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Activation of RNA Polymerase III Transcription of Human *Alu* Repetitive Elements by Adenovirus Type 5: Requirement for the E1b 58-Kilodalton Protein and the Products of E4 Open Reading Frames 3 and 6

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We found that transcription of endogenous human *Alu* elements by RNA polymerase III was strongly stimulated following infection of HeLa cells with adenovirus type 5, leading to the accumulation of high levels of *Alu* transcripts initiated from *Alu* polymerase III promoters. In contrast to previously reported cases of adenovirus-induced activation of polymerase III transcription, induction required the E1b 58-kDa protein and the products of E4 open reading frames 3 and 6 in addition to the 289-residue E1a protein. In addition, E1a function was not required at high multiplicities of infection, suggesting that E1a plays an indirect role in *Alu* activation. These results suggest previously unsuspected regulatory properties of the adenovirus E1b and E4 gene products and provide a novel approach to the study of the biology of the most abundant class of dispersed repetitive DNA in the human genome.

Alu elements are the single most abundant class of dispersed repeated sequences in the human genome, comprising 5 to 10% of the mass of human DNA (17, 19, 67, 70, 76, 77). These elements have a dimeric structure that consists of two related but nonidentical *Alu* monomers that are each homologous to an internally deleted 7SL RNA gene (Fig. 1C and D) (13, 86, 87, 97). *Alu* elements are mobile (51, 52, 93), and several lines of evidence suggest that they transpose through an RNA intermediate transcribed by RNA polymerase III (Pol III): they are flanked by direct repeats that vary in length and sequence between elements, end in a 3′ A-rich tract, and contain an internal RNA Pol III promoter which directs transcription initiation to the first residue of the *Alu* element (Fig. 1C) (for reviews, see references 17, 18, 37, and 90). *Alu* elements have no known function (34, 103); however, it has been suggested that they may provide *cis*-acting sequences that serve as modulators of chromatin structure (20), sites of initiation of cellular DNA replication (2, 41), hotspots for recombination (14, 38, 69), inhibitors of gene conversion (30), stabilizers of cytoplasmic RNA (11, 68), negative transcriptional regulators (72, 85, 100), or modulators of intranuclear processing of mRNA precursors (40). Alternatively, it has been proposed that they may encode the RNA component of a cytoplasmic ribonucleoprotein similar to the signal recognition particle (80).

Alu elements bear an internal Pol III promoter that resembles those found in tRNA and adenovirus (Ad) VA genes (24, 63). Although *Alu* promoters are active in vitro (20, 21, 31, 63), *Alu* elements do not appear to be efficiently transcribed by Pol III in vivo. Rather, the *Alu*-related transcripts found in HeLa cells are transcribed primarily by RNA Pol II, presumably reflecting the fact that many *Alu* elements are embedded within Pol II transcription units (11, 61, 62, 77). On the basis of studies of the promoter of the related 7SL RNA gene, it has been suggested that the inactivity of most *Alu* Pol III promoters in vivo stems from the absence of required upstream transcriptional control elements (62, 88, 89): the 7SL promoter is heavily dependent on sequences located upstream of the transcription initiation site, and 7SL RNA pseudogenes lacking these upstream sequences are apparently inactive in vivo. According to this hypothesis, only a limited subset of *Alu* elements that are located in proximity to favorable 5′ flanking sequences are transcriptionally competent, and the remainder are inactive pseudogenes. This hypothesis implies that flanking sequences could impose distinct patterns of regulation on individual *Alu* elements, a possibility that is supported by two observations. First, transcripts of a single *Alu* element are abundant in primate brain (53, 94). Second, Matera et al. have provided evidence that a small subset of *Alu* elements are transpositionally competent and that members of this class are preferentially transcribed in cultured human cells (51, 52).

Several nuclear DNA viruses encode regulatory proteins that modulate Pol III transcription (3, 23, 33, 79). In the best-characterized case, Ad infection activates transcription of transfected copies of class III genes in vivo (23) and stimulates in vitro transcription by RNA Pol III in HeLa cell extracts by altering the activity of transcription factor IIIC (TFIIIC) (32, 33, 101). The 289-residue protein encoded by Ad early region 1a (E1a) is required for activation (23, 33), and purified E1a is capable of stimulating Pol III transcription in vitro (16). E1a-induced activation of Pol III transcription in vivo appears to be largely restricted to genes that have been newly introduced into cells, inasmuch as transcription of most resident cellular class III genes is not affected (23). Recently, Jang and Latchman reported that herpes simplex virus induces Pol III transcription of endogenous *Alu* elements (39). In this report, we demonstrate that Ad5 infection also strongly stimulates Pol III transcription of human *Alu* elements in HeLa and 293 cells. In contrast to the cases of Ad5-induced Pol III transcriptional activation mentioned above, this process requires the E1b 58-kDa protein and the products of E4 open reading frames (ORFs) 3 and 6

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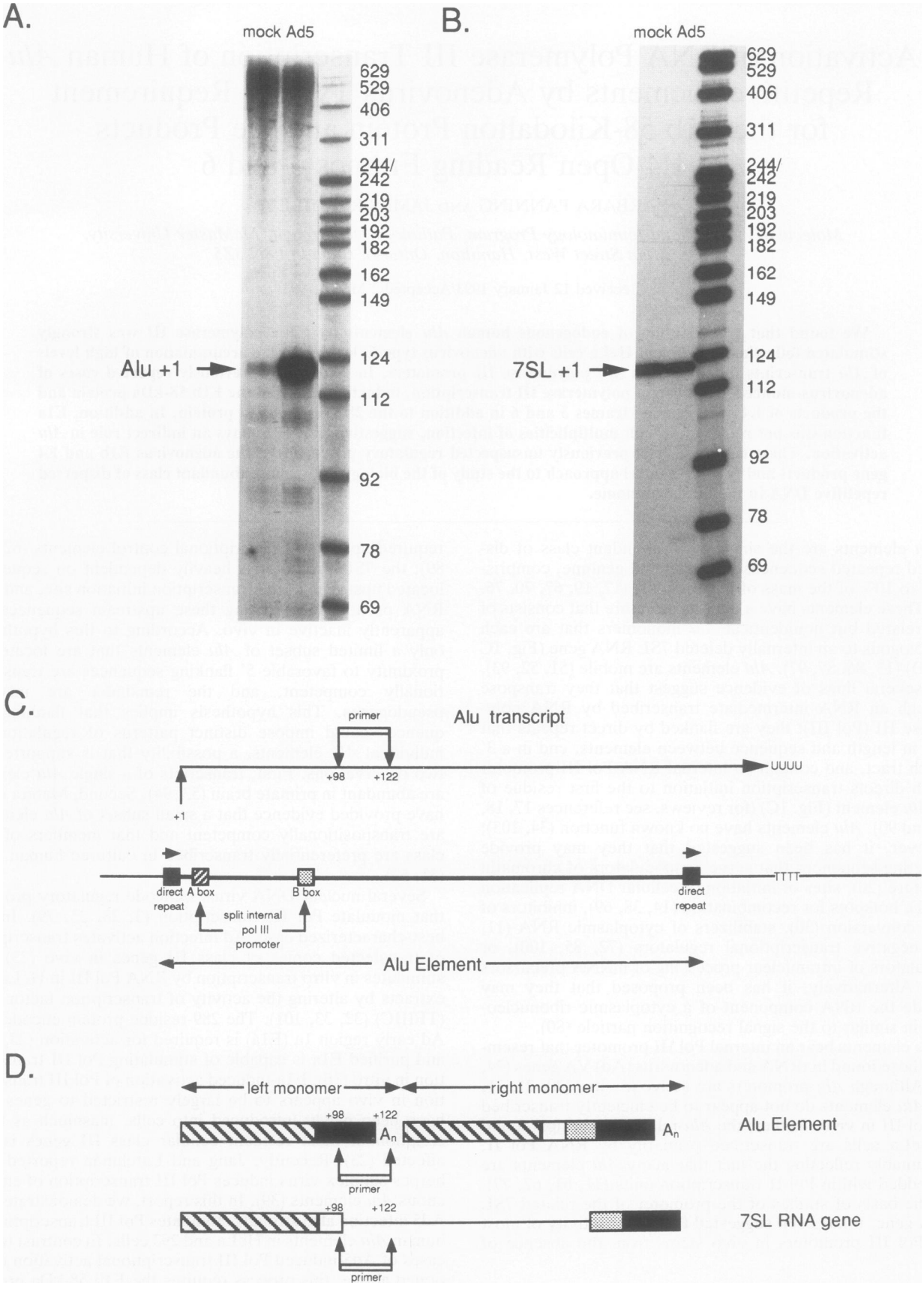
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C.
primer
Alu transcript
+98
+122
UUUU
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direct repeat
A box
B box
split internal pol III promoter
direct repeat
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Alu Element
D.
left monomer
right monomer
+98
+122
An
An
Alu Element
primer
+98
+122
7SL RNA gene
primer

