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Adenovirus Type 2 Preferentially Stimulates Polymerase III Transcription of *Alu* Elements by Relieving Repression: a Potential Role for Chromatin

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The number of *Alu* transcripts that accumulate in HeLa and other human cells is normally very low; however, infection with adenovirus type 5 increases the expression of *Alu* elements dramatically, indicating that the potential for polymerase III (pol III)-dependent *Alu* transcription in vivo is far greater than generally observed (B. Panning and J. R. Smiley, Mol. Cell. Biol. 13:3231–3244, 1993). In this study, we employed nuclear run-on in combination with a novel RNase H-based assay to investigate transcription from uninfected and adenovirus type 2-infected nuclei, as well as genomic DNAs from uninfected and infected cells. When performed in the presence of excess uninfected nuclear extract, such assays revealed that (i) the vast majority of transcriptionally competent *Alu* elements in nuclei are masked from the pol III transcriptional machinery and (ii) the induction of *Alu* expression upon adenovirus infection can be largely accounted for by an increased availability of these elements to the pol III transcription machinery. We also investigated the role of H1 histone for silencing of *Alu* genes and, in comparison, mouse B2 repetitive elements. Depletion of H1 led to an ≈17-fold activation of B2 repetitive elements but did not change *Alu* transcription relative to that of constitutively expressed 5S rRNA genes. These results are consistent with the view that *Alu* repeats are efficiently sequestered by chromatin proteins, that such masking cannot be accounted for by nonspecific H1-dependent repression, and that adenovirus infection at least partially overrides the repressive mechanism(s).

Within the human genome reside at least 500,000 copies of the *Alu* interspersed repetitive gene family (9, 45). These repeat elements possess bipartite RNA polymerase III (pol III) promoters and serve as active templates in vitro, yet despite their high copy number seldom give rise to high levels of pol III-transcribed RNAs in vivo. Important exceptions to this rule are provided by the recent findings that pol III-dependent *Alu* transcription can be strongly induced in HeLa cells following high-titer adenovirus or herpesvirus infection (36, 37). These findings confirm that *Alu* repeats have the potential to function as active templates in vivo under certain conditions but leave unanswered why such low *Alu* small-RNA levels accumulate under normal circumstances. One explanation which has been offered is that the vast majority of *Alu* repeat elements, by virtue of their insertion into the genome via retroposition, lack 5′-flanking sequences requisite for efficient capture of transcription factors (43). According to this idea, since *Alu* elements are unable to compete for pol III transcription factors (which are presumed to be present in limiting amounts within the cell), they are progressively packaged into chromatin structures with concomitant transcriptional silencing. The differential developmental regulation of *Xenopus* somatic and oocyte 5S genes has served as a paradigm in this regard. It appears that oocyte 5S genes are subject to chromatin-mediated repression at least in part because they form less stable pol III transcription complexes than their somatic counterparts (18, 23, 40, 46). Chromatin-dependent repression of the oocyte 5S genes is presumed to involve histone H1, since H1 is required to block access of pol III transcription factors to these templates in vitro (1, 6, 38, 47).

There is no clear evidence that mammalian short interspersed elements (SINEs) possess a weaker affinity for pol III transcription factors than, for example, 7SL or tRNA genes, which are likewise type II pol III genes but are expressed constitutively in somatic cells. Nevertheless, previous studies of mouse B1 and B2, two SINE families in mammalian cells, do support the idea that the transcription rate and template availability of pol III repeat elements can vary as a function of cell proliferation or virus-mediated changes in pol III transcription factor activity (2, 4, 11, 13, 20, 22, 27, 34, 39, 41, 44). Mouse B2 elements are almost completely sequestered by chromatin proteins in quiescent cells but become partially unmasked in growing or simian virus 40 (SV40)-transformed cells (4). Still greater unmasking and template activity can be accomplished in vitro by removal of histone H1 (and other uncharacterized proteins) from chromatin by using ion-exchange resins (3). Similar to the case for B2-encoded small RNAs, the levels of putative pol III-dependent transcripts from *Alu*-like mouse B1 repeats become elevated in certain undifferentiated embryonal carcinoma and SV40-transformed cell lines (3). However, no studies with respect to chromatin packaging have been reported for B1 repeats, in part because they are transcribed in vitro from genomic DNA (gDNA), and presumably also in vivo, at about a 20-fold-lower level than are B2 repeats (4).

In considering the above results and associated models for SINE regulation, we questioned whether they should be extrapolated to explain the apparent transcriptional silencing of *Alu* elements in human cells. One reason for our circumspection was the recent finding that pol III-dependent *Alu* transcription in vitro can be completely repressed not only by reconstitution of an *Alu* template with histone octamers, but also by H3-H4 tetramer particles when the template is meth-

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ylated at CpG dinucleotide residues (12). This novel result suggested to us the possibility that histone H1 might not be required in vivo to maintain efficient transcriptional silencing of *Alu* repeats. We were further influenced by evidence that transcriptional induction of *Alu* sequences during adenovirus infection requires not only the viral E1A gene product, as had previously been suggested for rodent SINEs, but also expression of adenovirus E1B (58-kDa), E4 (ORF3), and E4 (ORF6) products (36). The latter result suggested that limited availability of general pol III transcription factors such as TFIIIB and TFIIIC was unlikely to be the sole determinant of *Alu* inactivity and that adenovirus infection might provide an excellent opportunity to gauge the role of chromatin proteins and/or sequence-specific DNA-binding proteins in the repression of *Alu* expression.

MATERIALS AND METHODS

Cell culture and virus infections. HeLa S3 and NIH 3T3 cells were grown as monolayer cultures in Dulbecco modified Eagle medium (Gibco/BRL) with 10% fetal calf serum, 2 mM glutamic acid, and antibiotics (50 U of penicillin per ml and 50 μg of streptomycin per ml). For propagation of HeLa cells in suspension, minimal essential spinner medium (Quality Biological) was substituted for Dulbecco modified Eagle medium. Adenovirus type 2 (Ad2) was kindly provided by R. Padmanabhan (University of Kansas Medical Center). The infection of HeLa cells was performed as described by Panning and Smiley (36).

