DNA Sequences and Transcription Factor Interactions of Active and Inactive Forms of Mammalian 5 S RNA Genes*

(Received for publication, September 28, 1983)

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Mouse and human 5 S rRNA genes which are, respectively, active and inactive in a human cell-derived transcription system have been characterized. Both contain several base pair substitutions, relative to the gene encoding the prominent 5 S rRNA, indicating that they are variant genes. Both the mouse gene and a Xenopus 5 S rRNA gene are transcribed in systems reconstituted either with the Xenopus 5 S gene-specific factor (IIIA) and other factors from human cells or with the human IIIA equivalent and other factors from amphibian cells. This suggests that structural aspects of the factors relevant to their interactions with each other and with DNA have been conserved and that transcription of mammalian genes is mechanistically similar to that of amphibian genes which employ an internal control region (the site of interaction of IIIA). The latter is also indicated by the demonstrated interaction of the purified Xenopus IIIA factor with both the active mouse and the inactive human gene at intragenic sites corresponding to the internal control site of the amphibian gene; this is observed despite the presence of sequence variations in this region in both of the mammalian genes. Thus, the complete inactivity of the human gene and the lowered activity of the murine gene (relative to the amphibian gene) is not necessarily due to a failure to bind the 5 S gene-specific factor. It is suggested that some control region sequences may be directly or indirectly involved in other factor interactions and the possible roles of specific bases within this region in productive transcription complex formation is discussed. The sequence analyses also suggest alternate termination sequences for class III genes.

The reiterated families of 5 S ribosomal genes in higher eukaryotes are composed of transcriptional units diverse in sequence and, possibly, function. This has been demonstrated in Xenopus, where oocytic and somatic 5 S genes, differing slightly in sequence, are developmentally regulated (reviewed in Ref. 1) and in chicken, where variants of 5 S rRNA, located in the nucleus, appear to be expressed in a tissue-specific manner (2). We have recently characterized two mammalian 5 S genes which differ in their ability to serve as competent templates in an in vitro transcription system (3). One gene, derived from mouse, is transcriptionally active in the soluble system although its product has a sequence which is slightly altered from that of the major 5 S ribosomal RNA species. In contrast, a second gene, derived from humans, is transcriptionally inactive for a 5 S-sized RNA. Both 5 S genes are adjacent to middle repetitive, interspersed DNA sequences which are actively transcribed in vitro.

In the present report, we have determined the DNA sequence of both the transcriptionally active and inactive mammalian 5 S genes as a first step in identifying those nucleotides critical for expression. It has previously been shown for the Xenopus 5 S genes that the only sequences necessary for initiation of transcription in vitro lie within the coding regions between positions +50 and +83 (4, 5). Furthermore, a purified protein factor, isolated from Xenopus ovas and required for the in vitro transcription of Xenopus 5 S genes, can interact with 5 S DNA between residues +45 and +97 (6). We demonstrate that, despite some sequence variations between the transcriptionally active and inactive mammalian 5 S genes, both are capable of selectively interacting with the Xenopus transcription factor within positions corresponding to the intragenic control region found in the Xenopus 5 S genes. We discuss our findings in terms of possible base pair changes causing template inactivity and how they may affect factor-mediated transcription.

**EXPERIMENTAL PROCEDURES**

**DNA Sequencing**—Gel-purified DNA fragments were labeled at their 5' ends by dephosphorylation with calf intestine phosphatase (Boehringer Mannheim) and phosphorylation with [γ-32P]ATP (New England Nuclear) and polynucleotide kinase (British Research Laboratories). Labeling at 3' ends was accomplished by "filling-in" recessed restriction cleaved ends with Klenow DNA polymerase in the presence of [α-32P]dATP or dCTP (New England Nuclear) and each of the remaining unboxed deoxynucleosidetriphosphates. Labelled fragments were then cleaved with a secondary restriction enzyme, gel-purified, and extracted by electrodution in 0.5 × TBE (1 × TBE = 0.1 M Tris/borate, 2 mM EDTA, pH 8.3).

Sequencing was performed by the method of Maxam and Gilbert (7). Adenine > guanine reactions were performed in 0.1 M piperidine formate, pH 2.0, at 37°C for 10 min. Partial cleavage products were electroforeased on 6, 8, and 20% thin polyacrylamide, 7 M urea gels (8). Gels were autoradiographed on XR-5 Kodak film at −70°C with and without DuPont Cronex Lightning-Plus intensifying screens.

**In Vitro Transcription**—Preparation of cell-free soluble extracts from KB tissue culture cells was as described (9). Incubation conditions for in vitro RNA synthesis were the following: 10 mM Hepes,
pH 7.9, 70 mM KCl, 3 mM MgCl₂, 10% glycerol, 0.5 mM dithiothreitol, 0.6 mM ATP, CTP, and UTP, 25 μM [α-32P]GTP (2.5-12.5 Ci/mmol; Amersham), 6 μg/ml of α-amanitin, 20-40 μg/ml of DNA, and 20 μl of soluble extract in a total volume of 50 μl. Phosphocellulose column fractionated material from KB cell extracts was prepared according to the methods described elsewhere (10). Assays employing this material used 6 μl of the designated fractions per 50-μl reaction.