FIG. 1. Induction of *Alu* expression upon Ad5 infection. Cytoplasmic RNA, harvested from HeLa cells 24 h postinfection with 25 PFU per cell, was analyzed by primer extension using 5'-labeled synthetic 25-mers designed to detect *Alu* and 7SL transcripts. Samples were then resolved on an 8% sequencing gel. Markers were 3'-end-labeled *Hpa*II fragments of pBR322 DNA; fragment sizes (in nucleotides) are indicated. (A) Primer extension analysis of 20 μg of cytoplasmic RNA, using the *Alu*-specific primer. (B) Primer extension analysis of 1 μg of cytoplasmic RNA, using the 7SL-specific primer. (C) Diagram of an *Alu* element, indicating the location of the primer relative to site of initiation of Pol III transcription. (D) Comparison of *Alu* and 7SL sequences. The boxed regions indicate areas of homology between *Alu* and 7SL RNAs.

in addition to the E1a 289-residue product. These findings suggest novel regulatory properties of the Ad5 E1b and E4 proteins and raise the possibility that analogous cellular *trans*-acting factors serve to modulate *Alu* expression in vivo.

MATERIALS AND METHODS

Cells and virus. Table 1 lists the Ad5 mutants that were used in this study; detailed descriptions of the structure of each mutation are provided in the legends to Fig. 3, 6, 7, 8, and 9. Ad5 mutants bearing lesions in the E4 region were propagated and titered on the E4-complementing line W162 (95), and all other Ad5 mutants were grown on 293 cells (26). 293 cells were maintained in α-minimal essential medium supplemented with 10% newborn calf serum; W162 and HeLa cells were grown in α-minimal essential medium containing 5% fetal calf serum. With the exception of *ts*125, *pm*975 and *dl*520, all virus preparations were purified by cesium chloride equilibrium density gradient centrifugation (25). Where indicated, infections were carried out in the presence of 20 μg of cytosine arabinofuranoside hydrochloride (araC; Sigma) per ml or 100 μg of cycloheximide (Sigma) per ml.

The titers of stocks of E4 mutants that are obtained by plaque assays on complementing W162 cells cannot be directly compared with the titers of other Ad5 stocks determined on 293 cells, because W162 cells are of simian origin and support plaque formation by wild-type Ad5 at a substantially reduced efficiency relative to human cells. To estimate the true titer of E4 stocks, we measured the virion concentrations in purified wild-type and E4 mutant stocks by measuring their A_{260} and then titered the stocks on 293 cells and W162 cells in parallel. Wild-type and E4 mutant strain stocks had titers of approximately 10^9 PFU per optical density unit when titered on W162 cells, indicating that the E4 mutations did not greatly alter virus particle-to-PFU ratios; however wild-type stocks gave a 25-fold-higher titer on 293 cells. Therefore, the titers of E4 mutant stocks were estimated on the basis of their A_{260} relative to that of wild-type stocks titered on 293 cells.

TABLE 1. Mutant strains

Viral strain	Reference
*dl*309	43
*dl*E1,3	27
*dl*312	43
*dl*520	29
*pm*975	55
*dl*50	12
*dl*55	12
1969(−)	6
1893(−)	6
1772(−)	6
*pm*2015/2250	54
*dl*1007	10
*dl*355*	35
*dl*356*	36
E4*dl*ORF1-4	35
E4*in*ORF6,6/7	35
E4*in*ORF3	35
*dl*355*+E4*in*ORF3	35
*dl*E3	27
*ts*125	91

Primer extension analysis. HeLa or 293 cells were infected at a multiplicity of 25 PFU per cell, and cytoplasmic RNA was isolated 24 h later by the method of Berk and Sharp (7). Primer extension was performed as previously described (82). Synthetic oligonucleotide primers were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. The *Alu* primer, 5'-TTAGTAGAGA(C/G)GGGGTTTCACCATG-3', is predicted to give rise to a ca. 122-nucleotide (nt) extension product from transcripts initiated from the *Alu* Pol III promoter. The indicated degeneracy was incorporated into the primer at position 11 in order to maximize the number of *Alu* elements detected. The 7SL RNA primer, 5'-AACT TAGTGCGGACACCCGATCAAG-3', is predicted to give rise to a 122-nt product from 7SL RNA. Both primers were chosen to minimize cross-hybridization due to the homology between *Alu* RNAs and 7SL RNA (see Results).

Nuclear run-on transcription assays. Nuclear run-on assays were carried out as described previously (81), except that nuclei were isolated as follows. Infected or uninfected HeLa cells (3×10^6 to 5×10^6) were washed extensively in cold phosphate-buffered saline, pelleted, suspended in 1 ml of buffer 1 (0.32 M sucrose, 3.0 mM calcium chloride, 2.0 mM magnesium acetate, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM Tris-HCl [pH 8.0], 40 U of RNAsin [Promega Biotec Corp.] per ml), and disrupted in a Dounce homogenizer, using a wide-bore pestle. Two volumes of buffer 2 (1.85 M sucrose, 5.0 mM magnesium acetate, 0.1 mM EDTA, 1.0 mM dithiothreitol, 10 mM Tris-HCl [pH 8.0], 40 U of RNAsin per ml) was added to the cell lysate, and the mixture was layered over 1.8 ml of buffer 2 and centrifuged at 130,000 × *g* for 45 min in an SW50.1 rotor. The nuclear pellet was resuspended in 100 μl of nuclei storage buffer (5.0 mM magnesium acetate, 0.1 mM EDTA, 5.0 mM dithiothreitol, 50 mM Tris-HCl [pH 8.0], 200 U of RNAsin per ml, 25% glycerol) per 1.5×10^6 cells in the original cell pellet and stored in liquid nitrogen. Run-on transcription in isolated nuclei was carried out in the presence of α-amanitin (Sigma) at the concentrations indicated.

Alu run-on transcripts were detected by using M13mp18 and M13mp19 constructs bearing a 1,300-nt *Nco*I-*Pst*I fragment containing the 600-bp duplicated *Alu* element present in the human α-2 globin gene cluster (31). The *Nco*I end was filled in with Klenow DNA polymerase, and the blunt *Nco*I-*Pst*I fragment was cloned into M13mp18 and M13mp19 digested with *Hin*cII and *Pst*I. Construct *Alu*18 produces noncoding-strand *Alu* DNA and is therefore predicted to hybridize *Alu* Pol III transcripts, while *Alu*19 produces the

opposite strand of *Alu* DNA. To detect 7SL-specific transcripts, a 125-bp *Sau*3A fragment of 7SL DNA (+101 to +226) was cloned into the *Bam*HI site of pBR322. This fragment was generated by polymerase chain reaction-mediated amplification of 7SL cDNA, using the synthetic oligonucleotides 5′-GTGCAGTGGCTATTCACAGG (+245 to +269) and 5′-CTCTGCCGATCGGGTGTCCG (+93 to +112) followed by cleavage with *Sau*3A, and contains a region of the 7SL gene that is lacking from *Alu* elements (86). Transcription of the E1A gene was detected by using pKH101 (6), a plasmid bearing the Ad5 *Kpn*I H fragment (0 to 5.7 map units). VA RNAs were detected by using a pUC19 plasmid bearing the 100-bp *Bam*HI-*Xba*I fragment spanning the 5′ end of the Ad2 VA1 gene from −30 to +70. Human rRNAs were detected by using p2.0, a plasmid bearing the 5′ end of the rat rDNA cluster (71). Ten micrograms of denatured plasmid DNA or single-stranded M13 DNA was bound to nitrocellulose filters in a slot blot apparatus and immobilized by UV cross-linking with a Stratalinker 2400 (Stratagene) according to the manufacturer's instructions. Slot blots were hybridized as previously described (81) for 36 h at 58°C. Following hybridization, filters were washed extensively at 68°C and then treated with 10 μg of RNase A per ml for 30 min at 37°C.