Oligonucleotides. The following antisense oligonucleotides were synthesized (Applied Biosystem 380B DNA synthesizer) for use as probes for Northern (RNA) hybridizations and as primers for primer extension experiments, as well as for use with the biotin selection-RNase H technique: Alu-24, 5′-GGATG GTCTCGATCTCCTGACCTC-3′; Alu-25, 5′-TTAGTAGAGA(C/G)GGGGT TTCACCATG-3′ (36); B2, 5′-TGGTTGTGAGCCACCATGTGGTTGCTGG-3′; 5S, 5′-TAACCAGGCCCGACCCTGCTT-3′; and 7SL, 5′-ATATTGATGCC GAACTTAGTGCGGACACC-3′. Alu-24, B2, and 7SL oligonucleotides were kindly provided by R. Maraia (5, 28, 29). The biotinylated versions of the same oligonucleotides were synthesized by directly incorporating two biotin groups at the 5′ end of the oligonucleotides with Biotin-ON Phosphoramide (Clontech).

Preparation of gDNA and nuclei. gDNA was purified essentially as described previously (35). Nuclei were prepared from HeLa cells by first swelling the cells on ice in 20 volumes of hypotonic buffer A (1 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]) for 30 min (38). Triton X-100 and $MgCl_2$ were then added to final concentrations of 0.5% and 3 mM, respectively. Cells were disrupted in a Dounce homogenizer by applying 30 strokes of the B pestle. The resulting suspension was overlaid on an equal volume of buffer B (0.34 M sucrose, 10 mM Tris-HCl [pH 8.0], 3 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) and centrifuged for 5 min at 600 × *g*. The nuclear pellet was resuspended in buffer B and centrifuged again through a cushion of 1 M sucrose in buffer B for 10 min at 2,000 × *g*. Finally, the nuclei were resuspended in buffer B containing 50% glycerol and stored at −20°C. The nuclei from NIH 3T3 cells were prepared by the same procedure as described for HeLa cells, with the exception of the initial swelling step, i.e., cells were lysed directly in buffer B in the presence of 0.5% Triton X-100 and the nuclei were purified through a 1 M sucrose cushion as described above.

Preparation of nuclear transcription extract. Nuclear extract was prepared from approximately 2×10^9 suspension-propagated HeLa S3 cells by the method of Dignam et al. (10) except that extracted nuclear proteins were precipitated with 0.33 g of ammonium sulfate per ml and then dialyzed against 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9)–20 mM KCl–1 mM $MgCl_2$–2 mM DTT–17% glycerol. The nuclear extract was aliquoted (30 to 60 μl) into small tubes, quick-frozen in dry ice, and stored at −70°C. Each aliquot was thawed only once.

Preparation of chromatin. Mild micrococcal nuclease treatment was employed for preparation of chromatin (31). The reaction was stopped by the addition of EDTA to 10 mM, and digested nuclei were then dialyzed for 12 to 16 h against 8 mM Na_2HPO_3–1 mM EDTA-acid (pH 7.0)–1 mM DTT–0.4 mM PMSF (30). Insoluble material was removed by centrifugation at 1,000 × *g* for 5 min. Histone H1 was depleted from chromatin by utilizing ion-exchange resins, either Dowex 50 (modified procedure of Thoma and Koller [42]) or Bio-Rex 70 (both from Bio-Rad) (30). When the Dowex resin was used, the chromatin-containing solution was brought to a final concentration of 0.35 M NaCl–50 mM phosphate buffer (pH 7.0) and then mixed with the Dowex resin (preequilibrated in the same buffer) at a ratio of 1 g of resin per 2,500 μg of chromatin (measured as DNA). Incubation with resins was done for 1 h at 4°C on a rotating shaker. Resin beads were removed by centrifugation and washed twice with the respective buffers for incubation. The combined fractions were dialyzed against 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–1 mM DTT–0.4 mM PMSF overnight and used for in vitro transcription. That histone H1 had been removed was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of acid-soluble chromatin proteins (21). Both control and H1-depleted chromatin preparations were subjected to additional micrococcal nuclease digestion and checked on a 1.5% agarose gel to verify a regular pattern of nucleosomal repeats. No obvious sliding of nucleosomes was detected, and, as expected, histone H1-depleted mono- and dinucleosomes migrated slightly faster than control nucleosomes because of digestion of the linker DNA (unpublished data).

Transcription in vitro. The transcription reaction mix contained 1.5 to 3 μg of template (DNA, nuclei, or chromatin); 5 μl of 5× buffer (0.35 M KCl, 35 mM $MgCl_2$, 10 mM DTT, 50 mM HEPES; pH 7.9); 2 μg of α-amanitin per ml; 3 μl of nuclear extract (30 μg of protein); 500 μM (each) ATP, CTP, and UTP; 50 μM GTP; and 25 μCi of [α-^{32}P]GTP (NEN-DUPONT) in a 25-μl final volume (38). After a 30-min preincubation at room temperature with transcriptional extract in the absence of nucleotides, the cold and labeled NTPs were added and transcription was continued for 1 h at 30°C. Template DNA was digested by addition of 10 U of DNase I (RNase-free; Boehringer Mannheim) and incubation for 30 min at 37°C followed by addition of 200 μl of stop buffer (50 mM Tris-HCl [pH 7.9], 5 mM EDTA, 250 mM NaCl, 1% SDS, 50 μg of yeast RNA per ml) and proteinase K (250 μg/ml) and incubation for a further 30 min at 37°C. RNA was extracted sequentially with phenol-chloroform and chloroform and then precipitated with 2.5 volumes of ethanol. RNA was analyzed by using a 10% acrylamide gel containing 7 M urea in Tris-borate buffer. For run-on transcription, the same conditions were used except that no nuclear extract was added and larger amounts of nuclei (15 μg) and 100 μCi of [α-^{32}P]GTP were added to each reaction mixture.

Detection of *Alu* RNA. Purification of total cellular RNA and Northern blot hybridization were performed as described previously (5, 28, 29). Primer extension was done as described elsewhere (4). Mouse mammary tumor virus reverse transcriptase (RNase H-minus Superscript II; Gibco/BRL) was used at 200 U per reaction volume; extension was done for 15 min at 37°C and 1 h at 42°C. Products were fractionated on a 10% acrylamide gel containing 7 M urea in Tris-borate buffer.