Preparation of soluble extracts from unfertilized Xenopus laevis eggs and purification of the Xenopus transcription factor were previously described (6). Assay conditions, using the Xenopus extracts, were the same as above except that the KCl concentration was 65 mM and the MgCl₂ concentration was 5 mM. Assays employing this material used 30 μl of the egg extract and 0.1-0.3 μg of the transcription factor per 25-μl reaction. Methods for purification of RNA products and their subsequent analysis on polyacrylamide gels are detailed elsewhere (3). In all cases, the RNA products were denatured by glycoxidation prior to electrophoresis on 12% polyacrylamide gels. To quantitate the radioactivity in a specific transcript, the appropriate gel band was excised and measured by Cerenkov radiation.

DNase Protection Experiments—DNA-protein interaction was examined by a modification of the “footprinting” technique of Galas and Schmitz (11). Incubation conditions were as follows: 75-100 ng of end-labeled DNA fragment was incubated with approximately 100-200 pg of Xenopus transcription factor in 10 mM Hepes, pH 7.9, 65 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, and 0.1 mM dithiorethiol at 25 °C for 10 min in a final volume of 25 μl. Reactions were then treated with concentrations of DNase I ( Worthington, RNase-free) in the range of 0.25-5 μg/ml, depending upon the amount of factor used, and incubated at 25 °C for 1 min. Reactions were terminated by the addition of 15 mM EDTA, pH 7.5, 0.2% sodium dodecyl sulfate, and 40 μg/ml of sonicated salmon sperm DNA, final concentrations. DNA fragments were then purified by extraction with phenol/CHCl₃ and precipitation from ethanol. After resuspension in 9% formamide and heating at 95 °C for 2 min, the samples were electrophoresed through 6 or 8% polyacrylamide, 7 M urea sequencing gels (8) in a 0.5 x TBE buffer. A HaeIII digest of 5'-labeled pBR322 was used as size markers. Gels were exposed to Kodak XR-5 film with Dupont Cronex Intensifying Screens at -70 °C.

Preparation of DNA Fragments for Footprinting—Restriction maps of the three 5 S genes used in the footprinting experiments have been presented elsewhere (3, 12). The pXbs1 plasmid, containing a single repeat of the Xenopus borealis somatic 5 S gene, was cleaved with HindIII, 5'-labeled as described in the DNA sequencing section, subcleaved with Hpal, and gel-fractionated through 15% agarose in Tris/acetate buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.9). The fragment obtained was 425 base pairs in length with the labeled end approximately 200 base pairs from the midpoint (+60) of the 5 S gene. The mouse 5 S gene contained in the pM4 plasmid was excised by cleavage with HpalI, 5'-labeled, and subcleaved with TaqI. Upon gel purification, this produced a fragment of 350 base pairs with the labeled end located 130 base pairs from the midpoint of the gene. The human 5 S genes in the pH444 plasmid was initially cleaved with HpalI, 5'-labeled, and subcleaved with HindII. This generated a 430-base-pair fragment with the labeled end approximately 170 base pairs from the midpoint of the 5 S gene. 5'-labeling on alternate ends of the M4 and H4 genes containing restriction fragment was also performed in order to produce the same fragments only labeled on opposite DNA strands. All fragments were purified from agarose gels by the method of Vogelstein and Gillespie (13).

RESULTS
DNA Sequence of Transcriptionally Active and Inactive Mammalian 5 S rRNA Genes—The cloning and characterization of the transcriptionally active mouse 5 S gene, M4, and the inactive human 5 S gene, H4, have been described in detail in the accompanying manuscript (3). In order to examine the relationship between the primary structure of a gene and its ability to function as an active template in an in vitro transcription system, we have sequenced the 5 S genes from the M4 and H4 clones. The strategy outlined in Fig. 1 was employed in determining the sequence from these two clones.

A comparison was made of the variant residues between the two genes and the predominant form of 5 S rRNA from human KB cells (identical in sequence with mouse 5 S rRNA).

DNase Protection Experiments—The strategy outlined in Fig. 1 was employed in determining the sequence from these two clones. Preparativepolyacrylamide gels by the method of Vogelstein and Gillespie (13).

