TFIIIA binds with equal affinity to somatic and major oocyte 5S RNA genes

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Current models for the differential control of expression of *Xenopus* somatic and oocyte 5S RNA genes suggest that an impaired ability to bind TFIIIA contributes to the inactivation of oocyte 5S RNA genes in somatic cells. The somatic 5S RNA gene is transcribed more efficiently than the major oocyte 5S RNA gene in S-150 extracts of mature oocytes. However, this differential transcription efficiency is not determined simply by the relative affinity for binding of a positive transcription factor, TFIIIA. We have compared the abilities of somatic, major oocyte, and minor oocyte 5S RNA genes to bind TFIIIA using both a standard footprint competition assay and an indirect DNase protection assay. This indirect DNase protection assay permits the direct comparison of TFIIIA binding to two templates in one reaction. Both assay methods indicate that the major oocyte 5S RNA gene and the somatic 5S RNA gene bind TFIIIA with equal affinity. As a further control, we have confirmed earlier work indicating that the minor oocyte gene binds TFIIIA with a reduced affinity. Binding of TFIIIA to these three 5S RNA genes results in a different pattern of protection of each gene. We suggest that slight differences in the contacts between TFIIIA and the 5' border of the control region influence the ability of additional transcription factors to bind to the TFIIIA:5S DNA complex.

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The intragenic control region (ICR) of the *Xenopus borealis* somatic (Xbs) 5S RNA gene (Bogenhagen et al. 1980; Sakonju et al. 1980) has been shown to bind a 38,000-dalton protein, TFIIIA, that acts as a positive transcription factor (Engelke et al. 1980). This interaction plays a significant role in current models for the differential expression of *Xenopus* 5S RNA genes during development. Brown and co-workers (Brown 1984; Brown and Schlissel 1985) have suggested that the nearly exclusive expression of the somatic 5S RNA genes in somatic cells may result from a tighter binding of TFIIIA to the somatic gene combined with a reduced concentration of TFIIIA.

The most obvious difference between the intragenic sequences of somatic and oocyte 5S RNA genes is a cluster of oocyte-specific base pair changes at the 5' end of the ICR (Wormington et al. 1981). We have recently described a collection of point mutants of the Xbs 5S RNA gene with a number of transition mutations in this region, between gene positions 46 and 56 (McConkey and Bogenhagen 1987). A number of the mutants with transitions at the 5' boundary of the ICR exhibited significant increases or decreases in their ability to be transcribed but showed no apparent changes in their ability to bind TFIIIA as assayed by DNase protection. These observations suggest that contacts between TFIIIA and the 5' end of the ICR may contribute little to the binding affinity. Therefore, it was of interest to compare the abilities of somatic and major oocyte 5S RNA genes to bind

TFIIIA in relation to their relative transcriptional capacities.

Despite the widespread interest in the 5S RNA gene system as a model for differential gene regulation, no systematic comparison of the binding of TFIIIA to major oocyte and somatic 5S RNA genes has been published. Sakonju and Brown (1982) compared the relative affinity of TFIIIA for a somatic (Xbs) 5S RNA gene and the trace oocyte (Xlt) 5S RNA gene. The Xlt 5S RNA gene family constitutes only 10% of the oocyte genes (Peterson et al. 1980) and may not be representative of the major oocyte class. We have conducted experiments similar to those of Sakonju and Brown (1982) using the standard DNase protection assay to compare the affinity of TFIIIA for somatic (Xbs), major oocyte (Xlo), and trace oocyte (Xlt) *Xenopus* 5S RNA genes. In addition, we have developed a method to directly compare the binding of TFIIIA to two different templates. Both approaches indicate that TFIIIA binds equally tightly to somatic and major oocyte 5S RNA genes.