Detection of in vitro-synthesized *Alu* RNA by using RNase H. In vitro-transcribed ^{32}P-labeled RNA was mixed with 8 pM specific biotinylated oligonucleotide in a total volume of 20 μl in 0.3 M NaCl–10 mM Tris-HCl (pH 8.0)–1 mM EDTA, and the reaction mixture was overlaid with a thin layer of mineral oil. After a denaturation step (10 min at 90°C), the RNA and oligonucleotides were allowed to anneal (2.5 h at 56°C) and were then diluted with buffer containing 80 μl of 10 mM Tris-HCl (pH 8.0)–20 mM NaCl–0.5% SDS–5 mM EDTA. Streptavidine-agarose (SA) (Pierce) (100 μl of a 1:4-diluted slurry in 10 mM Tris-HCl [pH 8.0]–0.5 M NaCl–0.1% SDS–5 mM EDTA–1 mg of total yeast RNA per ml) was added to each reaction volume, which was then mixed for 1 h at room temperature on a rotating shaker (33). After incubation, the SA was pelleted (1 min at 2,000 rpm in a microcentrifuge) and the supernatant was decanted and immediately precipitated with ethanol. The SA pellets were washed three times with 10 volumes (200 μl) of high-salt buffer (10 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 0.1% SDS, 5 mM EDTA, 1 mg of total yeast RNA per ml) and then three times with low-salt buffer (10 mM Tris-HCl [pH 8.0], 20 mM NaCl, 0.5% SDS, 5 mM EDTA, 1 mg of carrier RNA per ml), with rotation for 5 min and spinning for 1 min at 2,000 rpm each time. Finally, the SA was washed three times with RNase H reaction buffer (20 mM Tris-HCl [pH 7.5], 0.1 M KCl, 10 mM $MgCl_2$, 5% sucrose) and resuspended in 100 μl of the same buffer with 0.1 mM DTT. RNA-DNA hybrids attached to SA were digested with RNase H (0.3 μg/ml; generously provided by Robert Crouch, National Institutes of Health) for 45 min at 37°C, with mixing at 15-min intervals. SA was pelleted, and the supernatant, which contained the solubilized RNA fragments, was transferred to new tubes. The SA beads were then treated with denaturing solution (10 mM Tris-HCl [pH 7.8], 10 mM DTT, 1 mM EDTA, 0.5% SDS, and 50 μg of carrier RNA per ml) for 10 min at 80 to 90°C to ensure complete elution of RNA. Combined supernatants were precipitated with 0.3 M Na acetate (pH 4.0) and 3 volumes of ethanol at −20°C. For 5S RNA analysis, in vitro-transcribed RNA was treated the same way, but no RNase H was applied, i.e., intact full-length 5S RNA was isolated from SA by incubation with denaturing solution. RNA was fractionated on a 10% polyacrylamide–7 M urea gel and exposed to film or placed in a phosphorimager cassette. Quantitative analysis was done by scanning dried gels in a phosphorimager (Molecular Dynamics).

RESULTS

In vivo detection of *Alu* RNA. The first step in this study was to verify previously published results that high-titer adenovirus infection induces a 20- to 100-fold increase in the pol III-dependent expression of endogenous *Alu* repeats (36). Infection of HeLa cells was performed as described by Panning and Smiley, except that Ad2 was used in place of the Ad5 strain. Both Northern blot analysis and primer extension were employed to quantify pol III-transcribed *Alu* transcripts. Primary *Alu* RNA products derived from pol III transcription should be ≈300 to ≈500 nucleotides (nt), whereas a 120-nt product corresponding to the *Alu* left monomer is released by RNA pro-

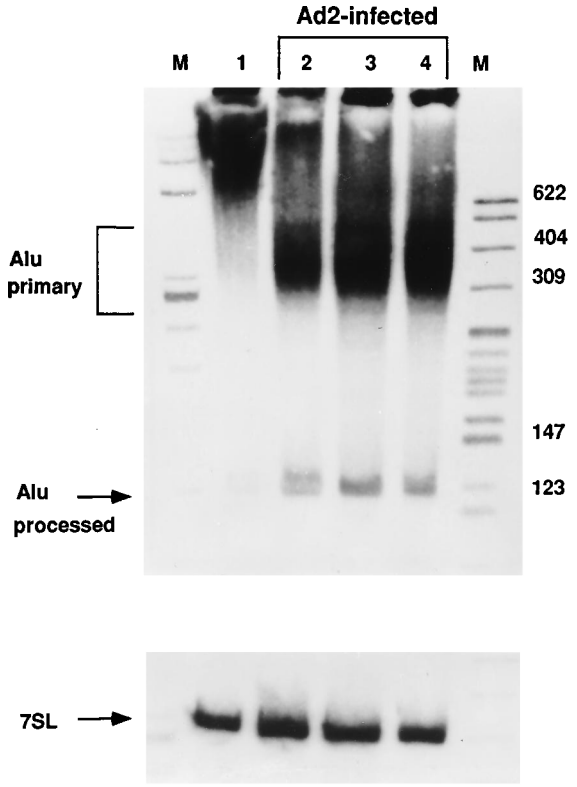

FIG. 1. Induction of *Alu* transcription by Ad2 infection: Northern blot of total cellular RNA isolated from mock-infected HeLa S3 cells (lane 1) and from cells infected with 25, 50, or 150 PFU per cell (lanes 2 to 4, respectively). The RNA blot was hybridized sequentially with several ^{32}P-end-labeled antisense oligonucleotides, including an *Alu*-specific oligonucleotide, Alu-24 (top panel), and a 7SL-specific probe (bottom panel). Markers (lanes M) were either ^{32}P-labeled ϕX174/*Hae*III (left) or pBR322/*Msp*I (right), with sizes indicated in nucleotides on the right.

cessing (28). As shown in Fig. 1, the 120-nt processed *Alu* transcription product could be detected at a very low level in uninfected HeLa cells, while primary pol III transcription products were obscured by the high background of *Alu* sequences present in pol II-transcribed heterogeneous nuclear RNA (hnRNA). Infection with Ad2 induced a dramatic increase in *Alu* RNAs of the size predicted for primary pol III-dependent transcription products. The level of 120-nt processed *Alu* transcripts also increased, albeit less markedly. It is noteworthy that the levels of 7SL RNA remained unaffected by adenovirus infection (Fig. 1, bottom); likewise, no changes in 5S rRNA or U6 small nuclear RNA levels were detected following Ad2 infection (data not shown). An additional observation from this Northern analysis is that Ad2 infection was associated with a substantial decrease in the amount of *Alu*-containing hnRNA. Similar results for inactivation of pol II-dependent transcription in adenovirus-infected cells have been reported elsewhere (17). Primer extension performed with two different oligonucleotides (Alu-24 and Alu-25; see Materials and Methods) confirmed that the putative Ad2-induced *Alu* pol III transcription products detected by Northern blotting were initiated at the normal *Alu* pol III start site (not shown).