Metabolic labeling of proteins. HeLa cells were infected with the appropriate virus in the presence or absence of 20 μg of araC per ml. Cells were then labeled with [^{35}S]methionine from 24 to 25 h postinfection, harvested, and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (1).

RESULTS

Activation of expression of human *Alu* elements by Ad5. We used a primer extension assay to monitor changes in the abundance of *Alu*-related transcripts in HeLa cells after infection with Ad5. To eliminate interference from cross-hybridization with the related 7SL RNA, we designed an *Alu*-specific primer complementary to nt 98 to 122 of the *Alu* consensus sequence (Fig. 1C) (44). This region extends into the A-rich sequences connecting the two *Alu* monomers (Fig. 1D) and displays only limited homology to the 7SL gene. As a control, we also used an oligonucleotide primer designed to specifically detect 7SL RNA. The 7SL RNA primer was complementary to residues 98 to 122 of 7SL RNA (Fig. 1D), the region immediately downstream of the breakpoint in homology between *Alu* elements and the 7SL RNA gene (86). Cytoplasmic RNA from uninfected HeLa cells gave rise to two types of primer extension products with the *Alu* primer: a heterogeneous smear that extended into the high-molecular-weight range of the gel, and a faint band of ca. 122 nt (Fig. 1A; note that the autoradiogram was overexposed to allow visualization of the 122-nt product). The heterogeneous primer extension products are most likely derived from *Alu* elements that are embedded within transcripts of Pol II-transcribed genes, as reported by other workers (61, 62), while the 122-nt product corresponds to that predicted for transcripts initiated at the *Alu* Pol III promoter (20, 21, 60) (Fig. 1A). We found that the 122-nt primer extension signal was greatly augmented following infection with Ad5, while the heterogeneous products remained relatively constant in abundance. In contrast, 7SL RNA levels did not change after Ad5 infection (Fig. 1B). These data indicate that Ad5 infection strongly stimulates the accumulation of cytoplasmic RNAs initiated from *Alu* Pol III promoters.

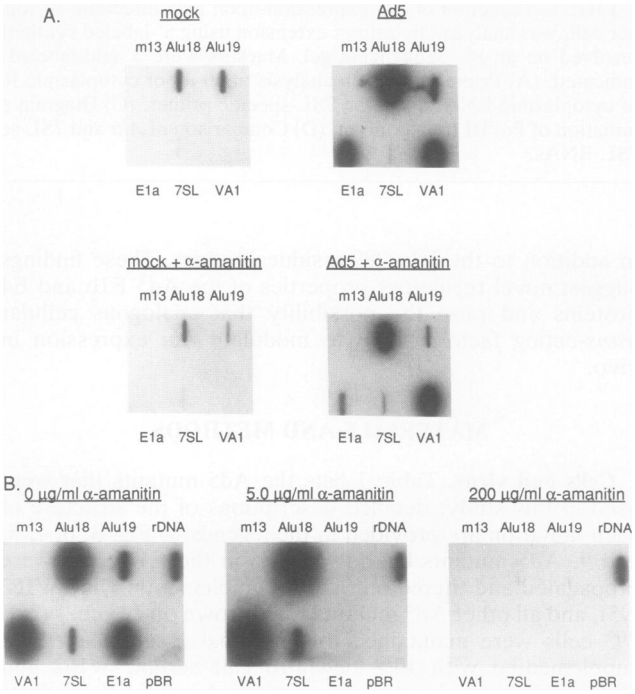

FIG. 2. *Alu* transcription in Ad5-infected cells. HeLa nuclei were harvested 24 h postinfection, and in vitro run-on transcription was carried out in the presence of α-amanitin at the concentrations indicated. Radiolabeled run-on transcripts were hybridized to 10 μg of M13 replicative-form DNA, single-stranded DNA from M13 constructs bearing *Alu* DNA (*Alu*18 and *Alu*19), and double-stranded plasmids encoding E1a, 7SL, and VA sequences and 50 μg of a plasmid bearing rDNA sequences fixed to nitrocellulose filters. *Alu*18 and *Alu*19 bear the *Alu* noncoding and coding strands, respectively.

Matera et al. (52) have reported that a distinct subset of *Alu* elements that have served as donors in recent retroposition events are transcriptionally active in HeLa cells, while the elements belonging to the major consensus class are silent. The *Alu* primer used in the present work is an exact match to the Alu consensus and does not cross-react with transcripts derived from the recently transposed class (60a). Our data therefore suggest that uninfected HeLa cells contain detectable levels of transcripts derived from *Alu* elements belonging to the major consensus class and that Ad5 infection activates expression of at least some of these elements. However, in other experiments, we have found that transcripts derived from the recently transposed class are also strongly induced during Ad5 infection (60a).

Increased Pol III transcription of *Alu* elements following infection with Ad5. We used the nuclear run-on transcription assay to determine whether the increase in cytoplasmic *Alu* RNAs seen after Ad5 infection reflected an increase in Pol III transcription of *Alu* sequences (Fig. 2). In the first experiment (Fig. 2A), run-on transcription assays were performed in the presence and absence of 2.5 μg of α-amanitin per ml to preferentially inhibit Pol II transcription (45, 48), and the labeled RNA products were hybridized to M13 clones bearing the *Alu* noncoding and coding strands (*Alu*18 and *Alu*19, respectively). As controls, the RNA samples were also hybridized to plasmids bearing the Ad5 E1a region (which is transcribed by Pol II) and a portion of the human 7SL gene and the Ad5 VA1 gene (both transcribed by Pol

III). Mock-infected nuclei displayed low levels of transcription from both *Alu* DNA strands, while infected nuclei displayed a large increase in *Alu* run-on transcripts that hybridized to the noncoding (*Alu*18)-strand probe. In contrast, no significant change in hybridization to the coding (*Alu*19)-strand probe was observed after infection. This asymmetric increase in *Alu* transcription was resistant to 2.5 μg of α-amanitin per ml, consistent with the notion that Ad5 infection stimulates Pol III transcription of *Alu* elements. Controls indicated that, as expected, transcription of the Ad5 E1a region was sensitive to 2.5 μg of α-amanitin per ml, while transcription of the Ad5 VA gene was largely unaffected. To confirm that the *Alu* transcripts were produced by RNA Pol III (as opposed to RNA Pol I), we tested the sensitivity of Ad5-induced *Alu* transcription to higher concentrations of α-amanitin (Fig. 2B). As expected, transcription of *Alu* elements and the Ad5 VA gene was resistant to 5 μg of α-amanitin per ml, while transcription of the E1a gene was strongly inhibited. However, the *Alu* and VA signals were eliminated by 200 μg of α-amanitin per ml, while RNA Pol I transcription of rDNA was not affected. These data therefore indicate that Ad5 infection stimulates transcription of *Alu* elements by RNA polymerase III.

Because of the extensive homology between *Alu* elements and the 7SL RNA gene, the *Alu* noncoding-strand probe (*Alu*18) should also detect 7SL transcripts. To determine whether the increased *Alu*18 hybridization signal was due primarily to *Alu* transcripts, a probe corresponding to the region of the 7SL RNA gene that is deleted from *Alu* elements was used to monitor the rate of transcription of this gene (and its pseudogenes) following infection. We found that the 7SL signal did not differ between infected and uninfected nuclei (Fig. 2A). These results suggest that the sequences detected by the *Alu* noncoding-strand probe in this assay are predominantly *Alu* transcripts.

