Nucleosome Interactions with a Human Alu Element
TRANSCRIPTIONAL REPRESSION AND EFFECTS OF TEMPLATE METHYLATION

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Ella W. Englander, Alan P. Wolfe†, and Bruce H. Howard§

From the Laboratory of Molecular Growth Regulation and §Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

Alu interspersed repetitive elements possess internal RNA polymerase III promoters which are strongly transcribed in vitro, yet these elements are nearly silent in somatic cells. To examine whether repression by chromatin proteins could contribute to the low level of Alu expression, a conserved Alu element from the fourth intron of the human α-fetoprotein gene was reconstituted with purified octamer or tetramer particles. Analysis of reconstitutes revealed that this Alu element directed translational and rotational positioning of octamers as well as tetramers. In vitro transcription experiments with reconstituted templates demonstrated that RNA polymerase III-dependent transcription of the Alu element was profoundly repressed by positioned octamer particles. Furthermore, complete CpG methylation of this template enhanced the capacity of tetramers to repress transcription.

Gene silencing can be effected in vertebrate cells not only by sequence-specific DNA-binding proteins, but also by packaging of transcription control regions into nucleosomes and by DNA methylation. Nucleosomes serve a dual role in suppressing gene expression. First, they provide the basis for assembling DNA into 30-nm fiber and higher order structures that are opaque to transcription factors. Second, when appropriately positioned, they act as single particles to block transcription factor binding. This latter mechanism has been extensively documented in the case of the ribosomal 5 S genes of sea urchin and Xenopus species (1–7). As a consequence of nucleosome positioning signals, nucleosomes bound to 5 S genes strongly repress the capacity of those genes to serve as templates for RNA polymerase III (pol III)dependent transcription. Recent studies on several promoter regions in Saccharomyces cerevisiae (8–10) and on the mouse mammary tumor virus long terminal repeat (11–14) indicate that certain RNA polymerase II-transcribed promoters are also subject to regulation by nucleosome positioning.

DNA methylation serves a complementary role to nucleosome formation in gene silencing, and likewise has been demonstrated to mediate transcriptional repression in at least two ways. Binding of factors to their cognate response elements can be inhibited directly by CpG methylation (15–17). Alternatively, methylcytosine-specific binding proteins (18–20), and perhaps other as yet uncharacterized chromatin proteins (21), can sequester DNA regulatory regions which contain 5′-methyl CpG.

We have been investigating control of transcription of the human Alu family by RNA polymerase III. This family comprises about 5% of the human DNA, with at least 500,000 Alu copies interspersed throughout the genome (22). Recently, considerable progress has been made in elucidating questions relating to Alu subfamily structure and evolution (23). On the other hand, a central problem remains with respect to regulation of Alu expression: whereas these elements are readily transcribed in in vitro transcription reactions (24, 25), they are almost silent in HeLa and other somatic cells (26, 27). Here, we asked whether Alu sequences possess nucleosome-positioning signals which could contribute to their transcriptional silencing in vivo. Since the Alu consensus sequence contains a strikingly high CpG content (25 in 283 bases), and since there is evidence that most Alu sequences are heavily methylated in somatic cells (28–30), we further asked whether nucleosome positioning, if found, might be influenced by the methylation state of the Alu template.

EXPERIMENTAL PROCEDURES

Construction of DNA Templates—The Alu element (AFP5.5) was derived from the fourth intron of the human α-fetoprotein gene (31). pHAFFPS5.5, a clone carrying the 5.5-kb proximal portion of the α-fetoprotein gene, was obtained from Dr. A. Dugaiczyk. A 1.2-kb fragment (Fig. 1) centered around the Alu element was cut out with BamHI and KpnI and subcloned into the corresponding sites in the polylinker of pUC20 (Boehringer Mannheim). To create a shorter fragment carrying the Alu element, the 1.2-kb insert was cut with PstI at position +119 in the 5′-flanking region of the gene, trimmed with T4 DNA polymerase to form a blunt end, and ligated to a filled-in BamHI site in the polylinker. The region flanking the Alu element on the 3′ side was cut with SmaI at position 512 and ligated to the EcoRV site in the polylinker. In the resulting 650-bp fragment (Fig. 1), the Alu element, flanked by direct repeats is situated in the center; it has 119 bp of native upstream flanking sequence, a pol III termination signal (TTTT) approximately 120 bp downstream, and an additional 100-bp segment of native 3′-flanking sequences. Following excision with EcoRI and HindIII, the 650-bp fragment was 5′-end-labeled with polynucleotide kinase and used as a template for nucleosome assembly and for in vitro transcription. Additional templates truncated in the 3′ region were prepared by cutting with: (i) AflIII at position 317 to yield a 450-bp fragment truncated just 3′ to the Alu element (ii) HpaII at position 157 to yield a 290-bp fragment truncated within the right monomer of the gene.

Methylation of DNA Templates—Cytosines in the CpG sequences were methylated with the CpG methylase M. SssI (New England Biolabs, Beverly, MA). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Laboratory of Molecular Growth Regulation and §Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, Bethesda, Maryland 20892. Tel.: 301-496-9038.

§ The abbreviations used are: pol III, polymerase III; kb, kilobase(s); bp, base pair(s); Exo III, exonuclease III; nt, nucleotide(s).

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Biolabs). Incubation was as recommended by the manufacturer except that enzyme concentration was increased 5-fold. The extent of methylation was monitored by resistance to cleavage with HpaII and HinfI (New England Biolabs). Methylation reactions were carried out on complete resistance to cleavage was achieved, then phenol–chloroform extracted, and ethanol precipitated. Mock methylation reactions were carried out under identical conditions.