***Alu* transcription in vitro.** In the study cited above (36), nuclear run-on experiments were used to show that the elevated *Alu* RNA levels observed following Ad5 infection could be accounted for by an activation of transcription. In considering those results, we posed several questions. First, are endogenous *Alu* elements available to the pol III transcription apparatus in uninfected HeLa cells, or are they largely masked by chromatin proteins or other sequence-specific DNA-binding proteins? Second, if endogenous *Alu* sequences are not available, is the increase in pol III-dependent *Alu* expression following adenovirus infection associated with a comparable change in *Alu* template availability? The latter question is of some interest, since most past work on stimulation of pol III transcription by adenovirus focused on factors in cell extracts, in particular increases in the activity of the transcription factor TFIIIC that can be induced by the 289-residue form of the adenovirus E1A gene product (8, 14–16, 49). Conversely, Panning and Smiley (36) have argued that the role of E1A in endogenous *Alu* activation is likely to be indirect. Insofar as the preferential expression of *Alu* elements in adenovirus-infected cells may not require augmentation in pol III transcriptional capacity, the possibility of virus-induced derepression of these elements must be examined. *Alu*-silencing mechanisms that might be affected include chromatin proteins, sequence-specific DNA-binding proteins, and/or modifications at the DNA level, e.g., methylation (19, 26).

To investigate these issues, we carried out in vitro transcription with HeLa nuclei or gDNA, using HeLa nuclear extract/*Alu* template ratios demonstrated previously to ensure pol III transcription factor excess; from such experiments, estimates regarding the relative availability of transcriptionally competent *Alu* templates to the pol III machinery in intact nuclei and naked DNA (the term "availability" is used here in a strictly functional sense, i.e., the capacity to support productive pol III transcription) could be made. Figure 2A shows the results of PAGE of total RNA synthesized in vitro from templates consisting of purified gDNA or cell nuclei. The amounts of DNA in these reaction mixtures were equal, and 2 μg of α-amanitin per ml was included to inhibit pol II-dependent transcription. The main discrete transcripts synthesized, 5S rRNA and tRNAs, were produced at similar rates from gDNA and nuclei. When gDNA was used as a template, an additional smear of higher-molecular-weight RNAs was observed. On the basis of size, this heterogeneous material was predicted to contain pol III-transcribed *Alu* RNAs.

To compare pol III *Alu* products encoded by gDNA and chromatin templates, we first applied Northern blot analysis. RNA synthesized by using unlabeled ribonucleotide triphosphates was purified, separated by gel electrophoresis, and hybridized with a ^{32}P-labeled *Alu* probe. Figure 2B shows that *Alu*-hybridizing material transcribed from gDNA formed a smear similar to that seen in Fig. 2A for total RNA. Importantly, no signal above the background (i.e., above the level observed with nuclear extract alone; compare lanes 1 and 4 in Fig. 2B) was detected when nuclei were transcribed. This result is consistent with the view that chromatin proteins render the vast majority of *Alu* sequences inaccessible to pol III transcription factors. The finding that 5S and tRNA genes were transcribed approximately equally from gDNA and nuclear templates (Fig. 2A) confirmed that nuclei used in these experiments were fully competent templates for in vitro transcription. Moreover, the possibility that *Alu* RNAs were being selectively degraded was effectively excluded by experiments in which free *Alu* plasmid DNA was mixed with nuclei prior to incubation. In such experiments, no significant inhibition of transcription or degradation of *Alu* RNA synthesized from plasmid templates was found (not shown).

One limitation inherent in Northern analysis was that it did not provide detailed information concerning the 5′ ends of *Alu*-containing transcripts. Although α-amanitin was present during transcription, such molecules could have arisen from

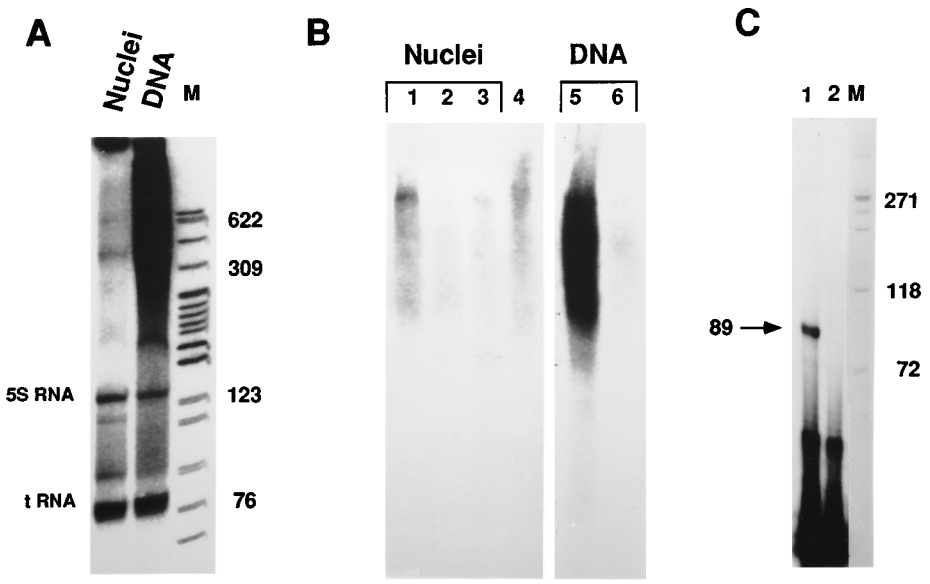

FIG. 2. *Alu* transcription with nuclei or gDNA used as templates. (A) Total RNA transcribed from nucleus or gDNA templates by using HeLa nuclear extract. The major transcripts detected, 5S RNA and tRNA, are indicated. Lane M, ^{32}P-labeled ϕX174/*Hae*III molecular size marker (sizes indicated in nucleotides on the right). (B) Northern blot analysis of *Alu* RNA. RNA was synthesized in vitro from reaction mixtures which contained nuclei plus HeLa nuclear transcription extract plus nucleotide triphosphates (NTPs) (lane 1), nuclei plus NTPs (lane 2), nuclei plus buffer (lane 3), transcription extract alone (lane 4), DNA plus transcription extract plus NTPs (lane 5), or DNA plus NTPs (lane 6). After transfer, RNA was hybridized with the antisense Alu-24 oligonucleotide probe. (C) Primer extension analysis. Transcription reactions with gDNA as a template were carried out either in the presence (lane 1) or in the absence (lane 2) of nuclear extract; products were annealed to the Alu-24 oligonucleotide and incubated with reverse transcriptase. Lane M, ϕX174/*Hae*III marker (sizes indicated in nucleotides). The position of the extended product (arrow) is indicated.

nonspecific transcription from pol I promoters or non-*Alu* pol III promoters in the gDNA template. To address this possibility, we employed primer extension analysis. Figure 2C shows that when a labeled *Alu*-specific oligonucleotide was annealed to in vitro-synthesized RNA and extended by reverse transcriptase, the only extended product obtained had the expected length of 89 nt. This supports the interpretation that transcription of *Alu* RNA from gDNA in our in vitro transcription reactions was initiated by pol III from correct *Alu* start sites.