Requirement for the Ad5 E1A 289-aa gene product. We surveyed the ability of a variety of Ad5 mutants to induce *Alu* expression to determine which Ad5 functions are required for this process. The products of the E1a gene seemed likely candidates, because they have been previously shown to be required for stimulation of transcription of Pol III-transcribed genes in vivo and in vitro (16, 23, 32, 33, 101). The E1a gene produces two major proteins of 289 and 243 amino acids (aa) by differential splicing (5, 64). To determine whether either or both of these products mediate activation, HeLa cells and 293 cells were infected with viruses bearing lesions in the E1A gene and then assayed for levels of *Alu* RNA by primer extension (Fig. 3A). Deletion mutants that lack either the E1a gene (*dl*312) or the entire E1 region (*dl*E1,3) did not induce *Alu* expression in HeLa cells, suggesting that one or more E1a products are required. Further analysis indicated that *pm*975, which cannot produce the 243-aa E1A gene product, induced wild-type levels of *Alu* RNAs, while *dl*520, which does not produce the 289-aa protein, was defective. Taken in combination, these data establish that the E1a 289-aa product is required for activation of *Alu* elements by Ad5 in HeLa cells and that the 243-aa protein is dispensable.

293 cells constitutively express E1a gene products (46), but uninfected 293 cells displayed the same low levels of *Alu* transcripts as do uninfected HeLa cells (Fig. 3A). However, *Alu* transcripts were induced to much higher levels when 293 cells were infected with either wild-type or E1-deficient Ad5. The simplest interpretation of these data is that the E1a (and E1b) proteins produced in 293 cells are not sufficient for high-level expression of *Alu* elements and that the superinfecting Ad5 provides one or more additional required proteins. Direct evidence supporting this hypothesis is presented below.

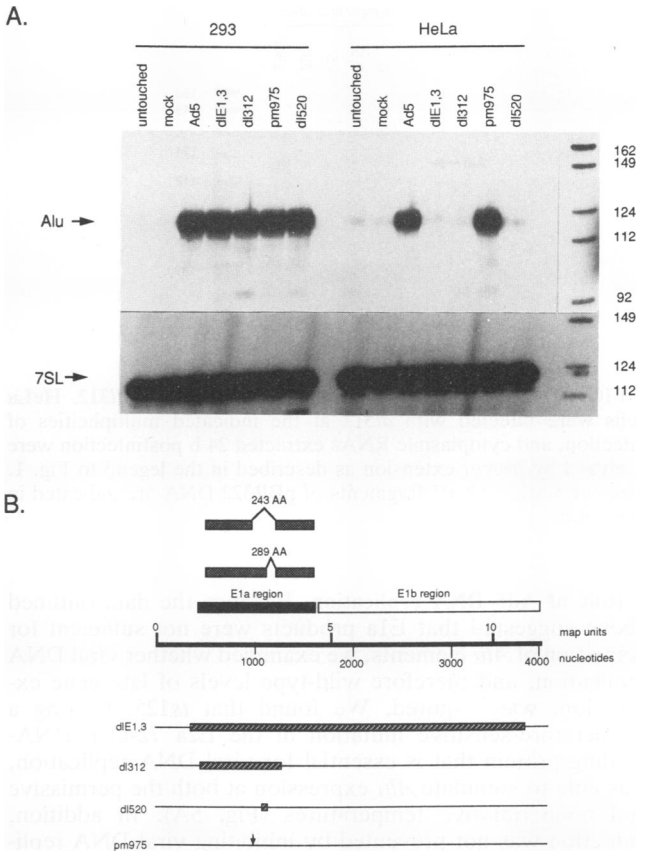

FIG. 3. Role of E1a gene products in activation of *Alu* expression. Cytoplasmic RNA was isolated from cells infected with the indicated Ad5 mutants and then analyzed by primer extension as described in the legend to Fig. 1. Sizes of marker fragments (3′-end-labeled pBR322 cleaved with *Hpa*II) are indicated in nucleotides. (A) Primer extension analysis of *Alu* and 7SL RNAs from 293 or HeLa cells infected with various E1a mutants. (B) Diagram of mutants used, showing the E1 region and indicating the locations of the lesions. Mutant *dl*E1,3 (27) bears deletions extending from 1.0 to 10.6 map units and from 78.5 to 84.7 map units, eliminating E1 and E3 coding regions. *dl*312 (43) lacks nt 448 to 1349 (1.2 to 3.7 map units), eliminating the E1a region. *dl*520 (29) has a deletion in the splice donor of the 13S E1a message and produces only the 243-aa 12S product. *pm*975 (55) encodes a point mutation in the splice donor of the E1a 12S species and as a result specifies only the 13S 289-aa polypeptide.

The 289-aa E1a protein is required for the efficient expression of the other Ad early genes (42). It was therefore possible that E1a plays only an indirect role in *Alu* activation, by stimulating the expression of additional required Ad function(s). To address this question, we took advantage of the observation that Ad early gene expression can be achieved in the absence of E1a during infection at high multiplicity (56). We found that the E1a deletion mutant *dl*312 was able to induce Alu expression following infection of HeLa cells with 2,500 PFU per cell (Fig. 4). Although not definitive, these data raise the possibility that the E1a 289-aa protein is required primarily to activate expression of other viral gene products, which in turn serve to stimulate *Alu* transcription.

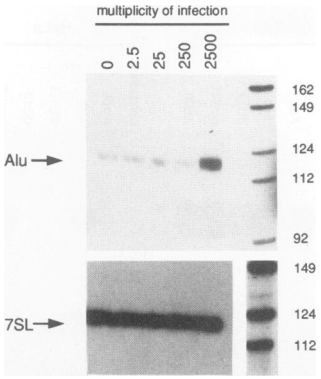

FIG. 4. Effects of high-multiplicity infection with *dl*312. HeLa cells were infected with *dl*312 at the indicated multiplicities of infection, and cytoplasmic RNAs extracted 24 h postinfection were analyzed by primer extension as described in the legend to Fig. 1. Sizes of marker *Hpa*II fragments of pBR322 DNA are indicated in nucleotides.

Role of Ad5 DNA replication. Because the data outlined above suggested that E1a products were not sufficient for activation of *Alu* elements, we examined whether viral DNA replication, and therefore wild-type levels of late gene expression, was required. We found that *ts*125, bearing a temperature-sensitive mutation in the E2a 72-kDa DNA-binding protein that is essential for viral DNA replication, was able to stimulate *Alu* expression at both the permissive and nonpermissive temperatures (Fig. 5A). In addition, induction was not prevented by inhibiting viral DNA replication with araC (Fig. 5B) or hydroxyurea (data not shown). Control experiments confirmed that the treatment with araC effectively blocked Ad5 DNA replication (Fig. 5C). Taken together, these results indicate that viral DNA replication, and therefore wild-type levels of late gene expression, is not required for activation of *Alu* transcription.

Requirement for E1b and E4 gene products. The simplest interpretation of the data presented above was that one or more Ad5 early proteins in addition to E1a were required for activation of *Alu* expression. To test this hypothesis, we infected HeLa cells with viral mutants bearing deletions in early regions E1b, E3, and E4 (Fig. 6). *dl*E3, bearing a large deletion in the E3 region, showed wild-type levels of *Alu* RNA, indicating that E3 products are not required. This observation is consistent with the wild-type activity of *dl*E1,3 in 293 cells (Fig. 3; *dl*E1,3 bears the same E3 deletion as does *dl*E3). In contrast, mutants bearing large deletions in E1b (*dl*50) or E4 (*dl*1007) were strongly impaired, indicating that one or more products of each of the E1b and E4 genes are necessary for efficient induction of *Alu* expression. Additional control experiments confirmed that *dl*50 and *dl*1007 complemented each other for *Alu* activation during coinfection of HeLa cells and that *dl*50 was able to efficiently induce *Alu* expression during infection of 293 cells, while *dl*1007 was inactive in this cell type (data not shown).

The E1b gene products act as posttranscriptional regulators of viral gene expression (4, 65, 99), while the E4 region encodes at least one protein that acts as a transcriptional regulator and at least two others that serve as posttranscriptional modulators of gene expression (10, 28, 35, 36, 50, 66, 73, 96). Analysis of run-on transcription in nuclei isolated from HeLa cells infected with *dl*1007 or *dl*50 showed that there was no detectable increase in Pol III transcription of *Alu* elements upon infection with either mutant strain (Fig. 7). These data therefore indicate that products of the E1b and E4 genes are required for transcriptional activation of *Alu* elements. The levels of expression of the viral E1A and VA genes seen upon infection with *dl*1007 and *dl*50 were comparable to those seen in nuclei from wild-type-infected cells, demonstrating that comparable multiplicities of infection were used.