**Histone Purification and Nucleosome Reconstitution**—Core histones were purified from adult chicken erythrocytes according to the procedure described by Simon and Felsenfeld (32). In short, nuclei were disrupted, chromatin was sheared by sonication, dialyzed, and loaded onto a hydroxyapatite column. Elution of histone H$_2$A was at 0.65 M NaCl, pH 8, 10 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride. Elution of H$_4$A + H$_4$B was at 0.35 M NaCl, and histones H$_2$B-H$_4$ were eluted with 2 M NaCl. The profile of the eluted material as analyzed in a SDS-18% polyacrylamide gel has shown the expected histones (33). Quantification was by measuring absorbance at 230 nm.

Assembly of nucleosomes onto the end-labeled DNA fragments was by dialysis from high salt and urea with purified histones (34). The reconstitution reactions were in a total volume of 180 μl. DNA concentration was 55 μg/ml and was made up of 0.2–0.3 μg of end-labeled Alu template supplemented with carrier pUC DNA restricted to 0.3–0.8-kb fragments. If the reconstituted templates were to be used in an *in vitro* transcription reaction, 3.5 μg of cold Alu (650 bp) template replaced the equivalent amount of carrier DNA. DNA and histones were mixed at a ratio of 0.4, 0.5, 0.6, 0.7, or 0.9 mass of histone/mass of DNA in 2 M NaCl, 3 M urea, 10 mM Tris, pH 8, 1 mM EDTA, 1 mM β-mercaptoethanol. The reactions were dialyzed 12 h against the same buffer but with 5 mM urea. Salt gradient dialysis was carried out by successive changes of 80 min each with decreasing NaCl concentrations of 1.2, 1, 0.8, and 0.6 M, and then 4 h dialysis at 0.6 M NaCl without urea. Dialysis was continued with two further changes over the next 16 h against 10 mM Tris, pH 8, 1 mM EDTA, 1 mM β-mercaptoethanol. The extent of reconstitution was monitored by gel retardation analysis of the histone–DNA complexes in 0.7% agarose gels in 0.5 X TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) (35).

**DNase I and Exonuclease III Analyses**—To obtain DNase I digestion, approximately 25 fmol of end-labeled templates, free or reconstituted with histone octamers/tetramers were mixed on ice with DNase I diluted in 5 mM CaCl$_2$, 10 mM MgCl$_2$; assays with free DNA templates were supplemented with carrier DNA and bovine serum albumin. Following incubation for 1, 2, or 4 min, aliquots of the reaction mixtures were transferred into stop solution; the samples were then phenol–chloroform extracted, ethanol precipitated, and resolved in a 6% sequencing gel. Exonuclease III digestion of the Alu template was used to analyze the downstream nucleosomal boundary. Ten fmol of the end-labeled 650-bp Alu template, free or reconstituted, were restricted with BstNI to create a 220-bp fragment with a 3′ recessed end. The reaction on ice was then cooled on ice for 30 min and incubated at 37 °C with EcoRII in buffer supplied by the manufacturer (New England Biolabs). Incubation time (5 min) and enzyme concentration, 8 units for free DNA and 25 units for reconstituted template were determined in preliminary experiments. Incubation was terminated by extraction with phenol, and the products were resolved in a 6% polyacrylamide denaturing gel.

**In Vitro Transcription**—The 650-bp linear DNA fragment carrying the Alu element flanked by a pol III termination signal was used as a template for *in vitro* transcription in a free form or reconstituted with histone octamers/tetramers. Nuclear transcription extract was prepared according to the procedure of Dignam et al. (35), modified by an addition of 2.5 μM magnesium sulfate precipitation step. *In vitro* transcription reactions were assembled in a final volume of 25 μl with 100 ng of Alu template in 10 mM HEPES, pH 7.9, 5 mM MgCl$_2$, 42 mM KCl, 1 mM dithiothreitol, 10% glycerol. The total amount of DNA was standardized to 300 ng with λ-DNA. Mixtures were incubated for 20 min on ice, and transcription was initiated by addition of NTPs: ATP, CTP, and UTP at 0.5 mM and GTP at 0.03 mM, and 5 μCi of [α-32P]GTP. Following incubation at 30 °C for 60 min, reactions were terminated, extracted with phenol–chloroform, and analyzed in 6% polyacrylamide denaturing gel. The transcripts were quantified using the Molecular Dynamics PhosphorImager with ImageQuant software.

**RESULTS**

*In vitro* nucleosome assembly reactions have proven to be extremely valuable as a means to investigate how nucleosome positioning can regulate pol III transcription. However, the great majority of studies employing this approach have focused on ribosomal 5 S RNA genes. Ribosomal 5 S genes are classified as type I pol III transcription units, since they contain a characteristic internal control region, and their expression requires TFIIIA in addition to the general pol III factors TFIIIB and TFIIIC. In contrast, tRNA genes and pol III-transcribed Alu repeats are classified as type II genes, having internal pol III promoters which consist of separate A box and B box domains. We reasoned that, among type II genes, Alu elements would be excellent candidates for repression by nucleosome positioning, since pol III-dependent Alu transcripts are present *in vivo* only at very low levels despite the high copy number of these elements within the human genome. As a starting point to investigate this possibility, we selected an Alu element (AFP Alu) derived from the fourth intron of the human α-fetoprotein gene (31). This repeat element has the expected dimeric structure, pol III bipartite promoter, A/T-rich spacer, and >20-bp poly(A) tract characteristic of the typical Alu element (Fig. 1).