MOUSE M4 55  HPAI
| -50 | +120 |
| -30 |

HUMAN H4 55
| -50 | +70 |
| +120 | +250 |

FIG. 1. DNA sequencing strategy for the mouse M4 and human H4 5 S genes. In this diagram, the heavy line denotes the 5 S gene within the DNA fragments whose sequence was determined. The 5' and 3' notations indicate which DNA strands were labeled at a particular restriction site and the arrow gives the extent of the sequence determined. For the M4 5 S gene, a 350-base-pair HpalI/TaqI fragment was sequenced from the HpalI end on both DNA strands. For H4 5 S gene, a 270-base-pair BstNI/HindII fragment was sequenced from the BstNI end on both DNA strands and a 430-base-pair HpalI/HindII fragment was sequenced from the 5'-labeled HpalI end. The restriction maps of these two genes are given elsewhere (3).
when incubated in the presence of either the M4 5 S gene or the somatic 5 S gene from *X. borealis* *Xbs1* in a human KB cell extract. In the competition experiment shown (Fig. 3), the concentration of the 5 S DNA template, either M4 or *Xbs1*, was held constant at 10 μg/ml and 4 μg/ml, respectively, while the concentration of competitor DNA was varied between 5 and 36 μg/ml. The total DNA mass per 50-μl reaction was kept constant at 40 μg/ml with additions of pBR322 DNA being made as required.

The ability to compete for 5 S RNA synthesis from the mouse and *Xenopus* genes was measured using two different competitor DNAs, H444 and H460. The H444 clone contains an inactive 5 S gene and active *Alu* sequences, whereas the H460 clone contains active *Alu* sequences but no regions homologous to 5 S tRNA; the *Alu* sequences in each clone have essentially equivalent transcriptional activity *in vitro*. Use of these clones, therefore, should allow us to distinguish between competition from the *Alu* or 5 S RNA; the *Alu* sequences present in either human clone, but was most likely the result of 5 S sequences present in the H444 clone. This suggests that, despite base pair substitutions within the 5 S coding region which presumably render it inactive, the gene still retains the capacity to interact with a transcriptional component(s) required for 5 S RNA synthesis.

**Functional Equivalence of Human and Amphibian 5 S Factors**—Previous studies with chromatographically fractionated human cell extracts have shown that 5 S gene transcription requires a 5 S gene-specific factor (designated IIIA) as well as two other factors (designated IIIB and IIIC) which are apparently commonly required by genes encoding tRNA and adenovirus VA RNA (10). Similar results have been demonstrated for extracts derived from various amphibian (*Xenopus*) cell types (16). The 5 S gene-specific factor, designated TFIIIA, has been purified from *Xenopus* oocytes and is a protein of 38,000 daltons. It has been shown to interact specifically, and in the absence of other proteins, with both oocyte and somatic type 5 S genes between residues +45 and +96 (6), a region which includes the internal control region essential for transcription initiation (4, 5).

Given the evolutionary sequence conservation of 5 S genes and the ability of amphibian 5 S genes to be transcribed in mammalian cell extracts (9, 10), it seemed probable that the readily available *Xenopus* factor (TFIIIA) might be used in lieu of the homologous factor to determine possible effects of sequence alterations in mammalian genes on interactions with the 5 S-specific factor. To ascertain this, the possible mechanistic similarity of the *Xenopus* 5 S factor (TFIIIA) and the semipurified human 5 S factor (KB fraction A) was tested by interchanging them in reconstituted systems containing human or *Xenopus* components which alone would not support the synthesis of 5 S RNA from cloned templates. In this experiment, the synthesis of 5 S RNA from the *Xbs1* and M4 templates was tested in an unfractionated human KB extract and in a fractionated KB extract containing phosphocellulose column fractions B and C complemented with either the 5 S-specific factor(s) in KB fraction A or with the purified *Xenopus* TFIIIA. In addition, the *Xbs1* and M4 5 S genes were transcribed in an unfertilized *Xenopus* egg extract (containing factors IIIB and IIIC but depleted of TFIIIA (6)) complemented with either TFIIIA or KB A. The autoradiograph in Fig. 4 shows the results of this experiment. In lanes 1–3, a KB cell extract was incubated with *Xbs1* (lane 1), M4 (lane 4, lane...
Fig. 3. Transcription competition between active and inactive 5 S genes. In this experiment, the concentration of template DNA, Xbs1, or M4 was held constant while the concentration of competitor DNA, H4u, or H4o was varied. The total DNA per 50-μl reaction was maintained at 40 μg/ml by the addition of pBR322 DNA as required. Incubation conditions, using a KB cell extract, and subsequent electrophoretic analysis on 12% polyacrylamide gels were as described under “Experimental Procedures.” The autoradiograph in A represents a competition experiment in which the *Xenopus* somatic 5 S gene (Xbs1) was used as template at 4 pg/ml, in the presence of competing H4u, or H4o, DNA, whose concentration was varied between 6 and 36 pg/ml. In lanes 1–6, H4o DNA was present in the following amounts: 0 (lane 1), 6 (lane 2), 16 (lane 3), 26 (lane 4), 30 (lane 5), and 36 pg/ml (lane 6). In lanes 7–11, H4o DNA was used in the following amounts: 6 (lane 7), 16 (lane 8), 26 (lane 9), 30 (lane 10), and 36 pg/ml (lane 11). The extent of transcriptional competition by each template on Xbs1 was quantitated by excising regions of the gel containing Xbs1 5 S RNA and measuring the Cerenkov radiation. These values were then plotted against the concentration of competitor DNA employed. A quantitation of the data in A is shown in B; curve 1 represents competition by H4o, curve 2 represents competition by H4u, and curve 3 represents nonspecific competition by pBR322 (gel not shown). In C, the M4 5 S gene was used as template, at 10 pg/ml, in the presence of competing H4o or H4u DNA whose concentration was varied between 5 and 30 pg/ml. Lanes 1–7 contained H4o DNA in the following amounts: 0 (lane 1), 5 (lane 2), 10 (lane 3), 15 (lane 4), 20 (lane 5), 25 (lane 6), and 30 pg/ml (lane 7). In lanes 8–13, H4u DNA was used in the following amounts: 5 (lane 8), 10 (lane 9), 15 (lane 10), 20 (lane 11), 25 (lane 12), and 30 pg/ml (lane 13). D represents a quantitation of the data in C. Competition by H4o, H4u, and pBR322 (gel not shown) is indicated by curves 1, 2, and 3, respectively.