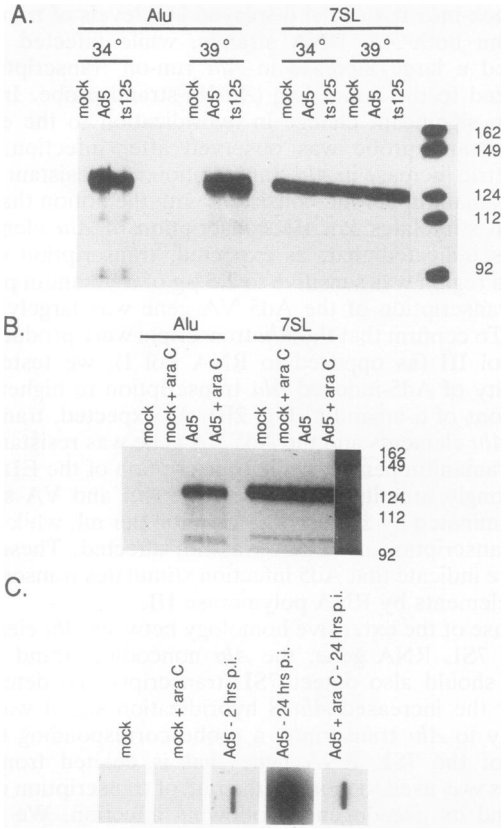

FIG. 5. Effect of inhibition of DNA replication. (A and B) HeLa cells were infected with 25 PFU of the indicated virus strain per cell, and cytoplasmic RNA extracted at 24 h postinfection was analyzed by primer extension as described in the legend to Fig. 1. Sizes of marker DNA fragments (*Hpa*II fragments of pBR322 DNA) are indicated in nucleotides. (A) Analysis of *Alu* and 7SL transcripts from cells infected with Ad5 or *ts*125 (91) at the permissive (34°C) and nonpermissive (39°C) temperatures. (B) Levels of *Alu* and 7SL cytoplasmic RNAs in HeLa cells infected with Ad5 in the presence or absence of 20 μg of araC per ml. (C) Effect of araC on accumulation of viral DNA. HeLa cells were infected with Ad5 at 25 PFU per cell in the presence and absence of 20 μg of araC per ml, and DNA was isolated 2 and 24 h postinfection (p.i.). Ten micrograms of DNA from each sample was affixed to a nitrocellulose filter and probed with radiolabeled pKH101, a plasmid bearing sequences from the left end of the Ad5 genome.

Requirement for the E1B 496-aa protein. The E1B region encodes two major products, a 19-kDa, 176-aa protein and a 58-kDa, 496-aa protein (8, 64). To determine which of these proteins was required to activate *Alu* expression, we analyzed RNA extracted from cells infected with *pm*2015/2250, which does not produce the 58-kDa protein, and the viruses 1969(−), 1893(−), and 1772(−), which fail to specify the 19-kDa protein (Fig. 8). Strains 1969(−), 1893(−), and 1772(−) induced levels of *Alu* RNA similar to those observed during infection with wild-type Ad5, while *pm*2015/2250 was strongly impaired. As expected, *dl*50 and *dl*55 (defective for

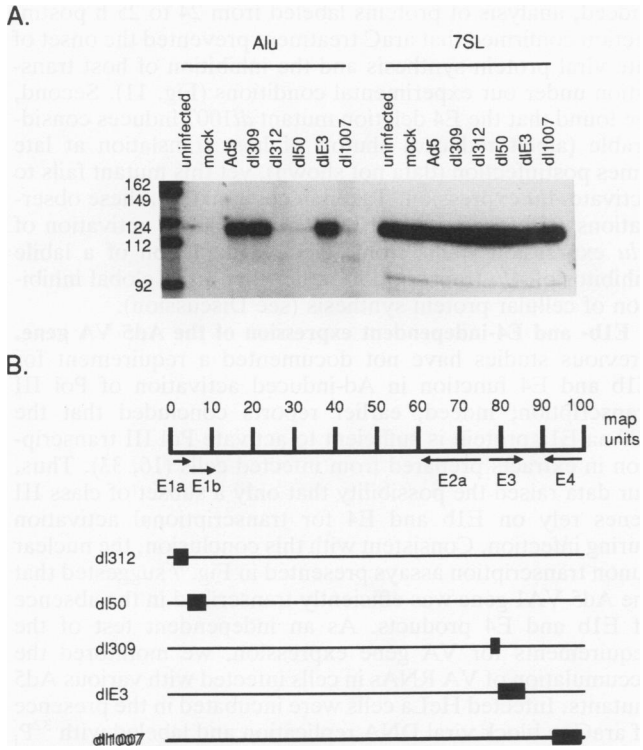

FIG. 6. Role of early gene products in activation of *Alu* expression. HeLa cells were infected with 25 PFU of the indicated Ad5 mutants per cell, and *Alu* and 7SL expression was assayed by primer extension analysis of cytoplasmic RNA as described in the legend to Fig. 1. Size markers were 3'-end-labeled *Hpa*II-cleaved pBR322 size markers (lengths are indicated in nucleotides). (A) *Alu* and 7SL RNA levels detected after infection with various early region mutants. (B) Locations of the lesions in the mutant viruses used. *dl*312 produces no E1a proteins. *dl*50 (12) produces no E1b gene products because of a deletion in the E1b coding region between nt 1770 and 3641 (4.8 to 10.0 map units). *dl*E3 (27) lacks E3 sequences between 78.5 and 84.7 map units and therefore produces no E3 products. *dl*309 (43) contains a small deletion between 83 and 85 map units in the E3 region. *dl*1007 (10) fails to produce any E4 gene products as a result of a deletion of sequences between 93.3 and 98.4 map units in the E4 coding region.

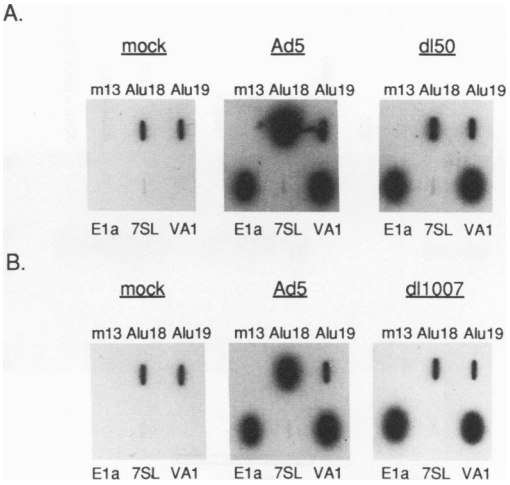

FIG. 7. Run-on assays for *Alu* transcription following infection with E1b and E4 mutants. HeLa cells were infected with 25 PFU of *dl*1007 or *dl*50 per cell, and nuclei were isolated 24 h later. Nuclear run-on transcription was performed with [α-^{32}P]UTP, and the resulting RNAs were hybridized to nitrocellulose filters bearing *Alu*, 7SL, E1a, and VA DNA as described in the legend to Fig. 2.

both E1b products) also failed to induce *Alu* RNAs. Controls indicated that *dl*50, *dl*55, and *pm*2015/2250 could be efficiently complemented by coinfection with the E1a deletion mutant *dl*312, demonstrating the presence of biologically active virus in the mutant stocks. These data indicate that the 58-kDa 496-aa E1b protein is required for the activation of *Alu* transcription. Although both *dl*50 and *pm*2015/2250 displayed greatly reduced activity relative to that of wild-type Ad5, they did show slight induction above the levels seen in uninfected or mock-infected cells in some experiments (data not shown), suggesting that other viral proteins are able to induce low levels of *Alu* expression in the absence of the 58-kDa protein.