**In Vitro Nucleosome Assembly on a DNA Fragment Containing the AFP Alu Element**—Initially the AFP Alu was tested as a template for nucleosome assembly within its natural DNA context, located near the center of a 1.2-kb DNA fragment (Fig. 1). This DNA fragment was end-labeled and reconstituted by salt-urea dialysis (34) with purified core histones from adult chicken erythrocytes. Assembly products were analyzed by nucleoprotein gels to verify complete reconstitution (see "Experimental Procedures").

**FIG. 1.** Schematic representation of the Alu templates used for nucleosome assembly. The structural features of the dimeric Alu element are shown. a, open bars represent the left (~120 bp) and the right (~150 bp) monomers. Solid boxes represent pol III promoter motifs: the A box (GGCGCGGTGG) and the B box (GATCGAGC). Central A denotes the A/T-rich spacer and AA denotes the poly(A) tail, TTTT is the pol III termination signal. The 650-bp template and 290-bp template were obtained as a restriction of the 1.2-kb fragment with PstI and SnaBI. The 450- and 290-bp templates were generated by further 3'-truncations with ClaI and EcoRI, respectively. b, CpG methylation sites within the dimeric Alu element are indicated by solid circles.
To determine whether selected regions within this fragment could direct the majority of nucleosomes to bind in a preferential position and orientation, free and reconstituted templates were subjected to limited cleavage with DNase I and the products resolved in 6% sequencing gel (Fig. 2). Comparison of the two footprinting patterns (Oct and Free) reveals several regions (indicated by brackets) with obvious differences; these differences are suggestive of nucleosomal DNA. In more limited regions preferential cutting at ≈10-bp intervals is evident with the reconstituted samples, indicating rotationally positioned nucleosomes. The central portion of the fragment, corresponding to the Alu element, spans two such regions. One region centers over the start of the Alu gene, including a portion of the left monomer; the second, less clearly defined region is located directly over the right Alu monomer, i.e. situated between the A/T-rich spacer and the poly(A) tract (Fig. 2).

Two points bear consideration with respect to the localization of DNase I footprints on the AFP Alu element. First, the spacing between putative nucleosomes associated with the left and right monomer regions is greater than is commonly observed with in vitro reconstitutes. Second, the nucleosomal region which overlaps the left monomer occludes the proximal A box component of the Alu bipartite promoter, whereas the distal B box component of the promoter is located just at its downstream boundary. Earlier it was reported that both A and B box elements are required for Alu transcription in vitro (36), with the B box presumably functioning as the primary binding site for TFIIIC (37). Studies of the 5’ S genes have shown that suppression of transcription follows when binding of TFIIIA to the internal control region is prevented by appropriately positioned nucleosomes (1, 4, 38). To the extent that TFIIIA and TFIIIC are functionally analogous (i.e. serve as primary DNA binding factors for type I and II genes, respectively), the precise position of nucleosomes relative to the Alu B box may be expected to influence the degree of transcriptional repression.

We next decided to examine in more detail what DNA sequences were required for positioning of nucleosomes over the Alu left monomer. To this end, portions of the original 1.2-kb template were deleted, generating a series of truncated templates (Fig. 1). In the 650-bp template, the Alu element remains near the center, with 120 bp of upstream flanking sequence and a downstream sequence of 220 bp that includes the original pol III termination signal. This template was further truncated at the 3’ end of the gene to yield a 450-bp fragment carrying the complete Alu element but lacking the pol III termination signal. Finally, a deletion was made within the Alu gene to yield a 290-bp template in which a large portion of the Alu right monomer was missing. The resulting templates were end-labeled and assembled with histones as described previously. The corresponding DNase I analyses revealed that removal of 3’-Alu-flanking sequences did not alter the positioning of the two nucleosomes incorporated over the Alu element; moreover, deletion of most of the right monomer did not affect the positioning of the nucleosome over the remaining portion of the gene (Fig. 3). This indicates that the left monomer can position a nucleosome independently of the right monomer. Nevertheless, based on the findings of Prunell (39) it remains possible that nucleosome binding to the right monomer facilitates precise binding of a second nucleosome over the left monomer. The position of the right monomer-associated nucleosome would be predicted to be dictated by flanking adenine-rich spacer and poly(A) tracts (39). This placement creates a spacing between the two octamers of approximately 200 bp, which is optimal for maximum stability of paired nucleosomes (40).

Assembly of the Alu Element with Histone Tetramers—Nucleosome positioning in vivo during S phase is believed to occur by initial binding of (H2A/H2B), tetramer particles and subsequent assembly of complete octamers (41). Accordingly, we asked whether both histone tetratramers and octamers recognize the same nucleosomal positioning signal within Alu. The end-labeled 650-bp fragment was reconstituted by salt/urea dialysis with histone tetramers, then subjected to DNase I digestion. The resulting DNase I pattern (Fig. 4b) appears similar to that obtained with histone octamers. Likewise, analysis of the DNase I-generated fragments from the 290-bp Alu template reconstituted with histone tetramers reveals a pattern which is almost identical to that obtained following octamer reconstitution. These results suggest that histone tetramers recognize a nucleosome-positioning signal, which is similar to that recognized by histone octamers, and organize the DNA into an identical rotational position. Indeed, the similarity in positioning of octamer and tetramer complexes on the Alu templates is in agreement with results obtained for the 5 S genes (42, 43).