Results

Relative transcription efficiencies of three 5S RNA genes in an S-150 extract

The relative transcription efficiencies of three naturally occurring 5S RNA genes were compared in an S-150 ex-

tract made from mature oocytes. The genes used in this study have all been used in previous studies of the transcription of 5S RNA genes. These 5S RNA gene clones are described in Materials and methods and are diagrammed in Figure 1A. To ensure equal concentrations of

Figure 1. Diagrams of 5S RNA genes. (A) The three genes used in this study are diagrammed to scale. The arrow denotes the coding region extending from 5' to 3'. XbsΔ3' + 132 is the *X. borealis* somatic 5S RNA gene flanked by HindIII (H) and BamHI (B) restriction sites. The EcoRI (E) restriction site is actually in the plasmid pBR322 but is shown to denote the site at which the template was ³²P-labeled. XloΔ3' + 158 is the major *X. laevis* oocyte 5S RNA gene flanked by HindIII and EcoRI restriction sites. F denotes the Fnu4HI restriction site. Xlt191 is a *X. laevis* trace oocyte 5S RNA gene flanked by HindIII and BamHI restriction sites. As is the case of the Xbs gene, the EcoRI restriction site used for end labeling occurs in the adjacent pBR322 sequence. (B, C) Diagrams (not drawn to scale) of pOMSI and pTMSI constructed as described in Materials and methods. The orientation of the genes and the relevant restriction enzyme sites are labeled. The lines terminated in asterisks (*) depict the primers and their respective hybridization sites. pOMSI is a pBR322 derivative that contains the Xlo 5S RNA gene and a multilinker cloning site with the Xbs 5S RNA gene in a tandem orientation, as shown. pOMSII (not shown) has the two 5S RNA genes in an opposed orientation. pTMSI is a pBR322 derivative that contains the Xlt 5S RNA gene and a multilinker cloning site with the Xbs 5S RNA gene in a tandem orientation, as shown. pTMSII (not shown) has the two 5S RNA genes in an opposed orientation.

the genes in transcription assays, plasmids were constructed containing the Xbs 5S RNA gene along with either of the two oocyte 5S RNA genes (Fig. 1B,C). These constructions, described in Materials and methods, allow the transcription strengths to be determined with a fixed relative concentration of the two templates. Plasmids containing both orientations of the two 5S RNA genes (pOMSI and pOMSII; pTMSI and pTMSII) were transcribed to demonstrate that the transcription efficiencies are independent of the relative orientation. Mixtures of the Xbs and oocyte 5S RNA genes cloned in separate vectors were also transcribed to rule out possible *cis*-acting influences on transcription. The transcription reactions were performed in an oocyte S-150 extract (Glikin et al. 1984) and the RNAs were separated on a partially denaturing polyacrylamide gel capable of resolving the oocyte and somatic 5S RNAs (Wakefield and Gurdon 1983). Figure 2 shows the RNAs synthesized from the various combinations of oocyte and somatic 5S RNA genes. The Xlo 5S RNA gene is transcribed poorly compared to the somatic gene. The RNAs synthesized from either pOMS construct (lanes 4 and 5) or an equimolar mixture of the XbsΔ3' + 132 and XloΔ3' + 158 5S RNA genes (lane 8) are almost exclusively the somatic 5S RNA. In contrast, the Xlt gene is transcribed more efficiently than the Xlo gene (lanes 6, 7, and 9 of Fig. 2).

Our measurement of the transcription efficiency for the Xlo 5S RNA gene contrasts with previous results obtained with germinal vesicle extracts. Wormington et al. (1981) have shown that both the Xlt and Xlo 5S RNA genes compete only 25% as well as the Xbs 5S RNA gene in competitive transcription assays. Using an S-150 extract, we find that the Xlo 5S RNA gene is only transcribed with 2.5–3.0% of the efficiency of the Xbs 5S RNA gene (Fig. 2, lanes 4, 5, and 8). The Xlt 5S RNA gene is transcribed in the S-150 extract with approximately the same efficiency, relative to the Xbs 5S RNA gene, as in the germinal vesicle extract. These exact relative transcription efficiencies may depend on the source of the extract. However, in contrast to the results of Kmiec et al. (1986), we find that the relative transcription efficiencies are not significantly altered by the addition of a 2- to 32-fold excess of free TFI_{II}A to this extract. Other immunoblotting experiments have shown that even the unsupplemented extract is a rich source of TFI_{II}A (data not shown). Peck et al. (1987) have also recently published results similar to ours indicating that the access of TFI_{II}A to the oocyte-type 5S RNA genes is not a limiting factor for in vitro transcription in these extracts.

