Ad3 together presented a unique opportunity to examine *trans* autoregulation of the gene, since the E1A transcripts were so readily distinguished by nucleic acid hybridization. Although these viruses have diverged substantially in their evolution, the autoregulatory features of their E1A gene expression are remarkably similar. This is reminiscent of their strikingly conserved genome organization in contrast to their limited DNA sequence homology and known biological differences (39). The elevated capacity of Ad3 to express its E1A gene early after infection may reflect the response of Ad3 to selective pressures associated with the evolutionary divergence and epidemiological differences among the B and C subgroup human adenoviruses. The essential E1A gene is the first early gene to be expressed during productive infection, and expression of other early genes is clearly dependent on E1A transactivation. Effective autoregulation of the E1A gene may thereby provide a critical advantage to the adenovirus during natural infection in the field.

The dual autoregulatory features of E1A gene expression may each predominate at different stages of productive viral infection. The initial activation of the E1A promoter on incoming viral DNA molecules may be facilitated by the multiple enhancers associated with the Ad5 E1A gene. Homologous or identical DNA sequences are associated with the Ad3 E1A promoter, and these may also function as enhancer sequences for this virus. Once the gene is actively transcribing, the E1A gene products return to the nucleus to enable autoamplification of E1A gene expression and to induce transcription of other early E1A-dependent adenovirus genes. As viral DNA begins to replicate, however, the initial early transcription complexes are likely to turn over. Abundant E1A gene products may now act to delay or inhibit early gene transcription from progency DNA templates. This process would contribute to the early-to-late shift in the overall pattern of gene expression during a productive infection.

It was suggested above that both the positive and negative aspects of E1A autoregulation may be focused at the E1A promoter. The molecular basis of E1A autoregulation may be associated with mechanisms involved in the slow, complex process of transcription initiation from RNA polymerase II promoters (6, 7, 15, 34, 36, 43). Multiple sequential steps are required for the assembly of stable transcription complexes, their coordination with the polymerase, and the actual initiation, capping, and elongation of nascent RNA chains. The overall pathway can be influenced both positively and negatively by E1A products through interactions at different steps (15, 34) in the development of an E1A transcription complex. The repressive effects of E1A products are correlated with enhancer-associated promoters which may indicate that E1A products impede enhancer-mediated association with critical cellular factors (4, 36). Once beyond such an E1A repression-sensitive stage, some promoters, including that of the E1A gene itself, may permit another type of interaction with E1A protein which increases the frequency of initiation of transcription or the stability of a preinitiation-active complex.

The positive and negative autoregulatory effects of E1A appear to be temporally distinct and may also have physically separated sites of action. The negative effects appear targeted to the enhancer regions, and it is plausible that the positive effects are determined closer to the site of initiation

of RNA transcripts. This is supported by efforts to distinguish those DNA sequences required for basal versus E1A-induced transcription of other early genes, where it appears that such sequences overlap substantially (8, 13, 19, 21, 24). The DNA sequences proximal to promoters of class II genes may influence the course of transcription initiation by affecting the interactions of multiple, juxtaposed cellular transcription factors. With this in mind, the negative and positive effects of E1A proteins on transcription may relate to specific *cis*-acting DNA sequences, albeit in an indirect fashion via interactions with elements of a developing transcription complex.

Recognizing both negative and positive autoregulatory elements of the E1A gene should facilitate ongoing efforts to discern functional domains within the E1A proteins and within the regions of DNA proximal to the variety of promoters regulated by E1A. This will probably require experimental designs that effectively segregate the naturally concomitant mechanisms for stimulation and repression of transcription. The E1A promoter of Ad3 is observed to be both strong and inducible. These properties and comparisons with the Ad5 E1A promoter provide a useful model system for dissecting the roles of *cis*- and *trans*-acting factors which regulate gene expression at the level of transcription.

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