Reconstitution of Nucleosomes on a CpG-methylated Alu Template—A salient feature of the Alu consensus sequence is that it contains density of CpG dinucleotides about 9-fold higher than the average for the human genome. The 283-bp

**FIG. 2. Organization of nucleosomal regions on the 1.2-kb fragment assembled with histone octamers.** DNase I analysis: 5’-end-labeled 1.2-kb DNA fragment containing the Alu gene was used for analyzing the top strand. Digestions were carried out with DNase I at 0.75 μg/ml for 1, 2, and 4 min on ice. Digestion reactions for the free DNA templates were supplemented with carrier DNA and bovine serum albumin. Digestion products were resolved in 6% sequencing gel, and a photograph of an autoradiogram is shown. The end-labeled template is drawn to scale and the structural features of the dimeric Alu element are indicated; open bars represent the left and right monomers, and the solid bars are promoter motifs, the A box and the B box. Brackets denote regions of difference in DNase I footprinting pattern between the assembled (Oct) and the free (Free) DNA templates.
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Fig. 3. DNase I footprinting analyses of the Alu gene incorporated into nucleosomes assembled with 650- and 290-bp DNA fragments. The 5'-end-labeled 650- and 290-bp fragments (Fig. 1), free or reconstituted with histone octamers, were subjected to DNase I digestion. The assays were carried out as described in Fig. 2. Digestion products were resolved in a 6% sequencing gel and a photograph of a composite autoradiogram is shown. The schematic features of the Alu gene are indicated; in the case of the 290-bp template, a major portion of the right monomer has been truncated. Arrows denote the enhanced periodic cleavage sites on nucleosomal DNA detected with both templates (650 and 290 bp). The M lane is PBR/MspI digest, and the products of A+G sequencing reaction are shown for sequence alignment.

AFP Alu element has 21 CpGs (Fig. 1b), nine of which are clustered in two groups next to and over promoter elements in the left monomer. Recently, it has been demonstrated that CpG sequences in Alu repeats are often very heavily methylated in vivo (29, 30). Although we have no direct evidence that the AFP Alu locus is methylated in vivo, this element does not lie within a very CpG-rich genomic region, and thus appears unlikely to be protected from methylation in somatic cells (30). This led us to inquire whether methylation of all 21 CpGs in AFP Alu would alter its assembly into positioned nucleosomes. Several earlier studies have looked for a role of CpG methylation in modulating nucleosome formation with inconclusive results. These studies were limited, however, by the use of partly methylated bacterial or CpG-rich mammalian sequences (44, 45), or completely methylated (CpG) homopolymers (44).

To achieve complete modification of the AFP Alu element, the 650-bp Alu-containing fragment was subjected to M. Sss I methylase (46); in this fragment all CpG dinucleotides are contained within the Alu sequence. It was confirmed that methylation reactions had gone to completion by assaying the products for resistance to cleavage with the restriction enzymes HpaII and HinfI (together these enzymes recognize five sites in the 650-bp fragment; data not shown). The methylated fragment was then end-labeled and assembled with purified histone octamers as described above. Free and reconstituted templates were digested with DNase I and analyzed by comparison with DNase I digestion products from the unmodified (mock-methylated) 650-bp Alu template (Fig. 4a). Some alterations in the digestion patterns were observed between the methylated and non-methylated free templates. For example, a new hypersensitive site between positions 19 and 20 (horizontal arrow) probably results from enhanced cleavage of the bond 5' to the methylC at position 20. Similar effects on the pattern of DNase I digestion have been attributed to alterations in the orientation of the phosphodiester bond in methylated DNA (47). To assess whether methylation also affected DNase I digestion in a nucleosome-dependent manner, positions at which DNase I susceptibility was altered secondary to reconstitution were examined at the corresponding positions in non-methylated templates. Comparison of footprinting patterns in this way revealed subtle but reproducible differences consistent with additional histone-DNA interactions in the region adjacent to the B box in the methylated template (marked by vertical bars). An alternative explanation for the observed differences could be that methylation-induced alterations in DNA conformation occurred which, while not directly modifying histone-DNA interactions, nonetheless enhanced steric hindrance to DNase I selectively in reconstitutes. It should be noted that the region 5' to the B box spans a cluster of four CpG methylation sites (solid circles) that could potentially influence the structure of DNA near the edge of the nucleosome.

We asked further if the observed differences between non-methylated and methylated templates would be evident following reconstitution with histone tetramers. Comparison of DNase I footprinting data for methylated and non-methylated 290-bp fragments (Fig. 4b) shows the presence of distinct patterns in the region of the B box (marked by vertical bars) that can be observed extending through the B box. In addition, differences in footprinting patterns were detected in the region corresponding to a cluster of four methylation sites within and next to the A box. The methylation-modified footprinting pattern in this region (vertical bar) shows additional sites of increased sensitivity next to the previously observed, evenly spaced DNase I preferential cleavage sites.

To extend these observations, we asked whether the affinity of the methylated template for association with a histone octamer was changed. For this purpose, we carried out competitive reconstitution experiments with methylated and non-methylated 290 bp Alu templates (40). To date these experiments have not revealed significant differences between control and modified fragments (data not shown).