RNase H technique for detection of *Alu* transcripts. Although Northern and primer extension experiments enabled the detection of *Alu* transcribed from gDNA, these methods had several disadvantages. Foremost among these was that in vitro-synthesized RNA, being unlabeled, could not be distinguished from that preexisting in nuclei or the HeLa nuclear extract. In addition, *Alu*-containing hnRNA obscured the accurate quantification of the low level of pol III-transcribed primary *Alu* transcripts in uninfected HeLa cells. Finally, these methods could not be used to compare *Alu* RNA with 5S or 7SL RNA, because nuclei and nuclear extracts contained large amounts of the latter RNAs. To overcome these problems, a novel technique, employing RNase H, was used. In this technique (Fig. 3), in vitro-synthesized ^{32}P-labeled RNA was annealed to a biotinylated antisense *Alu* oligodeoxynucleotide; the resulting *Alu* RNA-oligodeoxynucleotide hybrid was reacted with a streptavidin-agarose (SA) matrix, and the attached hybrid was then treated with RNase H to cleave RNA in the region of the RNA-oligodeoxynucleotide duplex. By releasing free 5′ and 3′ *Alu* RNA fragments from the agarose solid support, the following advantages were gained: (i) only newly synthesized RNA was labeled and thus detectable; (ii) 5′ RNA ends were mapped by virtue of the length of 5′ fragments released by RNase H; and (iii) there were both a high level of sensitivity and a low background due to affinity purification of the RNA of interest. Note that in this particular case an additional increase of sensitivity resulted when RNase H treatment converted heterogeneous *Alu* pol III-transcribed RNAs to homogeneous 5′ fragments.

The RNase H technique was first applied to analyze the *Alu* products of nuclear run-on transcription in the presence of α-amanitin (Fig. 4). This allowed us to more accurately determine the relative levels of pol III-dependent *Alu* transcription in uninfected and Ad2-infected HeLa cells. RNase H treatment yielded 5′ fragments of expected lengths (the enzyme cuts inside the duplex between nt 65 and 89) together with a heterogeneous mixture of 3′ fragments originating from pol III termination at various distances from *Alu* 3′ ends. Figure 4 shows that correctly initiated *Alu* transcripts were readily observed in virus-infected nuclei. Conversely, in control nuclei the rate of *Alu* transcription was measurable only after overexposure of the gel. Since 5′ ends are mapped in the RNase H run-on protocol, this result confirmed and extended earlier data showing that the induction of *Alu* RNA after viral infection is due to an increased pol III-dependent transcription (36).

To evaluate the specificity of the *Alu* induction, 5S rRNA was purified from the same run-on RNA samples by annealing to a specific biotinylated oligonucleotide and SA chromatography. The results obtained (Fig. 4) indicated that the level of transcription of 5S rRNA genes was modestly elevated following Ad5 infection. Similar experiments indicated that such approximately two- to threefold differences in expression observed for 5S RNA were only slightly above the range of variation obtained with duplicate nuclear preparations from the same cell type.

Altered *Alu* template activity. We next used the RNase H method to compare *Alu* template activities in normal nuclei, Ad2-infected nuclei, and gDNA. In this case, in vitro transcription reactions were carried out with excess nuclear extract from uninfected cells to provide a measure of template availability

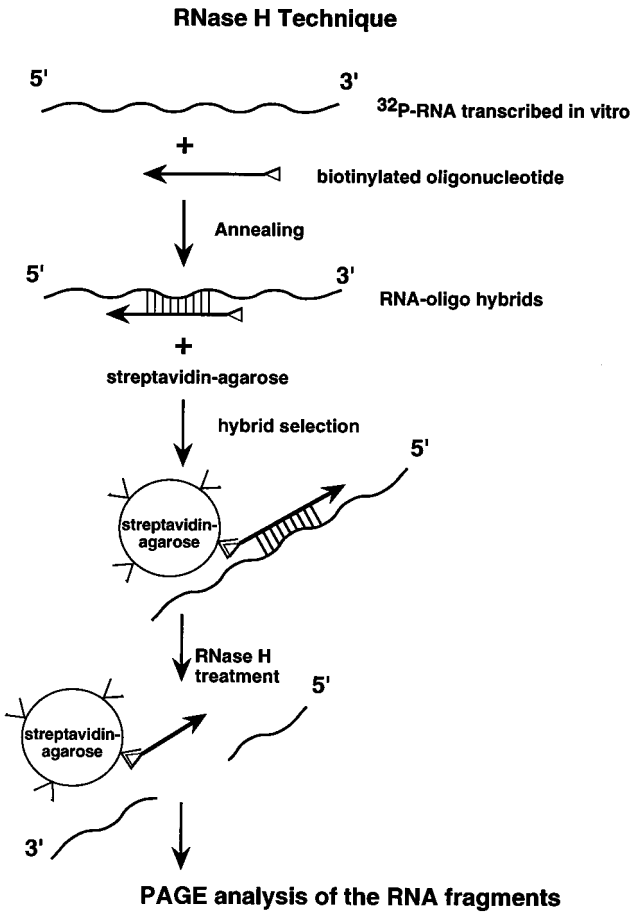

FIG. 3. Schematic representation of the RNase H procedure. In vitro-synthesized ^{32}P-labeled RNA is annealed to a biotinylated antisense oligodeoxynucleotide. The resulting RNA-DNA hybrid is incubated with an SA matrix. The attached hybrid is digested with RNase H, which cleaves the RNA only in the region of the RNA-oligodeoxynucleotide duplex. The released 5′ and 3′ RNA fragments are separated by denaturing PAGE.