Relative TFI_{II}A binding affinities of somatic and oocyte 5S RNA genes using the standard DNase protection assay

Previous studies have suggested that the relatively weak competitive transcription strength of oocyte 5S RNA genes results largely from a lower affinity for TFI_{II}A (Brown 1984; Brown and Schlissel 1985). Because the

Figure 2. Transcription of somatic and oocyte 5S RNA gene mixtures. Two exposures of a partially denaturing polyacrylamide gel analysis of RNAs synthesized in an oocyte S-150 extract are shown. XbsΔ3' + 132/pUC (lane 1), XloΔ3' + 158/pUC (lane 2), and Xlt191/pBR (lane 3) were transcribed and separated by electrophoresis to serve as markers for Xbs 5S RNA, Xlo 5S RNA, and Xlt 5S RNA, respectively. Note that Xlo 5S RNA is resolved into three differently migrating species. The more slowly migrating species result from inefficient termination at the first T cluster encountered by RNA polymerase III (Bogenhagen and Brown 1981). The other templates were pOMSI (lane 4), pOMSII (lane 5), pTMSII (lane 6), pTMSI (lane 7), an equimolar mixture of XbsΔ3' + 132/pUC and XloΔ3' + 158/pUC (lane 8), and equimolar mixture of XbsΔ3' + 132/pUC and Xlt191/pBR (lane 9). (Top panel) A 4-hr exposure; (bottom panel) a 20-hr exposure.

binding of TFIIIA to the major oocyte gene has not been investigated thoroughly, we performed DNase I footprinting experiments to compare the binding of TFIIIA to the three classes of 5S RNA genes diagrammed in Figure 1. The first assay method employed was a standard competitive DNase protection assay, essentially as described by Sakonju and Brown (1982). XbsΔ3' + 132 was singly end labeled with ^{32}P on the 5' end of its coding strand. Increasing concentrations of an unlabeled competitor DNA were mixed with the labeled XbsΔ3' + 132 5S RNA gene prior to the addition of TFIIIA. The minimal concentration of TFIIIA required to produce complete protection of the ICR of the labeled Xbs gene without a

competitor was maintained in all of the competitive binding reactions. The reactions were treated with DNase I according to the standard footprinting assay. Figure 3 shows an autoradiogram of one of the competition experiments. The lanes labeled zero (0) demonstrate that the labeled Xbs DNA is fully protected by 5 nM TFIIIA in the absence of competitor. Increasing concentrations of a second unlabeled 5S RNA gene compete for TFIIIA binding and reduce the protection of the ICR of the labeled gene. Quantitative densitometry of the autoradiograms was used to determine the relative competition strength, as diagrammed in Figure 3B.

The results shown in Figure 3 clearly indicate that TFIIIA binds with equal affinity to the somatic and major oocyte 5S RNA genes. We have performed additional footprint titrations of each gene separately to demonstrate that a direct exchange of TFIIIA (Kmieciak et al. 1986) does not affect the relative TFIIIA-binding affinities (data not shown). However, the sort of footprint competition results shown in Figure 3 require a comparison between samples that inevitably introduces some uncertainty regarding the relative affinity of TFIIIA binding. It is possible that contaminants in the competitor DNA or nonspecific binding could influence these results. Therefore, we have attempted to confirm the observation that TFIIIA binds equally tightly to the somatic and major oocyte genes with an additional approach.