2), and H4u (lane 3). In lanes 4–10, the KB phosphocellulose fractions B and C were incubated with Xbs1 plus TFIIIA (lane 4), Xbs1 alone (lane 5), M4 plus TFIIIA (lane 6), M4 alone (lane 7), Xbs1 plus KB fraction A (lane 8), M4 plus KB fraction A (lane 9), and KB fraction A with no DNA (lane 10). The data from this set of reactions indicate that the *Xenopus* 5 S factor, like the human factor, is functional for 5 S RNA synthesis on both the *Xenopus* and mouse templates.
when reconstituted with human components (IIIB and IIIC) which alone do not transcribe 5 S genes. In lanes 11–17, an unfertilized egg extract from *Xenopus* (containing factors B and C, and only trace levels of TFIIIA) was incubated with Xbs1 plus TFIIIA (lane 11), Xbs1 alone (lane 12), M4 plus TFIIIA (lane 13), M4 alone (lane 14), TFIIIA with no DNA (lane 15), Xbs1 plus KB fraction A (lane 16), and M4 plus KB fraction A (lane 17). The results indicate that the human 5 S factor, present in the KB fraction A, is able to transcribe 5 S genes from Xbs1 and M4 in a *Xenopus* system containing components which cannot themselves support significant 5 S RNA synthesis. It is also apparent that the “active” mouse gene is transcribed less efficiently than the *Xenopus* gene but this is true for the unfractionated human extract as well as for the heterologous reconstituted systems. Moreover, M4 is transcribed no more efficiently in systems reconstituted with homologous factors (lanes 9 and 13) than in systems reconstituted with heterologous factors (lanes 6 and 17). Whether the difference in transcriptional activity between the two genes reflects some peculiarity of the *Xenopus* gene or a restricted activity of the variant mouse gene, relative to that of the 5 S gene encoding the predominant 5 S rRNA, is unclear since the latter gene is not available.

This set of experiments demonstrates that the human and *Xenopus* 5 S gene-specific factors are functionally interchangeable for 5 S gene transcription in either human or *Xenopus* soluble systems (containing the other polymerase III factors) and suggests that their mechanism of action may be the same. In the following protein-DNA interaction studies with the mammalian 5 S genes, we have chosen to use the *Xenopus* 5 S factor, TFIIIA. This choice was based on the fact that TFIIIA is currently in a more highly purified form than its human counterpart, making it less likely to obscure interpretations of the data with nonspecific protein binding to the 5 S template.

**Interaction between a 5 S Transcription Factor and Active Versus Inactive 5 S Genes**—To determine whether the transcriptionally inactive H4 5 S gene is still capable of recognizing a 5 S-specific factor or whether its inactivity could simply be attributed to the fact that this ability is lost, a protein-DNA interaction study was performed. The ability of the transcriptionally active M4 5 S gene to recognize this factor was also examined at the same time. In this study, the footprinting technique of Galas and Schmitz (11) was used to monitor the interactions between these genes and the 5 S factor from *Xenopus laevis* ovaries (see above).