Requirement for the products of E4 ORF3 and ORF6. The E4 region of Ad5 contains at least six ORFs and appears to encode at least seven distinct polypeptides through differential RNA splicing (22, 84, 92). Three of the E4 polypeptides have been shown to serve as regulators of gene expression. The ORF6/7 polypeptide forms a complex with the cellular E2F transcription factor, thereby stimulating the activity of the E2 promoter (36, 50, 66), while the ORF3 and ORF6 proteins serve as functionally redundant posttranscriptional regulators of viral late gene expression (10, 35). The 34-kDa ORF6 protein forms a complex with the E1b 58-kDa protein (74), which is apparently required for the posttranscriptional regulatory activity of the 58-kDa protein (15). To determine which E4 gene products are required for stimulation of *Alu* expression, we analyzed cytoplasmic RNA isolated from HeLa cells infected with mutant viruses bearing lesions in various E4 ORFs (Fig. 9A). The E4 mutants fell into three categories with respect to the ability to induce *Alu* expression. One mutant, *dl*356*, showed wild-type activity. This result demonstrates that the ORF6/7 protein that binds the E2F transcription factor is not required. A second group of mutants were partially impaired but displayed significantly higher levels of *Alu* RNAs than did the E4 null mutant *dl*1007. This group included two mutants that inactivate ORF6 (*dl*355* and E4*in*ORF6,6/7), one mutant that deletes ORF1 through ORF4 (E4*dl*ORF1-4), and one that inactivates ORF3 (E4*in*ORF3). These data therefore implied the existence of at least two partially redundant E4 functions that are required for *Alu* activation, one encoded by ORF6 and the other specified by ORF3. To test this hypothesis, we examined a double mutant, *dl*355*/E4*in*ORF3, that bears inactivating mutations in both ORF3 and ORF6. This mutant showed the same extreme phenotype as did the E4 null mutant *dl*1007, suggesting that the ORF3 and ORF6 products together account for most or all of the activity of the E4 region. Control experiments demonstrated that all of the E4 mutants were capable of complementing the E1a deletion mutant *dl*312 for activation of *Alu* elements in coinfected cells, indicating that infectious virus was present in each E4 mutant stock. In summary, the data obtained in these experiments indicated that the products encoded by E4 ORF3 and ORF6 are both required for wild-type levels of *Alu* activation and suggest that these proteins display partial functional redundancy in this process.

Variable induction of *Alu* RNAs by cycloheximide. As described in Discussion, the E1b 58-kDa protein and the products of E4 ORF3 and ORF6 contribute to the shutoff of

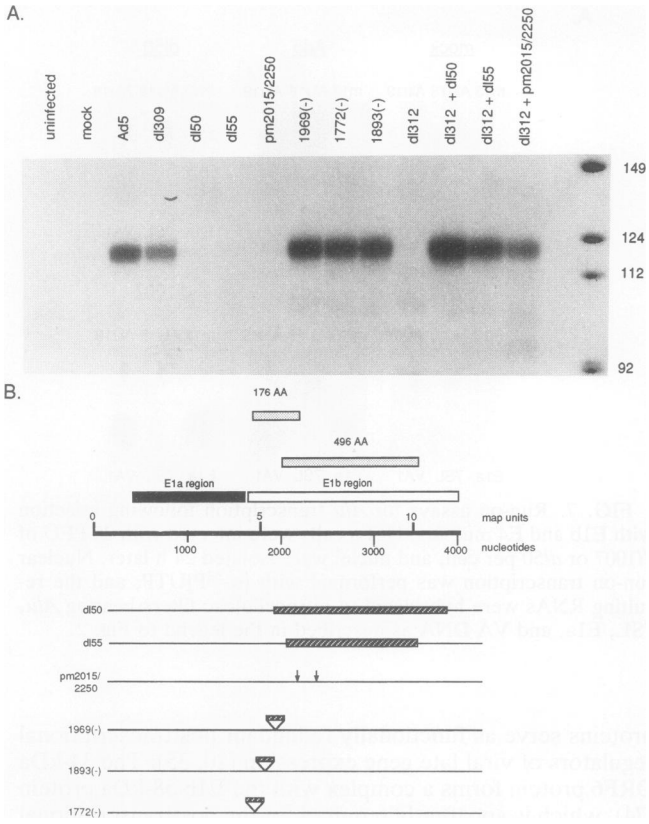

FIG. 8. Role of individual E1b gene products. HeLa cells were infected with 25 PFU of the indicated mutants per cell, and cytoplasmic RNAs were analyzed by primer extension as described in the legend to Fig. 1. Sizes of marker *Hpa*II fragments of pBR322 DNA are indicated in nucleotides. (A) Levels of *Alu* RNAs in cells infected with E1b mutant viruses. (B) Locations of mutations in the viruses tested. *dl*50 produces neither the 58-kDa 496-aa protein nor the 19-kDa 176-aa product. *dl*55 (12) fails to produce the E1b 58-kDa or 19-kDa protein as a result of a deletion of sequences between 5.3 and 9.0 map units (nt 1969 to 3330). *pm*2015/2250 (54) does not produce the 58-kDa product as a result of point mutations eliminating the AUG and introducing an additional stop codon downstream. *dl*309 contains a small deletion in the E3 region but grows like wild-type virus (43) and is the parental strain of *dl*50, *dl*55, and *pm*2015/2250. 1969(−), 1893(−), and 1772(−) (6) have stop codons inserted at aa 86, 51, and 21, respectively, of the 19-kDa 176-aa protein and produce only truncated products.

host protein synthesis that occurs late during Ad infection. We therefore examined whether the protein synthesis inhibitor cycloheximide induced *Alu* RNAs in uninfected cells. Treatment of HeLa cells with cycloheximide at 100 μg/ml resulted in a significant increase in the abundance of *Alu* RNAs in some experiments (for example, Fig. 10); however, the magnitude of the response varied between experiments and was in no case as great as that seen upon Ad5 infection (Table 2; note that Fig. 10 displays the results of the experiment which showed the greatest degree of induction by cycloheximide [Table 2, experiment 6]). The relatively low levels of induction by cycloheximide suggest that Ad5-induced *Alu* activation does not stem solely from global shutoff of host translation. Two additional observations support this conclusion. First, *Alu* elements were efficiently induced when viral DNA replication was blocked with araC (Fig. 5A), a condition that prevents host shutoff (49, 58, 59). Indeed, analysis of proteins labeled from 24 to 25 h postinfection confirmed that araC treatment prevented the onset of late viral protein synthesis and the inhibition of host translation under our experimental conditions (Fig. 11). Second, we found that the E4 deletion mutant *dl*1007 induces considerable (albeit reduced) shutoff of host translation at late times postinfection (data not shown), yet this mutant fails to activate *Alu* expression. Taken in combination, these observations raise the possibility that Ad5-induced activation of *Alu* expression stems from selective depletion of a labile inhibitor of *Alu* transcription rather than from global inhibition of cellular protein synthesis (see Discussion).

E1b- and E4-independent expression of the Ad5 VA gene. Previous studies have not documented a requirement for E1b and E4 function in Ad-induced activation of Pol III transcription; indeed, earlier reports concluded that the 289-aa E1a protein is sufficient to activate Pol III transcription in extracts prepared from infected cells (16, 33). Thus, our data raised the possibility that only a subset of class III genes rely on E1b and E4 for transcriptional activation during infection. Consistent with this conclusion, the nuclear runon transcription assays presented in Fig. 7 suggested that the Ad5 VA1 gene was efficiently transcribed in the absence of E1b and E4 products. As an independent test of the requirements for VA gene expression, we monitored the accumulation of VA RNAs in cells infected with various Ad5 mutants. Infected HeLa cells were incubated in the presence of araC to block viral DNA replication and labeled with $^{32}P_i$ from 2 to 18 h postinfection. Cytoplasmic RNA was then examined for the presence of labeled VA RNA by electrophoresis through an 8% sequencing gel. As previously reported, the E1a-deficient mutant *dl*312 failed to accumulate VA RNA (42). However, deletion of the E1b (*dl*50) or E4 (*dl*1007) regions did not prevent VA RNA synthesis (Fig. 12). These data confirm that E1b and E4 gene products are not required for all cases of activation of Pol III-transcribed genes in Ad5-infected cells.