Boundaries of the Left Alu Monomer Nucleosome—Next, to the previously observed information on the translational position of the nucleosome centered over the 5'-end of the left monomer, we employed restriction enzyme mapping and exonuclease III (Exo III) digestion. In preliminary experiments, the 650-bp Alu template was assembled with histones at decreasing ratios ranging from 1:0.9 to 1:0.4 DNA mass/histone mass. From these experiments, it was evident that the ratio of 1:0.5 was the lowest that supported complete assembly of the labeled template into a nucleosomal complex (data not shown). Using a lower DNA mass/histone mass ratio in these experiments minimized potential interference by adventitiously bound histones without substantially altering DNase I digestion patterns (data not shown). Accordingly, the end-labeled 650-bp
Fig. 4. Comparison of DNase I footprinting patterns of methylated and non-methylated Alu templates associated with histone octamers or tetramers. a, the 650-bp fragment was methylated at the CpG sites (Fig. 1b) with M. Sss I methylase. The methylated and non-methylated templates were assembled with histone octamers and subjected to DNase I digestion. The assays were conducted as described ("Experimental Procedures") and products analyzed in a 6% sequencing gel. A schematic structure of the Alu gene is shown; left and right monomers are depicted by open bars and promoter motifs by solid bars. Solid circles denote the positions of "CpG dinucleotides, and vertical bars mark the major regions of difference in the footprinting pattern, around the B box, between the assembled methylated and non-methylated templates. The horizontal arrow denotes a modification of the footprinting pattern for the free template following methylation. b, end-labeled 650- and 290-bp methylated or non-methylated Alu templates were assembled with histone tetramers and subjected to DNase I digestion. The products were resolved in a sequencing gel and visualized by autoradiography. Schematic structure of the Alu gene is shown next to the cleavage pattern for the 650- bp template assembled with tetramers. The positions of the "CpG dinucleotides are indicated by solid circles next to the cleaved methylated free 290-bp fragment. The differences in DNase I cleavage pattern between the methylated and non-methylated 290-bp assembled templates in the region of the B and the A boxes are denoted by vertical bars.

Alu templates, either methylated or non-methylated, were reconstituted with histone octamers or tetramers at the limiting ratio of 1:0.5. As expected the extent of reconstitution was found to be complete as assayed by gel retardation (Fig. 5a). The reconstituted templates were then subjected to restriction enzyme digestion (Fig. 5b). The BstNI site at position 87, approximately 5 bp downstream from the B box, was cut in all cases indicating that this site was outside of the protected nucleosomal core region. Cutting with BstYI at position −75 was largely blocked, supporting our initial assessment, based on DNase I and primer extension analyses, that places the upstream boundary of the nucleosome around position −80 (data not shown). Restriction with BstXI at position 37, a site which maps within the nucleosomal DNA, was mostly blocked for template assembled with histone octamers, whereas when the non-methylated template was assembled with tetramers, this site was susceptible to limited cleavage. Interestingly, the BstXI site was completely resistant to cleavage when a methylated template was assembled with tetramers. These results suggest that CpG methylation of the Alu template renders the template more efficient in directing the tetramer into a precise position that blocks BstXI access.
The end-labeled 650-bp Alu template non-methylated or methylated was assembled with histone octamers or tetramers at 1:0.5. a, the extent of assembly of the 650-bp template was confirmed by gel retardation assay in 0.7% agarose gel: oct is template assembled with histone octamers and tet with histone tetramers; the methylated templates are indicated. b, free and assembled (methylated and non-methylated) templates were subjected to restriction enzymes: BstNI restriction at position 87 yields a 220-bp fragment; MspI restriction at position 37 results in a 170-bp fragment. Restriction reactions for free templates were supplemented with carrier DNA and bovine serum albumin, and products were resolved in 2.0% agarose gels and visualized by autoradiography. Marker lane is an end labeled pBR322/MspI digest. c, exonuclease III digestion analysis of the downstream boundary for nucleosome associated with the left monomer of the Alu gene. Free (lanes 1 and 5) and assembled templates were restricted with BstNI at position 87 (horizontal arrow) to yield 220-bp long fragments. The restricted free or assembled templates were then incubated with Exo III for 5 min at 37 °C and products resolved in a 6% sequencing gel. A photograph of an autoradiogram is shown and a schematic representation of the 220 fragment is drawn in parallel with the resolved Exo III digest. The corresponding portion of the Alu gene (position 1–87) is denoted by an open bar, with promoter motifs (A and B) marked. The vertical arrow indicates the start and direction of pol III-dependent transcription for the Alu element. Lanes 2 and 6 show the products of Exo III digestion for the free template, non-methylated and methylated, respectively. Lane 3 is the Exo III digest of template assembled with histone octamers and lane 4 with tetramers. Lane 7 is digest of methylated template assembled with histone tetramers. The first Exo III stop site is observed at position 69 (horizontal arrow), and several additional internal stop sites at 10 bp intervals are also detected. By comparison, for the methylated template the corresponding first stop at position 69 is more pronounced. The marker (lanes M) is the end-labeled pBR322/MspI digest. d, schematic diagram of the putative translational position of the nucleosome associated with the left Alu monomer. Positions of restriction sites are indicated relative to the transcription start site.

This interpretation is corroborated by data obtained with Exo III. Reconstituted 650-bp templates (same as above) were digested with BstNI to create an entry site for Exo III in the vicinity of the nucleosome; this generated 220-bp fragments with 3'-recessed ends. The resulting fragments were subjected to Exo III digestion and the products analyzed on a 6% polyacrylamide denaturing gel. With both non-methylated and methylated reconstitutes, the first strong Exo III stop detected was at position 69 (indicated by a horizontal arrow), 5' to the B box (Fig. 5c lanes 3 and 4). Notably, with a methylated template this stop site was augmented (lane 7). In several further experiments, pairs of methylated and non-methylated templates were assembled into octamers by competitive reconstitution (40). In these experiments, the Exo III stop at position 69 was reproducibly more pronounced on the methylated template than on the control template (data not shown).