The DNA fragments employed in the “footprinting” experiments contained the 5 S gene from mouse M4, human H4, and *Xenopus* Xbs1 DNA. The fragments were constructed as described under “Experimental Procedures” and as diagrammed in Fig. 1. Each of the three 5 S gene-containing fragments were incubated with two concentrations of the purified 5 S factor, 0.1 and 0.2 μg, followed by a titration with DNase I in the range of 0.25–3 μg/ml. This titration was necessary in order to generate a ladder of end-labeled DNA fragments which adequately resolves any existing protected regions. If the proper DNase I concentration (which must be empirically determined at each new ratio of protein:DNA) is not employed, the DNA fragment will be under- or overdigested which will result in an inability to identify the correct endpoints of the protein-protected region. Since the distances from the labeled end to the midpoint of the 5 S gene within the three fragments are similar, the electrophoretic fractionation of subsequent DNase I digests should resolve any intragenic protected regions to comparable degrees. This would allow a more precise comparison to be made between the three genes examined.
The footprint pattern obtained when TFIIIA was incubated with a 5 S gene-containing fragment from either Xbs1, M4, or H4 is depicted in Fig. 5. A represents the DNase I protection pattern obtained with the Xbs1 gene which had been 5'-labeled on the coding strand. Lanes 1 and 2 show the ladders produced from a partial DNase I cleavage of DNA which had not been incubated with factor; the DNase I concentrations used were 0.5 and 1.0 μg/ml, respectively. The reaction in lane 2 was obviously overdigested by the amount of DNase I employed and in future reactions with deproteinized DNA the concentrations of DNase I were reduced to 0.25 and 0.5 pg/ml. The reactions in lanes 3 and 4 contained DNA incubated with 100 ng of purified TFIIIA and partially digested with 1.5 and 3.0 μg/ml of DNase I, respectively. The reaction in lane 5, which had been 5'-labeled on the noncoding DNA strand and, therefore, the results in B are not directly comparable with those in A, since the Xbs1 gene had been labeled on the opposite strand. Lanes 1 and 2 in B show control reactions in which DNase I ladders were produced from M4 DNA which had not been exposed to TFIIIA; the DNase I concentrations used were 0.25 (lane 1) and 0.5 (lane 2) μg/ml. The reactions in lanes 3 and 4 contained DNA incubated with 100 ng of TFIIIA and digested with 1.5 and 3.0 μg/ml of DNase I, respectively. These lanes reveal a protected region corresponding to posi-

![Figure 5](image_url)

**Fig. 5.** Xbs1, M4, and H4 5 S gene footprint analyses with purified transcription factor. DNA fragments containing the 5 S genes from the mouse (M4), human (H4), and *X. borealis* somatic (Xbs1) plasmids were purified and 5'-labeled at one end as described under "Experimental Procedures." Fragments bearing the mouse and human 5 S genes are diagrammed in Fig. 1. A 350-base-pair HpaII/TaqI fragment containing the M4 5 S gene was 5'-labeled at the HpaII site which is approximately 130 base pairs from the midpoint of the gene (+60). A 430-base-pair HphI/HindII fragment bearing the H4 5 S gene was 5'-labeled at the HphI site, approximately 170 base pairs from the middle of the gene. The Xbs1 5 S gene is contained on a 425-base-pair HindIII/HhaI fragment, described previously by Engels et al. (6), which was 5'-labeled at the HindIII site; the labeled end is approximately 200 base pairs from the midpoint of the 5 S gene. The M4 5 S fragment was labeled on the noncoding strand of the gene while the H4 and Xbs1 5 S fragments were labeled on the coding strand. 5'-end-labeled DNA fragments containing the 5 S genes from Xbs1 (A), M4 (B), and H4 (C) were incubated with the purified Xenopus transcription factor, digested with DNase I, and analyzed on 6% (A and C) or 8% (B) polyacrylamide, 7 M urea gels according to the methods described under "Experimental Procedures." Lanes 1 and 2 in each panel represent DNA ladders generated by partial digestion with DNase I in the absence of added factor. In A, the DNase concentrations used were 0.5 and 1.0 μg/ml in lanes 1 and 2, respectively; in B and C, 0.25 (lane 1) and 0.5 (lane 2) μg/ml of DNase I were used. Lanes 3 and 4 in each panel contain reactions in which DNA fragments were incubated with 100 ng of purified factor and digested with 1.5 and 3.0 μg/ml of DNase I, respectively. Lanes 5 and 6 in A and B represent reactions in which 200 ng of purified factor was added, followed by digestion with 1.5 and 3.0 μg/ml of DNase I, respectively; the reaction in lane 5, C, contained 200 ng of factor and was digested with 3.0 μg/ml of DNase I. Lane 7 in A and B and lane 6 in C represent a HaeIII digest of 32P-labeled pBR322 DNA as size markers. The numbers on the right-hand side of each panel indicate the intragenic boundaries of the region protected from DNase I digestion and the arrow denotes the direction of transcription.
tions +45 through +96 within the M4 5 S gene which is similar to that found for Xbs1 (A). Lanes 5 and 6 represent reactions in which 200 ng of TFIIIA were incubated with M4 DNA and digested with 1.5 and 3.0 µg/ml of DNase I, respectively. As with Xbs1, these conditions failed to adequately define the endpoints of the protected regions. To achieve maximum resolution of the protected region within each gene, the entire coding and flanking sequences could not be shown on a single gel; however, an extension of the sequences shown in Fig. 5 on other gels did not reveal any further areas of DNase I protection, nor did fragments labeled on the opposite DNA strand (18).