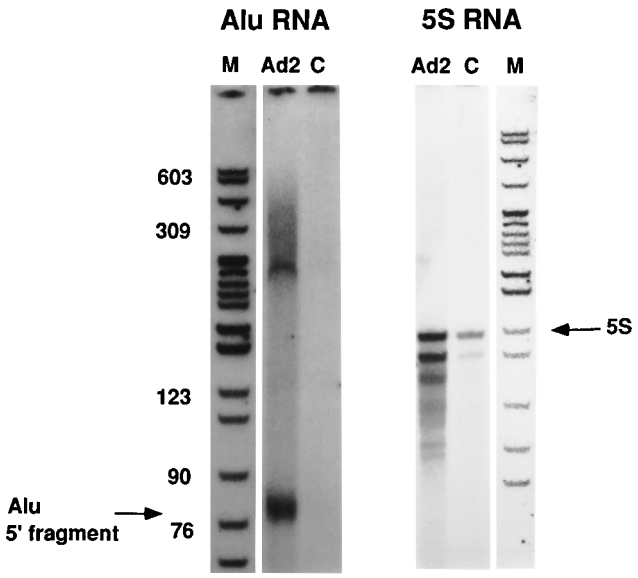

FIG. 4. Nuclear run-on transcription of *Alu* and 5S RNA genes. Nuclei from control untreated (lanes C) and Ad2-infected (lanes Ad2) HeLa cells were incubated without the addition of exogenous nuclear transcription extract. RNA products were hybridized to *Alu* (Alu RNA) or 5S (5S RNA) biotinylated antisense oligonucleotides and purified by using SA. RNase H digestion was used to release 5′ and 3′ *Alu* RNA fragments. 5S RNA was eluted with low-salt buffer without RNase H treatment. RNA fragments were separated by denaturing 10% PAGE. Lanes M, ^{32}P-labeled pBR322/*Msp*I marker (sizes in nucleotides indicated on the left).

and/or competence. Total ^{32}P-labeled RNA products from such reactions (Fig. 5A) were processed as before by being annealed to antisense *Alu* (Fig. 5B) or antisense 5S RNA (Fig. 5C) probes and released from streptavidin-biotinylated deoxynucleotide matrices by use of RNase H (*Alu*) or denaturing (in the case of 5S RNA). Remarkably, quantification of *Alu* transcription rates from uninfected nuclei and gDNA revealed a disparity of 70- to 100-fold (Fig. 5B). This result once again demonstrated that the vast majority of transcriptionally competent *Alu* sequences are masked within the nucleus. Ad2 infection increased *Alu* template activity substantially; however, transcription reached only 7 to 15% of that observed with gDNA, suggesting that adenovirus infection may affect only a subset of transcriptionally competent genomic *Alu* loci. As before, estimation of tRNA gene transcription (Fig. 5A) as well as measurement of affinity-purified 5S RNA (Fig. 5C) revealed only relatively small changes in the template activities of those genes.

DNA methylation is known to inhibit pol III-dependent *Alu* template activity in vitro (12, 19, 25, 26) and in vivo (unpublished results), although the effect is a rather modest two- to threefold. Notwithstanding the demonstration that DNA replication is unnecessary for the Ad5-mediated induction of *Alu* expression (36), unanticipated changes in *Alu* templates at the DNA level, e.g., demethylation and/or nicking, could in principle account for altered *Alu* template activity. This possibility was examined in transcription experiments comparing gDNAs from uninfected and Ad2-infected cells. DNA from the infected cells exhibited template activity equal to or less than that of control gDNA prepared in parallel from uninfected cells (data not shown). This indicated that Ad2-mediated induction of *Alu* expression is unlikely to be due to changes at the DNA level; rather, the observed increase in *Alu* template activity appears in fact to reflect an unmasking of these elements.

Histone H1 repression of B2 versus *Alu* repeats. Chromatin-mediated repression of two other repetitive pol III templates, *Xenopus* oocyte 5S RNA genes and mouse B2 SINEs, has been attributed to nonspecific masking by histone H1 (3, 38, 47). Conversely, both in vitro (12) and in vivo (11a) studies have provided evidence that nucleosome core particles may play a major role in *Alu* silencing. To investigate whether *Alu* elements conform to the generally accepted model of histone H1-dependent repression (7, 48), we extracted H1 from HeLa chromatin preparations, using ion-exchange resins. When such H1-depleted material was compared with control untreated chromatin in parallel transcription reactions, the degree of *Alu* template activation due to H1 removal averaged only 2- to 2.5-fold in several experiments, comparable to the changes observed in 5S rRNA and tRNA expression (data not shown).

Because of the reported strong activation of mouse B2 repetitive elements following a similar chromatin treatment (3), we were concerned that our experimental procedures could be yielding false-negative results. To exclude this possibility, experiments were performed with chromatin preparations from HeLa and mouse NIH 3T3 cells, either alone or mixed in a 1:1 ratio prior to the H1 extraction procedure. Analysis of total RNA products synthesized from these templates indicated that