In Vitro Transcription from an AFP Alu Template—Collectively, our results have demonstrated that the Alu gene positions histone contacts in a stable and precise manner over and next to promoter elements that are critical for its transcriptional activity. This led us to investigate whether the AFP Alu gene, when incorporated into positioned nucleosomes, is transcriptionally repressed. The AFP Alu element within its natural DNA context was found to be an efficient template for in vitro transcription when contained within a supercoiled plasmid (not shown) or as a linear fragment (Fig. 6, lane 1); in both cases the natural pol III termination signal was utilized, and the expected 420-nt transcript was the major transcription product. The 650-bp linear template (Fig. 1), which retains the natural pol III termination signal, was used for in vitro transcription experiments. This fragment was end-labeled, assembled with purified histones, and the extent of reconstitution determined as before; transcription assays were then carried out and the RNA products analyzed in denaturing gels (Fig. 6). First, it was shown that the free linear Alu template is readily transcribed (lanes 1, 2, and 8). As a control to verify the integrity of end-labeled reconstituted templates, these complexes were incubated under transcription reaction conditions, but with omission of [α-32P]GTP (lanes 3, 9, 12, and 16). As a further control, mixtures of free and reconstituted templates were coincubated; this ensured that reconstituted templates did not nonspecifically inhibit the capacity of pol III to transcribe either a free linear template (lanes 5 and 11) or a free supercoiled template (lane 6). With these controls in place, it could be shown that
assembly of the Alu fragment with histone octamers at DNA mass/histone mass ratios of either 1:0.9 or 1:0.5 completely and specifically repressed template activity (lanes 4 and 10).

This result prompted us to ask whether equally efficient transcriptional repression could be achieved by reconstitution of the Alu element with histone tetramers. The 650-bp fragment was assembled with histone octamers or tetramers at the 1:0.5 ratio; complete reconstitution was verified (Fig. 5a), and the template was tested for transcriptional activity. With histone tetramers, despite complete incorporation of the template into subnucleosomal particles, there was escape from repression, with the expected 420-nt transcript detected at about 40% of the level of the free template (compare lane 13 with lane 8 and data not shown). To determine the effect of methylation under these conditions, the 650-bp fragment was treated with M.SsaI methylase. It was found that transcriptional activity of the free methylated template is reduced by 50–60% relative to the unmodified template (lane 15 and data not shown). Since an additional reduction of 60% is expected following assembly with histone tetramers, the Alu transcript from a reconstituted template should be detected at 15–20% of its initial level. The results revealed however, that unlike the non-methylated template, transcription from the methylated CpG-modified tetramer-reconstituted Alu template was completely repressed, i.e. less than 2% of the initial signal (lane 17). We conclude that: (i) octameric and tetrameric particles repress transcription from the Alu template; and (ii) a tetrameric subnucleosomal particle positioned on a methylated Alu element is more efficient in blocking transcription than a tetrameric particle positioned on a non-methylated template.

**DISCUSSION**

**Nucleosome Positioning on the Alu Template**—Alu elements, being remarkably silent in vivo despite their vast copy numbers in the human genome, are inherently interesting templates for the study of nucleosome assembly and transcriptional repression. We have found that in vitro a nucleosome centers over the start site of the human α-fetoprotein Alu element, and that this nucleosome retains its position regardless of successive truncations which extend into the right Alu monomer. It has also been found that histone octamers and tetramers organize the Alu template into an identical rotational phase, in agreement with previous results obtained with the 5 S Xenopus and sea urchin rRNA genes (42, 43). The ability to position a nucleosome is not unique to the Alu AFP element, since a very similar DNase I protection pattern was obtained using a synthetic major Alu which is flanked on its 5’-end by prokaryotic sequences. In addition, less detailed evidence from another study is consistent with non-random interactions of Alu elements with nucleosomes (48).

An obvious question is whether Alu sequences position nucleosomes in vivo. While our experimental results do not answer this question, it can be partially addressed by examining whether Alu elements possess a sequence structure which predicts the observed pattern of nucleosome binding. Extensive studies have in fact been done to define sequence patterns which influence rotational orientation and translational position of DNA on nucleosomes (40, 45, 49–53). The rules which have been discerned can be applied to a given sequence using one of several computer algorithms (49, 52). When the Alu consensus sequence was examined in this way, several features emerged: (i) a strong rotational positioning signal occurs between the A and B box pol III promoter motifs in the most highly conserved region in Alu repeats (54); that this rotational positioning signal resides entirely within the repeat element sequence is confirmed by the finding that AFP.