DISCUSSION

The data presented in this report demonstrate that Ad5 infection activates Pol III transcription of endogenous human *Alu* elements in HeLa and 293 cells, resulting in the accumulation of high levels of cytoplasmic RNAs initiated from *Alu* promoters. Induction occurred in the absence of viral DNA replication, suggesting that activation is mediated by one or more early gene products. Further analysis revealed that at least four Ad5 early polypeptides are required for this process: the E1a 289-aa protein, the E1b 58-kDa protein, and the products of E4 ORF3 and ORF6. Although it is not yet clear what proportion of the ca. 500,000 *Alu* elements residing in the human genome are induced upon Ad5 infection, data to be presented elsewhere indicate that at least two distinct subclasses of Alu elements are stimulated (60a), suggesting that Ad5 gene products provoke a relatively global activation of *Alu* transcription.

Previous reports have established that the 289-aa E1a protein activates in vitro transcription of class III genes in the absence of other Ad5 proteins, by modifying the activity of TFIIIC (16, 32, 33, 101). However, although the 289-aa E1a protein was required for activation of *Alu* transcription under our standard conditions of infection, additional proteins encoded by the E1b and E4 regions were also required. These results indicate that E1a-induced modification of TFIIIC is not sufficient for activation of Alu elements in vivo. Consistent with this conclusion, 293 cells did not show

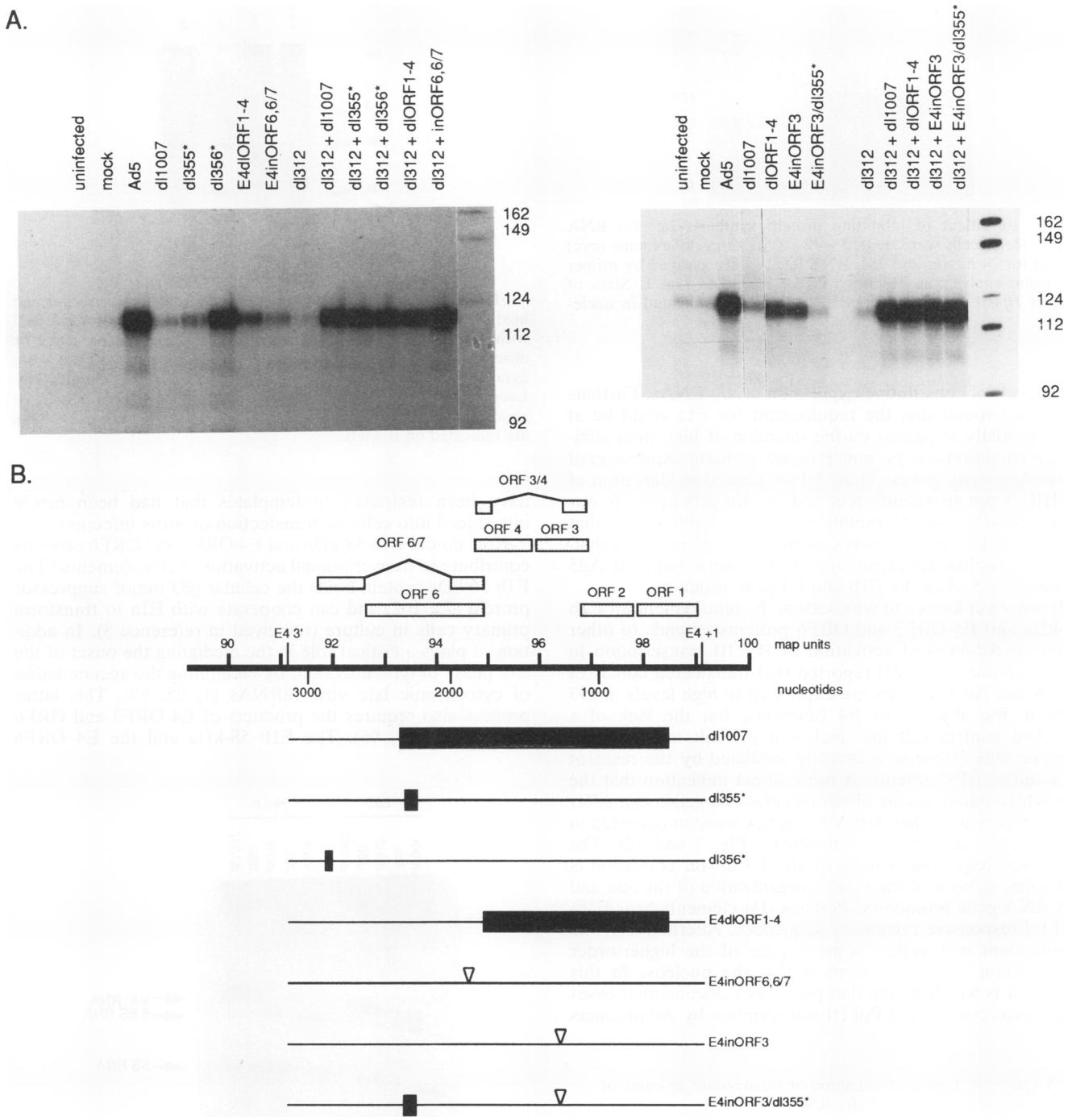

FIG. 9. Effects of deletion of various E4 gene products on activation of *Alu* expression. Cytoplasmic RNA isolated from HeLa cells 24 h after infection with mutants bearing lesions in the E4 coding region was analyzed by primer extension as described in the legend to Fig. 1. Single infections were done at a multiplicity of infection of 25, while coinfections used 25 PFU of each virus per cell. Sizes of marker *Hpa*II fragments of pBR322 DNA are indicated in nucleotides. (A) Levels of *Alu* transcripts after infection with E4 mutants. (B) Locations of the mutations used in these experiments. *dl*1007 contains a deletion eliminating expression of all E4 gene products. *dl*355* (35) has a 14-bp deletion at 93.5 map units in the sequences unique to the ORF6 coding region and fails to produce this gene product. *dl*356* (36) contains a 12-bp deletion at 91.8 map units and fails to produce the ORF6/7 protein product. E4*dl*ORF1-4 (35) lacks sequences between 94.9 and 98.4 map units and does not produce the products encoded by ORF1 through ORF4. Mutant strain E4*in*ORF6,6/7 (35) contains a 2-bp insertion at 94.6 map units, a region common to both ORF6 and ORF6/7, and thus fails to produce both these proteins. E4*in*ORF3 (35) introduces 8 bp at 96.4 map units, in sequences encoding ORF3, and as a result fails to produce the ORF3 gene product. *dl*355*/E4*in*ORF3 (35) combines the lesions in *dl*355* and E4*in*ORF3 and therefore produces neither the ORF3 nor the ORF6 protein product.

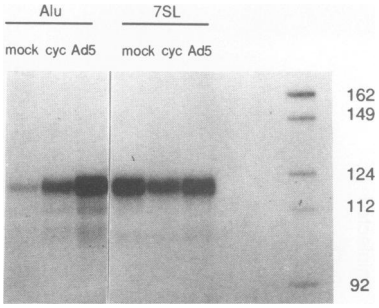

FIG. 10. Effect of inhibiting protein synthesis on *Alu* RNA levels. HeLa cells were treated with 100 μg of cycloheximide (cyc) per ml for 24 h. *Alu* and 7SL RNA levels were assayed by primer extension analysis as described in the legend to Fig. 1. Sizes of marker *Hpa*II fragments of pBR322 DNA are indicated in nucleotides.

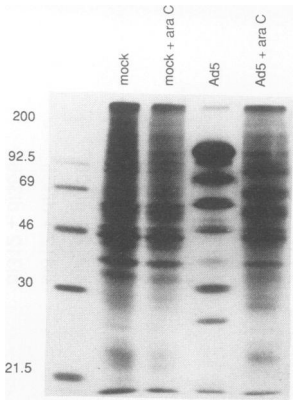

FIG. 11. Effects of inhibition of DNA replication on viral and host protein synthesis. HeLa cells were mock infected or infected with Ad5 at a multiplicity of infection of 25 in the presence or absence of 20 μg of araC per ml. Levels of protein synthesis were assayed by incorporation of [^{35}S]methionine 24 to 25 h postinfection. Labeled polypeptides were displayed on an SDS–10% polyacrylamide gel. Sizes of protein molecular weight markers in kilodaltons are indicated on the left.

high levels of constitutive expression of *Alu* RNAs. Furthermore, we found that the requirement for E1a could be at least partially bypassed during infection at high multiplicities, a condition that permits E1a-independent expression of other Ad5 early genes. Thus, E1a-mediated modification of TFIIIC is not stringently required for *Alu* activation. In our view, the simplest interpretation of the available data is that the 289-aa E1a protein plays an indirect role in *Alu* activation, by facilitating expression of additional required Ad5 proteins, including the E1b and E4 gene products.