The reactions in C represent the DNase I protection patterns obtained when the transcriptionally inactive human 5 S gene, H4, was incubated with TFIIIA. The fragment containing the H4 5 S gene had been 5' labeled on the coding strand and, therefore, the results obtained with this gene should be directly comparable to those of the Xbs1 gene, also labeled on the coding strand. Lanes 1 and 2 show the control ladders of DNase I-cleaved H4 5 S DNA without added factor, at 0.25 (lane 1) and 0.5 (lane 2) µg/ml of DNase I. Lanes 3 and 4 contain reactions in which H4 DNA was incubated with 100 ng of TFIIIA and digested with 1.5 and 3.0 µg/ml of DNase I, respectively. The reaction in lane 5 contained a 2-fold higher concentration of TFIIIA, 200 ng, incubated with H4 DNA and digested with 3.0 µg/ml of DNase I. Surprisingly, the results indicate that incubation of the inactive H4 5 S gene with TFIIIA can produce a DNase I-protected region at the same intragenic site (+45 to +96) as found for the transcriptionally active 5 S genes, Xbs1 and M4. The inactivity of H4 5 S, then, is apparently not simply a matter of being unable to interact with this 5 S-specific transcription factor or local factor interaction since deletion studies have shown that certain base pair changes within the region of +50 to +83 (defined by deletion mutations as the intragenic control region of the Xenopus 5 S genes) can be tolerated without a qualitative loss of transcriptional activity in vitro. These changes include those at positions +53, 55, 62, and 79 in the two Xenopus genes and at +68 and 79 in the M4 5 S gene. Base pair changes in this region which are specific to the H4 5 S gene include those at positions +64, 74, 79, and 81. These alterations, either individually or in combination, could be responsible for the inactivation of the H4 5 S template. Position +64 may be in an area important for TFIIIA binding but not absolutely required for transcriptional activity since mutations at +58, 65, and 66 in Xbo1 gene 3 completely abolish binding from residues +46 to +74 but leave the gene functional (although to a lesser degree than Xbs1) in vitro (6). It is curious that the base pair deletion at +81 in the H4 5 S gene does not appear to cause a serious disruption in overall or local factor interaction since deletion studies have shown that residues +81 to +83 are crucial to TFIIIA binding.

A comparison of the sequence changes within the DNase I protected regions of the Xenopus and mammalian 5 S genes considered here is shown in Table I. The Xbs1, Xbo1/gene 1 and M4 5 S genes are transcriptionally active in vitro, while the H4 5 S gene is not; KB 5 S refers to the DNA sequence predicted from that of the predominant form of 5 S rRNA found in human KB cells. The data from this table indicate that certain base pair changes within the region of +50 to +83 (defined by deletion mutations as the intragenic control region of the Xenopus 5 S genes) can be tolerated without a qualitative loss of transcriptional activity in vitro. These changes include those at positions +53, 55, 62, and 79 in the two Xenopus genes and at +68 and 79 in the M4 5 S gene. Base pair changes in this region which are specific to the H4 5 S gene include those at positions +64, 74, 79, and 81. These alterations, either individually or in combination, could be responsible for the inactivation of the H4 5 S template. Position +64 may be in an area important for TFIIIA binding but not absolutely required for transcriptional activity since mutations at +58, 65, and 66 in Xbo1/gene 3 completely abolish binding from residues +46 to +74 but leave the gene functional (although to a lesser degree than Xbs1) in vitro (6). It is curious that the base pair deletion at +81 in the H4 5 S gene does not appear to cause a serious disruption in overall or local factor interaction since deletion studies have shown that residues +81 to +83 are crucial to TFIIIA binding.

![Diagram of DNA sequence comparison](attachment:image.png)

**Fig. 6. Comparison of factor-protected regions in several 5 S genes.** The sequence shown is that of Xbs1 from position +40 to +100 within the 5 S gene; sequence alterations in other 5 S genes are indicated, with the notation d signifying a deleted base pair. The upper and lower DNA strands represent the noncoding and coding strands, respectively. The solid line above or below the DNA sequence identifies the factor-protected region on a particular strand; in Xbs1, the protected regions of both strands are shown. Positions of enhanced DNase cleavage are indicated by an arrow, with the uncertainty being within 1 and 3 nucleotides. Portions of the data used in this figure were taken from Engelke et al. (6).
Specifically, a deletion of these three residues completely abolishes factor binding to any region of the control site (17). Position +74 is, of course, a likely candidate for being a critical residue because of the enhanced DNase I cleavage found in this region of the TFIIIIA-protected sequence, relative to that of XbaI. Whether this difference should immediately suggest a nonfunctional mode of protein-DNA interaction is difficult to determine since, as previously noted, productive binding may result in several slightly different patterns of protection. However, it is worth noting that none of the active 5 S genes included in Table I or the 5 S gene variants from hen (as described in Ref. 2) have base pair changes in the region +69 to +78.