**FIG. 6. Analysis of in vitro transcription from the AFP Alu gene.** The 650-bp Alu template, non-methylated or methylated, was assembled with histone octamers or tetramers and tested for in vitro transcription. Transcription mixtures were resolved in 8% (a) or 6% (b) sequencing gels, visualized by autoradiography, and transcripts quantified using the Molecular Dynamics phosphorimager with ImageQuant software. Transcription assays were assembled in a final volume of 25 μl with HeLa nuclear extract and 100 ng template as follows. a, the template was assembled with histone octamers or tetramers at the ratio of 1:0.9 DNA mass/histone mass. Lane 1, a transcript resulting from free template is visualized at 420 nt (RNA). Lane 2, a transcript from a mixture of free cold and radioactive templates; the radioactive template is visualized at 650 bp (DNA). Lane 3, a control to confirm the integrity of the assembled template where [α-32P]GTP was omitted from the assay. Lane 4, transcript is not detected when the 650-bp Alu template (DNA) is assembled with histone octamers. Lanes 5 and 6, free Alu templates (Alu AFP and AluV, respectively, are included in the assay) in addition to the assembled labeled Alu AFP template. Lane 7, transcription from the free AluV template (included in the assay shown in lane 6); the expected transcript is visualized at 300 nt (RNA). b, analysis of transcription from templates assembled at a 1:0.5 ratio with: histone octamers (lanes 9–11), histone tetramers (lanes 12–14), and from methylated template (the specific activity for the end-labeled methylated template is 2-fold greater than the non-methylated) assembled with histone tetramers (lanes 16–18). In lanes 8 and 15, transcription is from a free cold template, non-methylated and methylated (the transcript is reduced to ~30%), respectively. Lanes 9, 12, and 16 are control assays in which the [α-32P]GTP was omitted. In lanes 10, 13, and 17, the assays were with templates assembled with octamers, tetramers, and methylated template with tetramers, respectively.
Alu and a synthetic Alu (55) exhibit the same expected rotational and translational positioning, although the latter element is flanked at its 5'-end by a prokaryotic sequence; (ii) the A-rich regions between the left and right monomers and downstream from the right monomer are predicted to form barriers to the movement of a nucleosome, if one is bound to the right monomer (the spacing of these A-rich regions, ~145 bp, closely matches the 146 bp bound in a nucleosome core); and (iii) the calculated energy cost for binding nucleosomes to both left and right monomers is low. As pointed out earlier, short arrays of nucleosomes (two or three particles) position more stably than single nucleosomes. The dimeric structure of Alu repeats would appear to allow a pair of nucleosomes to be positioned with a ~200 bp spacing, which is optimal for stable binding (40).

**Methylation of the Alu Gene Modulates in Vivo Transcription**—Methylation has been strongly correlated with repression of pol II-transcribed genes in eukaryotes (19, 56, 67), whereas pol I-transcribed genes encoding *Xenopus* 40S rRNA have not been found to be affected (58). To date, only a few pol III transcribed genes have been examined with respect to the effects of methylation on gene expression. These results reveal that transcription from the methylated type IIS rRNA template is not affected upon injection into *Xenopus* oocytes, whereas transcription of the methylated type II tRNA gene is partially repressed under identical experimental conditions (59). Similarly, another pol III transcribed type II RNA template, the adenoviral VAI gene, is transcriptionally repressed by methylation upon transfection into mammalian cells (60). We and others (30, 61) have found that pol III-mediated in vitro transcription of an Alu gene can be reduced by CpG methylation, although in our hands the magnitude of this effect, 2.5-fold, is relatively small.3

In part to enhance the efficacy of this DNA methylation effect, we decided to investigate the interplay of DNA methylation and nucleosome formation on the human α-fetoprotein Alu template. Our results reveal that an unmethylated Alu template is transcriptionally inactive in vitro when assembled with histone tetramers. The results also indicate that the methylated type IIS rRNA gene is subject to competition by two alternative pathways: (a) the effects of methylation on gene expression. These results suggest that selective pressure may exist for a conserved Alu subfamily member possesses nucleosome positioning information. Earlier reports of low pol III-dependent Alu transcription were interpreted as sup-}