It is not yet known to what extent the requirement for E1b 58-kDa and E4 ORF3 and ORF6 proteins extends to other cases of Ad-induced activation of Pol III transcription in vivo. Gaynor et al. (23) reported that transfected copies of tRNA and Ad VA genes are expressed to high levels in 293 cells in the absence of E4 functions, but the lack of a matched control cell line makes it difficult to determine whether this response is directly mediated by the resident E1a and/or E1b proteins. A more direct indication that the regulatory requirements of various class III genes can differ is the observation that Ad5 VA gene expression occurred in the absence of E1b or E4 products (Fig. 7 and 12). The additional requirement for E1b and E4 in *Alu* activation is intriguing in view of the similar organization of the *Alu* and VA RNA gene promoters. Perhaps *Alu* elements bear E1b- and E4-responsive regulatory sequences. Alternatively, the requirement may reflect some feature of the higher-order organization of *Alu* elements within the nucleus. In this regard, it is worth noting that previously documented cases of in vivo activation of Pol III transcription by Ad products have been restricted to templates that had been newly introduced into cells by transfection or virus infection.

How do the E1b 58-kDa and E4 ORF3 and ORF6 proteins contribute to transcriptional activation of *Alu* elements? The E1b 58-kDa protein binds the cellular p53 tumor suppressor protein (75, 102) and can cooperate with E1a to transform primary cells in culture (reviewed in reference 8). In addition, it plays a critical role in the mediating the onset of the late phase of lytic infection, by facilitating the accumulation of cytoplasmic late viral mRNAs (4, 65, 99). This latter process also requires the products of E4 ORF3 and ORF6 (28, 35, 73, 95, 96). The E1b 58-kDa and the E4 ORF6

TABLE 2. Levels of induction of steady-state amounts of *Alu* RNA

Expt	Relative amt of *Alu* RNA[a]	
	Cycloheximide/ mock	Ad5/ mock
1	1.1	18.6
2	1.8	20.5
3	2.6	26.5
4	2.9	71.0
5	6.6	28.9
6	10.9	50.4

[a] Determined by primer extension followed by quantitation with a PhosphorImager (Molecular Dynamics).

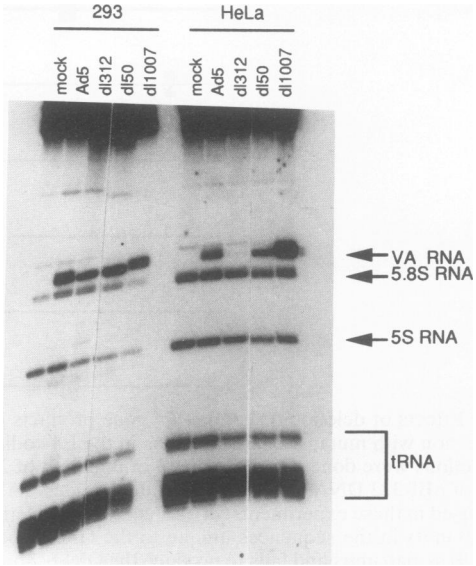

FIG. 12. Role of early gene products in transcription of VA RNA. HeLa or 293 cells were infected with 25 PFU of the indicated virus per cell in the presence of 20 μg of araC per ml and then labeled with $^{32}P_i$ from 2 to 18 h postinfection. Cytoplasmic RNA was subjected to electrophoresis through an 8% sequencing gel to determine levels of VA RNA.

products associate to form a complex that localizes to the nucleus (74, 83), and mutations that eliminate the 58-kDa protein or E4 region result in similar defects in the accumulation of cytoplasmic late mRNAs, viral DNA replication, and shutoff of host protein synthesis (4, 28, 65, 95, 96, 99). The E4 ORF3 and ORF6 proteins appear to be functionally redundant, in that mutations that inactivate either protein have relatively minor effects, while mutations that simultaneously eliminate both display an exaggerated phenotype with profoundly reduced levels of virus replication (10, 35). These data have been interpreted to indicate that the E1b 58-kDa protein functions as a complex with ORF6 and that E4 ORF3 performs a similar or partially overlapping function. The precise mechanism by which these proteins stimulate the accumulation of cytoplasmic late viral mRNA remains unknown; however, the available evidence indicates that they function within the nucleus at the posttranscriptional level to facilitate the transport or processing of mRNA precursors. Thus, mutants lacking the 58-kDa E1b protein display defects in the transit of late mRNA precursors through nuclear subcompartments, leading to reduced levels of cytoplasmic mRNAs (47), while mutants lacking all of the E4 region exhibit reduced stability of intranuclear late mRNA precursors (73). In addition, evidence has been presented that E4 products may act at the level of mRNA processing (57). The striking correlation between the Ad5 gene products required for the accumulation of late viral mRNAs, suppression of host protein synthesis, and activation of *Alu* elements suggests that these processes are mechanistically related.

One possibility is that activation is a part of cellular response to virus infection that is induced by events well downstream of the immediate targets of E1b and E4 activity: for example, the accumulation of late viral mRNA or the attendant global shutoff of host protein synthesis. Although this possibility cannot be discounted, several observations appear to exclude some of the more obvious potential inducing signals. First, activation was not affected by blocking viral DNA replication (Fig. 5), a condition that prevents late viral protein synthesis and the suppression of host translation (Fig. 11), and the accumulation of double-stranded RNA capable of activating the DAI protein kinase (49, 58, 59). Second, mutant strain E4*dl*ORF1-4 shows wild-type levels of host translational shutoff (35) but is partially impaired for induction of *Alu* expression (Fig. 9). A second possibility is that activation is the indirect consequence of the same intranuclear events that stimulate the transport and/or processing of late viral mRNA precursors. For example, these alterations might block the export of an unstable cellular mRNA encoding a labile repressor of *Alu* transcription (note that this hypothesis can account for the stimulation of *Alu* expression by cycloheximide and the observation that *Alu* activation can be uncoupled from global translational shutoff). Alternatively, activation might result from Ad5-induced changes in intranuclear architecture. It has been proposed that the E1b 58-kDa protein acts by increasing the number of intranuclear sites from which newly transcribed mRNA precursors can engage the RNA transport machinery (47). Perhaps this alteration involves changes in intranuclear organization or chromatin structure that allow *Alu* elements to gain access to required transcription factors or Pol III. In this context, it is interesting to note that the Ad12 E1b 55-kDa protein plays a role in decondensing a limited number of specific sites in human metaphase chromosomes, termed Ad12 modification sites (78). Although this phenomenon is not observed during infection with Ad5, these observations provide a precedent for E1b-dependent alterations in chromatin organization. Finally, it is possible that the E1b and E4 proteins exert their effects on the transport and/or processing of viral mRNA precursors by inducing transcription of one or more endogenous cellular genes. Perhaps the relevant targets are transcribed by Pol III, and *Alu* elements are coincidentally induced; alternatively, it is conceivable that *Alu* elements play some role in mediating E1b and E4 function. Further work is required to distinguish between these possibilities.

The ability of Ad5 gene products to induce abundant expression of *Alu* transcripts offers a novel approach to the study of the biology of this important class of retroposons. For example, we have found that *Alu* elements falling into several distinct subclasses are induced, implying that many *Alu* elements are transcriptionally competent, given the presence of appropriate *trans*-acting factors (60a). RNA polymerase chain reaction-based approaches should allow the identification of a large number individual *Alu* elements that respond to Ad5 infection, and it will be interesting to learn whether these elements share common features that might account for their activation. In addition, Ad5-infected cells can be used to examine whether *Alu* transcripts are assembled into ribonucleoproteins similar to the signal recognition particle. Finally, it is possible that Ad5 infection increases the frequency of *Alu* transposition, by increasing the concentration of the presumed RNA transposition intermediate.

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