There also exists the strong possibility that TFIIIIA does interact in a functional manner with the H4 5 S gene but that, as a result of altered DNA sequences, other transcriptional components do not. As discussed previously, the in vitro transcription of 5 S genes with either TFIIIIA or the human KB A factor(s) requires the addition of other components. These components, in addition to RNA polymerase III, are present in the Xenopus egg extract and in two separate chromatographic fractions of a KB cell extract (KB B and C). If any one of these factors, including polymerase, failed to productively interact with the H4 5 S template, the gene would not be transcribed. These components could interact at other sites within the gene, including the internal control region, or at gene-flanking regions. The importance of 5' flanking regions to the transcription of rRNA genes in homologous and heterologous in vitro systems has been previously demonstrated (19, 20). Although DNA interaction experiments using components other than TFIIIIA have not been attempted with the H4 5 S gene, previous studies employing Xenopus RNA polymerase III and the factors present in an egg extract failed to generate DNase I protected regions on Xenopus 5 S genes (6). This may indicate that the template interaction with these components is too weak to be detected by this method, that the interaction is largely with the protein component of the TFIIIIA-5S gene, or, more likely, that the additional interactions are simply not quantitative (equimolar with respect to the 5 S DNA-TFIIIIA complex).

**DISCUSSION**

We have examined two mammalian 5 S genes, one of which is active and one of which is inactive in an in vitro transcription system. Comparative DNA sequence analyses and DNA interaction studies with a purified 5 S transcription factor from Xenopus were used in the attempt to explain the inability of one of the genes to be expressed. DNA sequencing revealed the presence of base pair substitutions occurring throughout both the active mouse 5 S gene, M4, and the inactive human 5 S gene, H4, relative to the 5 S rRNA sequence from human KB cells (Table I); this indicated that they were variant forms of this gene. The sequence alteration in the M4 5 S gene correlates well with the fingerprint pattern obtained from RNase T1-generated oligonucleotides of in vitro synthesized M4 5 S RNA (3). Moreover, the occurrence of a deletion mutation, plus the influence of 5' flanking sequences on the exact initiation site, could explain the observation that the M4 5 S transcript is slightly smaller than in vivo 5 S rRNA (120 bases). The M4 5 S gene also contains a 3' thymine stretch, characteristic of transcriptional termination sites in many prokaryotic and eukaryotic genes, which is distinguished by two features not found in the other genes examined: a contiguous stretch of 23 residues, rather than 4-8, and the localization of this cluster on the coding rather than noncoding DNA strand. The efficient termination observed with the M4 5 S gene in vitro suggests that the current hypothesis (21) of RNA strand displacement occurring through an unstable U-dA hybrid may be only one of several possible mechanisms and not applicable in this particular case where an A-dT hybrid would exist. Termination of class III eukaryotic genes in vitro, then, could be independent of the strand polarity of the thymine-rich region, unless transcription proceeds beyond this point and the RNA is subsequently processed.

A comparison of the base pair substitutions in the M4 and H4 5 S sequences revealed that some were common to both genes and could be eliminated from a consideration of those positions critical to transcription; other substitutions, however, were gene-specific. It had previously been demonstrated, by deletion analysis, that the only DNA sequences required for the initiation of transcription in vitro from the Xenopus 5 S genes were between positions +50 and +83 within the coding region (4, 5). If an analogous mechanism of 5 S transcription initiation exists in mammals, then the base pair changes in similar regions of the M4 and H4 5 S genes could provide a more refined analysis of functionally important residues.

In spite of the inactivity of the human 5 S template, it can still serve as an effective competitor for transcription of the 5 S genes from mouse and Xenopus in a human KB soluble extract. This indicates that the base changes which rendered the gene incapable of transcription still leave intact the DNA interaction site of at least one transcriptional component. To explore this observation further, a DNase I protection experiment was performed which utilized the "footprinting" technique of Galas and Schmitz (11). The protein used in this experiment was the highly purified 5 S-specific transcription factor from Xenopus ovaries, which had been employed previously in similar studies of interaction sites on Xenopus 5 S genes (6). This is functionally equivalent to the corresponding 5 S-specific factor(s) found in human KB cell extracts, as demonstrated by the ability of each factor to function in conjunction with the other factors from either human or amphibian cells to transcribe both Xenopus and mammalian 5 S genes. These results are consistent with the notion that 5 S transcriptional mechanisms in Xenopus and mammals may be the same and thereby validate the use of the amphibian transcription factor to probe functional interactions on the mouse and human 5 S genes.

The DNase I protection experiments revealed that both the active and inactive mammalian 5 S genes are capable of interacting with the factor within the intragenic control region previously defined for the Xenopus 5 S genes. The
transcriptional inactivity of the human H4 5 S gene, therefore, is not simply correlated with the inability to interact with this factor. A comparison of the DNase I protected regions between similar DNA strands of the human H4 and Xenopus somatic 5 S genes revealed one major distinction: a sharply enhanced DNase I cleavage in the region of +72 to +75 on the coding strand. This region of the H4 5 S sequence contains a base pair substitution at +74, one of the four substitutions within positions +50 to +83 which is specific to the inactive gene (Table I). This altered pattern of DNase I protection, relative to that of the Xenopus somatic 5 S gene, does not necessarily indicate a nonproductive mode of binding since slightly varying patterns also occur in transcriptionally active 5 S genes (see "Results"); however, it does appear to offer one plausible explanation for the inactivity of the H4 5 S template, namely the sequence change at position +74 (Table I).