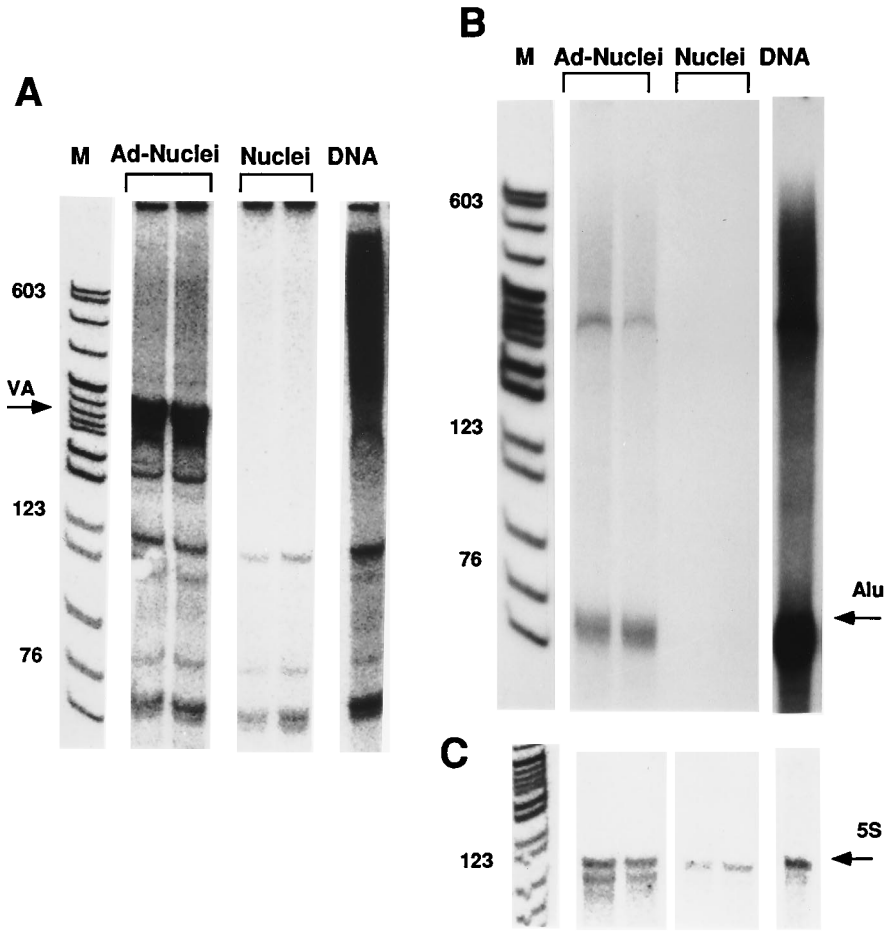

FIG. 5. Template availability of *Alu* sequences in uninfected versus Ad2-infected HeLa cells. Transcription reactions were performed in the presence of exogenous nuclear extract from uninfected HeLa cells as a source of transcription factors and pol III. (A) 10% PAGE of total ^{32}P-RNA transcribed in vitro. Templates were nuclei from uninfected HeLa cells (lanes Nuclei), Ad2-infected HeLa cells (lanes Ad-Nuclei), or genomic HeLa DNA (lane DNA). The positions of adenovirus-encoded virus-associated RNAs (VA) are indicated. (B) *Alu* RNA processed by the RNase H technique (from the total RNA preparations shown in panel A). The released 5′ *Alu* RNA fragment (arrow) is indicated. (C) 5S RNA isolated from total RNA transcripts shown in panel A by using biotinylated antisense oligonucleotide and SA. Lanes M, ^{32}P-labeled pBR322/*Msp*I markers (sizes indicated in nucleotides).

mouse, human, and mixed mouse-human chromatin preparations yielded similar overall patterns and levels of transcription (Fig. 6A); moreover, comparable results were obtained when 5S RNA transcription was measured (Fig. 6C). With these controls in place, *Alu* and B2 RNA products were measured by the RNase H technique. As seen in Fig. 6B, B2 expression in a mixed-template reaction was strongly induced in H1-depleted chromatin, whereas *Alu* transcription within the same reaction was only slightly enhanced. Similar data were obtained when unmixed HeLa and NIH 3T3 chromatin preparations were used (data not shown). Quantification of the results obtained with control and H1-depleted mixed chromatin preparations is presented in Fig. 6D.

DISCUSSION

Despite the fact that *Alu* repetitive elements constitute a major component of the human genome, a considerable amount concerning their transcriptional regulation remains to be learned. In this study, we examined the in vitro transcription level of genomic *Alu* elements relative to their overall transcriptional potential, as well as the mechanism(s) by which Ad2 infection induces endogenous *Alu* expression. We also examined whether, to the extent that chromatin proteins may mask *Alu* elements, such masking is dependent on the presence of the linker histone H1.

An additional aspect of this study worth comment is the utility of a novel RNase H-based assay to measure pol III-dependent *Alu* transcription rates and template availability. With standard approaches such as Northern blotting and primer extension analysis, background signals due to *Alu*-containing hnRNA prevented an accurate assessment of the pol III-dependent *Alu* template activity in uninfected HeLa cells. The RNase H assay revealed that the basal level of *Alu* transcription is <1% of that which would be observed if *Alu* family members were not subject to various forms of repression. We conclude that the fraction of *Alu* sequences which escape masking by chromatin proteins and/or sequence-specific DNA-binding proteins is extremely low. While this technique has not yet been applied in experiments designed to quantify pol I- and pol II-dependent transcripts, it should prove useful in those cases as well.

With respect to adenovirus induction of *Alu* transcription, evidence obtained in this study indicates that incubation of Ad2-infected nuclei with extracts from uninfected HeLa cells can reproduce the preferential expression of *Alu* elements;

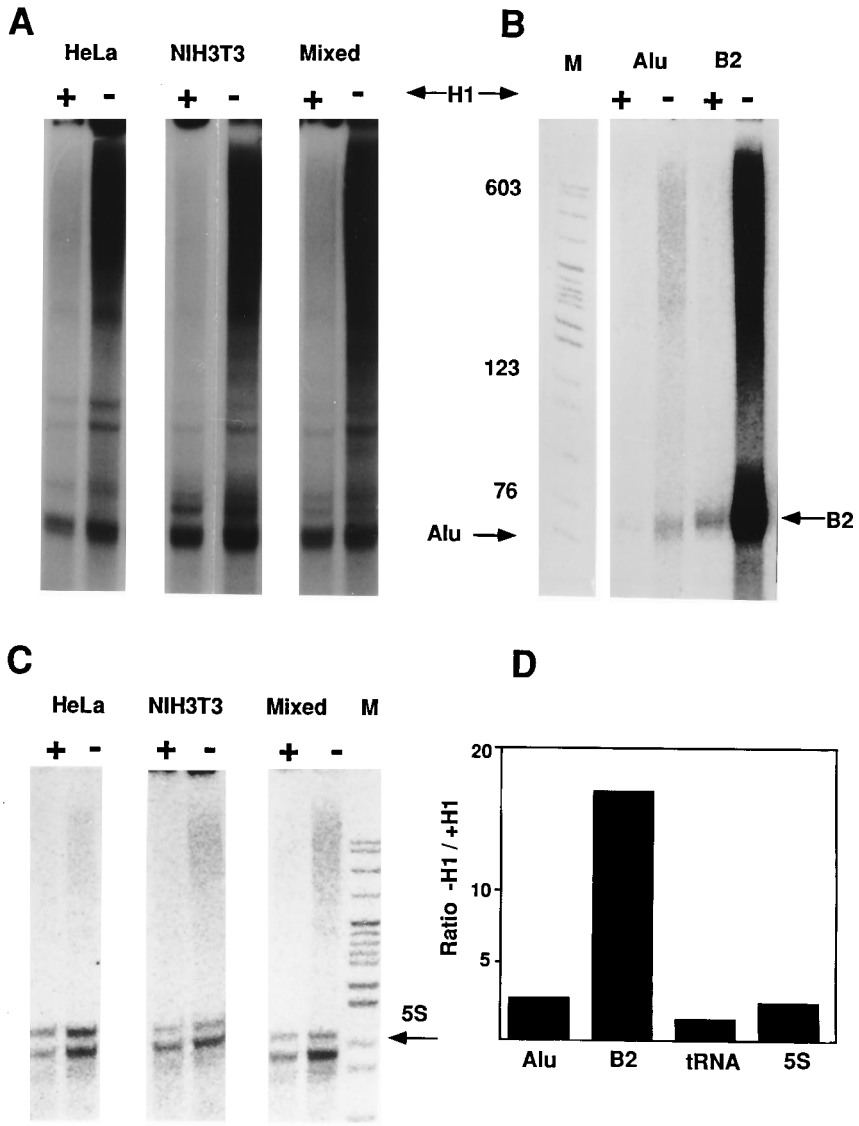

FIG. 6. *Alu* repression and the role of histone H1. (A) 10% PAGE of total RNA transcribed in vitro from H1-containing (+) and H1-depleted (−) chromatin preparations from HeLa and NIH 3T3 cells. For the mixing experiment, chromatin preparations from the two cell types were combined in a ratio of 1:1 before the H1 was depleted. (B) *Alu* RNA and B2 RNA fragments purified by the RNase H method (from mixed RNA samples shown in panel A). The *Alu* and B2 5′ fragments are indicated. Lane M, pBR322/*Msp*I marker (sizes indicated in nucleotides). (C) 5S RNA processed from the same total RNA samples shown in panel A. Lane M, pBR322/*Msp*I marker. (D) Relative RNA amounts measured by using phosphorimager scanning of gels shown in panels A through C and presented as ratios of RNA products from H1-depleted versus H1-containing templates.