3 R. Vorse, G. Humphrey, E. Englander, and B. Howard, unpublished results.

**REFERENCES**

1. Tremethick, D., Zucker, K., and Worel, A. (1990) *J. Biol. Chem.* 265, 5014-5023.
2. Wolfe, A. P., and Brown, D. D. (1987) *Cell* 51, 733-740.
3. Hayes, J. J. and Wolfe, A. P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 1229-1233.
4. Clark, D. J., and Wolfe, A. P. (1991) *EMBO J.* 10, 3419-3428.
5. Chipev, C. C., and Wolfe, A. P. (1992) *Mol. Cell. Biol.* 12, 45-55.
6. Wolfe, A. P., and Brown, D. D. (1988) *Science* 241, 1626-1632.
7. Felsenfeld, P. C., and Simpson, R. T. (1986) *J. Biol. Chem.* 260, 15318-15324.
8. Durrin, L. K., Mann, R. K., and Grunstein, M. (1992) *Mol. Cell. Biol.* 12, 1621-1629.
9. Simpson, R. T. (1991) *Prog. Nucleic Acid Res. Mol. Biol.* 40, 143-184.
10. Roth, S. Y., Shirwin, M., Johnson, L., Grunstein, M., and Simpson, R. T. (1992) *Genes & Dev.* 6, 411-425.
11. Bressnick, E. H., Rories, C., and Hager, G. L. (1992) *Nucleic Acids Res.* 20, 865-870.
12. Archer, T. K., Lefebvre, P., Wolford, R. G., and Hager, G. L. (1992) *Science* 255, 1573-1576.
13. Piña, B., Brüggemann, U., and Beato, M. (1990) *Cell* 60, 719-731.
14. Piña, B., Barettoni, D., Truss, M., and Beato, M. (1990) *J. Mol. Biol.* 216, 975-990.
15. Igeguchi, S. M. M., and Schaffner, W. (1989) *Genes & Dev.* 3, 612-619.
16. Koesch, M., Reichel, R., and Nivins, J. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 2110-2114.
17. Watt, F., and Moffly, P. C. L. (1988) *Genes & Dev.* 2, 1136-1143.
18. Boys, J., and Bird, A. (1991) *Cell* 64, 1132-1134.
19. Bird, A. (1992) *Cell* 70, 5-8.
20. Boys, J., and Bird, A. (1992) *EMBO J.* 11, 327-333.
21. Buschhausen, G., Witig, B., Gresmann, M., and Grassmann, A. (1987) *Nucl. Acids Res.* 15, 1107-1112.
22. Hwu, H. R., Roberta, J. W., Davidson, E. H., and Britten, R. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 3875-3879.
23. Schmid, C., and Marais, R. (1982) *Curr. Opin. Genet. Dev.* 2, 874-882.
24. Duncan, C., Biro, P. A., Choudary, P. V., Rider, J. T., Weng, R. R. C., Forget, B. G., De Hiei, J. K., and Weissman, S. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 5095-5099.
25. Perez-Stable, C., Ayres, T. M., and Shen, C. K. J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 3021-3025.
26. Paulson, K. E., and Schmid, C. W. (1988) *Nucleic Acids Res.* 16, 6145-6158.
27. Macphail, A. J., Hellmann, U., and Schmid, C. W. (1990) *Mol. Cell. Biol.* 10, 5424-5432.
28. Gama-Sosa, M. A., Wang, R. H. Y., Ko, K. C., Gehrke, C. W., and Ehrlich, M. (1983) *Nucleic Acids Res.* 11, 3087-3095.
29. Schmid, C. W. (1991) *Nucleic Acids Res.* 19, 5613-5617.
30. Kochanek, S., Herz, D., and Doi, F. (1993) *EMBO J.* 12, 1141-1151.
31. Gibbs, P. E. M., Zilinski, R., Boyd, C., and Dugaiczyk, A. (1987) *Biochemistry* 26, 1332-1343.
32. Suter, R. H. and Felsenfeld, G. (1976) *Nucleic Acids Res.* 6, 689-696.
33. Lee, D. Y., Hayes, J. J., Pruss, D., and Wolfe, A. P. (1993) *Cell* 72, 1-20.
34. Cabezón-Otero, R. D., Solner-Webb, B., and Felsenfeld, G. (1976) *Cell* 8, 353-367.
35. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* 11, 1475-1499.
36. Perez-Stable, C., and Shen, C. K. J. (1986) *Mol. Cell. Biol.* 6, 2041-2052.
37. Ger dushez, E. E., and Tocchini-Valentini, E. M., Zielinski, R., Boyd, C., and Dugaiczyk, A. (1987) *Methods Enzymol.* 26, 332-338.
38. Morse, H. (1989) *EMBO J.* 8, 2343-2351.
39. Pryor, W. D. (1982) *EMBO J.* 1, 173-179.
40. Shraeder, T. E., and Broders, D. M. (1990) *J. Mol. Biol.* 216, 69-84.
41. Wolfe, A. P. (1991) *J. Cell Sci.* 99, 201-206.
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42. Dong, F., and van Holde, K. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10596-10600
43. Hayes, J. J., Clark, D. J., and Wolfe, A. P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6829-6833
44. Felsenfeld, G., Nickol, J., Behe, M., McGhee, J., and Jackson, D. (1985) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 577-584
45. Drew, H. R., and McCall, M. J. (1987) *J. Mol. Biol.* **197**, 485-511
46. Renbaum, P., Abrahamove, D., Fainsod, A., Wilson, G. G., Rottem, S., and Razin, A. (1986) *Biochem. J.* **234**, 213-216
47. Fox, K. R. (1986) *J. Mol. Biol.* **197**, 485-511
48. Meersseman, G., Pennings, S., and Bradbury, E. M. (1992) *EMBO J.* **11**, 2951-2959
49. Piria, B., Truss, M., Ohlenbusch, H., Postma, J., and Beato, M. (1990) *Nucleic Acids Res.* **18**, 10596-10600
50. Shrader, T. E., and Crothers, D. M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 7418-7422
51. Calladine, C. R., and Drew, H. R. (1984) *J. Mol. Biol.* **178**, 773-782
52. Calladine, C. R., and Drew, H. R. (1986) *J. Mol. Biol.* **192**, 867-891
53. Satchwell, S. C., Drew, H. R., and Travers, A. A. (1986) *J. Mol. Biol.* **191**, 654-675
54. Kariya, Y., Kato, K., Hayashizaki, Y., Himeno, S., Tarui, S., and Matsubara, K. (1987) *Gene* (Amst.) **53**, 1-10
55. Sakamoto, K., Fordis, C. M., Corasco, C. D., Howard, T. H., and Howard, B. H. (1991) *J. Biol. Chem.* **266**, 3031-3038
56. Razin, A., and Cedar, H. (1991) *Microbiol. Rev.* **55**, 451-458
57. Doerfler, W., Toth, M., Kochanek, S., Achten, S., Freisem-Rahien, U., Belz-Krappa, A., and Orend, G. (1990) *FEBS Lett.* **268**, 329-333
58. MacLeod, D., and Bird, A. (1983) *Nature* **306**, 200-203
59. Besser, D., Götz, P., Schulz-Forster, K., Wagner, H., Kröger, H., and Simon, D. (1989) *FEBS Lett.* **269**, 358-363
60. Juttermann, R., Hosokawa, K., Kochanek, S., and Doerfler, W. (1991) *J. Virol.* **65**, 1735-1742
61. Liu, W. M., and Schmid, C. W. (1992) *Nucleic Acids Res.* **21**, 1351-1359
62. Aimouzzi, U., Mechali, M., and Wolfe, A. P. (1991) *Mol. Cell. Biol.* **11**, 555-565
63. Hansen, J. C., and Ausio, J. (1992) *Trends Biochem. Sci.* **17**, 187-191
64. van Holde, K. E., Lohr, D. E., and Robert, C. (1992) *J. Biol. Chem.* **267**, 2837-2840
65. Carey, M. P., and Singh, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7059-7063
66. Ullu, E., and Weiner, A. M. (1984) *EMBO J.* **3**, 3303-3310