A recent study by Sakonju and Brown (22) reports that eight guanine residues on the noncoding strand of the Xenopus somatic 5 S gene are important contact points for 5 S transcription factor interaction. These residues are located at positions +70, 71, 81, 82, 85, 86, 87, and 89. Moreover, base pair changes at positions +53 and +55 are thought to result in a 4-fold difference in binding affinity (for the factor) between the somatic and oocytic Xenopus 5 S genes.

An examination of the DNA sequence of the M4 and H4 5 S genes (Fig. 2) reveals no base pair changes in these positions except for a deletion in the H4 5 S gene at +81; the loss of this critical contact point may have altered the interaction of the 5 S factor with this template; however, no perturbation in this region is discernable from the DNase I protection pattern (Fig. 5). Positions +53 and +55 in the M4 and H4 5 S genes contain the same sequence as found in KB cell 5 S rRNA and, thus, cannot account for any differential affinity which may exist between the 5 S factor and the active and inactive 5 S templates.

The DNase I protection pattern observed with the transcriptionally active mouse M4 5 S gene differs from that of the Xenopus somatic 5 S gene (on similar DNA strands) by the absence of an enhanced cleavage near +75 on the noncoding strand and the presence of an unprotected region at +80 to +83 on the coding strand. Two M4 5 S-specific base pair changes which might account for these differences occur at positions +68 and +79; for example, the deletion at +79 could weaken the factor interaction at this position but not enough to abolish it completely, as observed with Xenopus 5 S genes deleted at residues +80 to +83 (17). These changes may account for the relatively lower level of transcription compared to the heterologous amphibian gene (in either the human or amphibian cell-free system) but whether this level is less than, or comparable to, that of the normal mammalian 5 S gene cannot at present be assessed.

These experiments indicate that the base pair substitutions specific to the H4 5 S gene, within the presumptive intragenic control region, are not critical positions for factor interaction per se, but may be critical to the overall transcription activity of the template. This suggests that either the 5 S-specific factor interacts in a nonfunctional manner on the inactive H4 5 S template, or that the defect lies with the interaction of another transcriptional component. Similar considerations apply to the base changes, and potentially reduced activity, of the M4 5 S gene. Since proteins other than the purified factor are required for the transcription of 5 S genes in vitro, but have not been shown by the footprint assay to bind to the genes or to disturb the 5 S factor-DNA interaction, it has been suggested that they might function by interacting with the protein component of the 5 S factor-DNA complex (6).

Thus, an altered interaction between factor and DNA might distort the site on the complex at which another transcriptional component normally binds. Alternatively, some regions of the template may also be important for direct interaction with other factors, either within regions "promoted" by the 5 S factor or at other sites within the gene. Thus, while interaction of the 5 S factor with the control region may be the most crucial protein-DNA contact, the possibility of other critical protein-DNA or protein-protein contacts should not be overlooked.

There also exists the possibility that the 5' flanking region of the H4 5 S gene, which has little homology with the M4 5 S flanking region, may act to repress transcription in vitro from this gene even though homologous extracts are used (see Sprague et al. (20)). The ability of 5' flanking regions to influence the efficiency of cell-free transcription has recently been demonstrated for the Drosophila RNAI gene, by switching these sequences between active and repressed templates (19). However, the possible importance of the 5' flanking regions in the transcription of 5 S genes is diminished by the overall lack of sequence homology observed among the Xenopus 5 S genes (14) and the mammalian 5 S genes, M4 and H4. In fact, a recently characterized hamster 5 S gene (23), which is transcriptionally active in vitro, shares no discernible base pair change with the 5' flanking region of either the active M4 or inactive H4 5 S template. Nevertheless, the possible existence of negatively acting sequences in the vicinity of either mammalian 5 S gene cannot be excluded.

Minimally, these experiments suggest that there are other, perhaps subtle, DNA-protein or protein-protein interactions which are important for transcription and which remain to be elucidated. The requirement for multiple components, including RNA polymerase III, in the transcription of 5 S genes in vitro (6, 10, 16), together with gene deletion studies (4, 5) may suggest the involvement of a multiprotein complex having its most crucial DNA interactions within the gene but also having contacts between the transcription factors and polymerase. Future studies will require the availability and analysis of the additional protein factors. Such factors, both from amphibian and mammalian cells, are currently being purified in an effort to answer these questions and to compare transcriptional mechanisms between the two species.

Acknowledgments—We would like to thank Dr. B. S. Shastry for his gift of purified transcription factor, TFIIIA, and Xenopus egg extract; Paul Martin for the phosphocellulose fractions of a human KB extract; and Dr. J. Doering for the recombinant plasmid, pXbs1.

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