indeed, there was little, if any, difference in the degree of *Alu* stimulation when extracts from either uninfected or Ad2-infected cells were used (unpublished results). These observations are of interest in view of past studies in which adenovirus induction of virus-associated (VAI) and 5S rRNA genes was ascribed to increased activity of general pol III transcription factor(s). According to those studies, extracts from adenovirus-infected cells contain a higher concentration of active form(s) of TFIIIC (15, 16, 49). Stimulation of pol III genes was found to depend on the function of the adenovirus E1A gene product (14, 16), and the 289-residue E1A gene product was shown to be capable of stimulating pol III transcription in vitro (8).

Against this background, Panning and Smiley (36) have held that E1A is unlikely to affect *Alu* transcription directly. Their view was based on three points: first, the requirement for E1A function is at least partially abrogated at sufficiently high multiplicities of infection (a condition under which expression of adenovirus early gene products becomes E1A independent); second, efficient *Alu* activation requires the E1B 58-kDa, E4 ORF3, and E4 ORF6 gene products; and third, 293 cells, which constitutively express E1A, do not exhibit high levels of endogenous *Alu* expression. The various alternative explanations for Ad5-mediated *Alu* induction offered by those authors left unresolved whether preferential transcription of *Alu* elements would be preserved upon transcription of infected nuclei in the presence of uninfected nuclear extracts. For example, if synthesis of an *Alu* transcriptional repressor was down-regulated during adenovirus infection, that factor should be supplied by uninfected nuclear extract, restoring repression. Likewise, if adenoviral E1B and/or E4 gene products operate directly or indirectly to overcome repression of endogenous *Alu* expression, templates in nuclei from infected cells could well require

factors from infected nuclear extracts to remain in an activated state. Our observations shift the emphasis away from alterations in soluble factors (general pol III factors, specific repressors, and/or activators) and instead point to changes in *Alu* templates as the major determinant of *Alu* transcriptional stimulation in adenovirus-infected cells.

To the extent that *Alu* template activity is increased during adenovirus infection, the question arises as to whether *trans*-acting mechanisms are involved (e.g., sequence-specific DNA-binding proteins or chromatin) or, alternatively, whether alterations occur at the DNA level in *Alu* templates. That DNA level modifications might play a role under these circumstances was suggested by evidence that methylation of CpG dinucleotide residues within *Alu* elements can reduce pol III-dependent *Alu* transcription both in vitro (12, 19, 26) and in vivo (16a, 25). Our failure to find a difference in template activity between gDNAs purified from uninfected and infected cells argues strongly against the interpretation that adenovirus induces demethylation, and thereby transcriptional activation, of genomic *Alu* elements. It remains possible, however, that methylation-dependent repression is compromised in the context of chromatin proteins.

A related issue concerns the relative roles of sequence-specific DNA-binding proteins and masking by chromatin in the normal repression of *Alu* expression. In this regard, whether one or both of these mechanisms may be impaired during adenovirus infection remains in question. Relatively little is known concerning the identities of sequence-specific DNA-binding proteins that associate with *Alu* elements in vivo; however, in vitro studies have revealed a factor in HeLa nuclear extracts that binds just 5′ to the *Alu* pol III internal promoter B box element to block transcription (16b). Interestingly, the activity of this potential repressor protein is reduced in extracts from Ad2-infected cells (16b), so the possibility that adenovirus infection alters *Alu* templates in such a way that this putative negative regulator fails to restore repression when uninfected nuclear extracts are incubated with nuclei from Ad2-infected cells cannot be discounted.

With respect to non-sequence-specific *Alu* repression mechanisms that appear to be relieved by adenovirus infection, it was essential to examine the potential role of histone H1. Similar experiments investigating mechanisms of *Xenopus* oocyte 5S rRNA repression in somatic tissues or the repression of mouse B2 repetitive elements in mouse fibroblasts revealed that histone H1 plays a major role in the transcriptional repression of those sequences (3, 38). In line with these findings, it might have been predicted that *Alu* template activity would be dramatically induced by ion-exchange resin-mediated removal of histone H1 and other chromatin proteins. On the other hand, differences between B2 and *Alu* elements in responsiveness to mitogenic stimulation of mouse or human fibroblasts, respectively, suggest that these two repetitive families are subject to distinct modes of regulation. Serum stimulation of mouse fibroblasts has been reported to induce a large increase in B2 expression (11). In contrast, we observed only a minor (<2-fold) elevation in the level of *Alu* expression following serum stimulation of quiescent human embryo fibroblasts (unpublished results). Likewise, elevated B1 repeat expression has been found in SV40-transformed murine fibroblasts (4), whereas we observed that *Alu* expression is induced only approximately two- to threefold by transient expression of SV40 large T and small t antigens (unpublished results).

Taken together, these observations raise the possibility that mechanisms in addition to histone H1 are operative in the repression of *Alu* transcription. If so, then preferential *Alu* transcription during adenovirus infection may be due to the ability of virus-encoded gene products to disrupt a mode of chromatin repression more specific than that mediated by histone H1. Such repression could entail nucleosome positioning (12), 5′-methyl-CpG-binding proteins (24, 32), or an as-yet-undefined aspect of higher-order chromatin structure (36). Members of the *Alu* family possess the capacity to position nucleosomes in in vitro reconstitution assays with histone octamers, and such positioning results in efficient repression of template activity (12). This in vitro evidence is bolstered by in vivo DNase I footprinting experiments which reveal in vivo nucleosome positioning on a readily detectable fraction of *Alu* repeats (11a). It is tempting to speculate that human *Alu* elements are more effectively repressed by core nucleosomes and/or other silencing mechanisms than are *Xenopus* oocyte 5S rRNA genes, mouse B2 repeats, and perhaps other classes of mammalian repetitive elements. To the extent that this is the case, *Alu* elements may provide a unique model system for studying transcriptional silencing mechanisms in higher eukaryotic cells.

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