Comparison of relative TFIIIA equilibrium binding constants by simultaneous footprinting of pairs of 5S RNA genes using an indirect DNase protection assay

The scheme for the indirect DNase protection assay is diagrammed in Figure 4A. An unlabeled template is incubated with a DNA-binding protein, (e.g., TFIIIA) and treated with DNase I. Following purification of the DNA, the footprint is visualized by annealing a ^{32}P -labeled primer to the DNase-treated template and extending the primer to the DNase-generated termini. The extensions are separated by denaturing polyacrylamide gel electrophoresis. One advantage of this technique is that binding of a protein to either of two different templates can be compared for a single binding reaction. Two distinct footprints can be resolved using primers that anneal to unique sites adjacent to each binding site for the protein. A titration with increasing concentrations of protein permits a direct comparison of the equilibrium binding constants.

To validate the indirect DNase protection assay as a method to study the binding of TFIIIA, the footprint generated by this method was compared to that generated by the direct DNase protection method, with the result shown in Figure 4B. The two footprinting patterns in Figure 4B are displaced with respect to one another by a small mobility difference that is predictable from the difference in methods. The extent of the protected regions is identical, although there is a slight loss of resolution generated by the indirect footprinting technique.

The binding of TFIIIA to two different pairs of 5S RNA

Figure 3. Competitive strengths of 5S RNA genes for TFIIIA assayed by the standard DNase protection assay. (A) One of the autoradiograms that was scanned to derive the competitive footprint graph in B. The first lane, labeled minus (–), contains the products of DNase treatment without the addition of TFIIIA. For all other reactions, 2 nM of an end-labeled Xbs 5S RNA gene was mixed with increasing concentrations of unlabeled Xbs, Xlo, and Xlt 5S RNA genes and 5 nM TFIIIA. The three sets contain 0, 1, 2, 4, 6, and 8 nM competitor DNA. (B) A graph of ICR accessibility to DNase I for the labeled Xbs DNA in the presence of increasing concentrations for nonradioactive competitor DNA calculated as in Materials and methods. Note that in the absence of competitor DNA, the coding strand of the ICR is fully protected from DNase I (0% ICR accessibility). With increasing concentrations of competitor, the fraction of labeled fragments bound to TFIIIA is decreased, resulting in an increasing frequency of DNase I cuts within the ICR, an increase in ICR accessibility. Titrations with the competitors pUC 9 and pXlt191 were extended to competitor concentrations of 16 nM. The competitor DNAs employed were pXbsΔ3' + 132/pUC (□), pXloΔ3' + 158/pUC (Δ), pXlt191/pBR (○), and pUC 9 vector (◇).

genes was assessed using the indirect DNase protection assay. The results of simultaneous footprinting of the somatic and major oocyte 5S RNA genes in the pOMSI construct (Fig. 1B) are shown in Figure 5. pOMSI was incubated with increasing concentrations of TFIIIA and treated with DNase I. A primer complementary to the DNA adjacent to the Xbs 5S RNA gene was annealed to one half of the DNase-treated templates and extended. An Xlo spacer-specific primer was annealed to the other half of the templates and extended. The XbsΔ3' + 132 5S RNA gene ICR is partially protected by 1 nM (Fig. 5A, lane 2) and maximally protected by 2 nM TFIIIA (lane 3). Some background bands are evident within the TFIIIA-protected region in Figure 5A (lanes 3–7). These bands are not the same bands that result from DNase cleavage of free DNA (as in lane 1), but appear to be due to premature termination of the primer extensions. The XloΔ3' + 158 5S RNA gene is also completely protected with 1–2 nM TFIIIA concentrations (Fig. 5B, lanes 2 and 3). By comparing the footprints on the major oocyte 5S RNA gene and the somatic gene, it is clear that both genes are fully protected at the same TFIIIA concentrations. This result has been obtained several times with the pOMSI plasmid using several preparations of TFIIIA or RNase-treated 7S particle (Hanas et al. 1983). We have also used different primers to visualize the DNase I protection on the noncoding strands. Again, no differences were observed in the affinity of TFIIIA for Xbs and Xlo 5S RNA genes. Results obtained with pOMSI were found to be the same as those obtained by performing the indirect footprinting assay with equimolar mixes of the Xlo and Xbs 5S RNA genes cloned in separate vectors (data not shown). The data from the indirect footprinting assay support the conclusion from the standard competition assay shown in Figure 3, i.e., that the Xbs and Xlo 5S RNA genes bind TFIIIA with essentially equal affinity.

As an additional control, we also used the indirect DNase protection assay to reinvestigate the relative affinities of TFIIIA for binding to the Xlt and Xbs 5S RNA genes. The results of a simultaneous footprint titration with the pTMSII plasmid (Fig. 1) are shown in Figure 6. These results reinforce the conclusion obtained above (Fig. 3) and by Sakonju and Brown (1982) that TFIIIA binds less tightly to the Xlt oocyte 5S RNA gene. Complete protection of the Xlt gene requires 4–6 nM TFIIIA. On first glance, it might appear that this result is at odds with the footprint competition data shown in Figure 3. However, we consider that the principal differences derive from the nature of the binding reactions. In the footprint competition experiment (Fig. 3), the binding reactions contained 5 nM TFIIIA and 1 nM Xbs 5S DNA, such that a relatively high concentration of the Xlt gene was required to sequester TFIIIA. In the footprint titration (Fig. 6), both genes are present at a concentration of 1 nM, and even the Xlt gene is saturated with TFIIIA at a 6 nM protein concentration.

The experiments presented above do not support a simple correlation between TFIIIA binding affinities and transcription efficiencies in the S-150 extract. Our control experiments with the trace oocyte 5S RNA gene

Figure 4. The indirect DNase protection assay. [A] Scheme for the assay. [B] An autoradiogram is shown of a denaturing polyacrylamide gel comparing the standard and indirect DNase I protection assays. (Lanes 1–4) Binding of TFI_{IIA} to XbsΔ3' + 132MT (McConkey and Bogenhagen 1987) as assayed by the indirect DNase protection assay. The intact plasmid (1 nM) was incubated with increasing concentrations of TFI_{IIA}: 0 nM (lane 1), 2 nM (lane 2), 4 nM (lane 3), and 8 nM (lane 4) and treated with DNase I, as described in Materials and methods. A ³²P-labeled *Eco*RI site pBR322 primer was annealed to the treated template and the primer extended to the DNase-generated termini. Note that the plasmid used in this experiment contains 5S maxigene that gives rise to an additional protected zone in the higher molecular weight range of the gel. (Lanes 5–8) Binding of TFI_{IIA} to XbsΔ3' + 132 ³²P-labeled at the *Eco*RI site preceding the gene as assayed by the standard DNase protection assay. The labeled DNA fragment (1 nM) was incubated with the same concentrations of TFI_{IIA} as the indirect assay: 0 nM (lane 5), 2 nM (lane 6), 4 nM (lane 7), and 8 nM (lane 8). (Lane m) ³²P-labeled pBR322 *Msp*I size markers.

agree with the data published previously by Brown and co-workers (Sakonju and Brown 1982; Brown 1984). The trace oocyte gene is transcribed with 14–25% of the efficiency of the Xbs 5S RNA gene and binds TFI_{IIA} with one-half to one-quarter of the affinity of the Xbs 5S RNA gene. In contrast, The Xlo 5S RNA gene does not show a correlation between the affinity of TFI_{IIA} binding and transcription efficiency. The Xlo 5S RNA gene binds TFI_{IIA} as tightly as the Xbs 5S RNA gene does, but is transcribed at 2.5–3.0% the efficiency of the Xbs gene in the S-150 extract. We suggest that other factors (i.e., transcription factors or RNA polymerase III) must be involved in the inefficient transcription of the Xlo 5S RNA gene.

Discussion

A principal goal of research into the transcription of 5S RNA genes by RNA polymerase III has been to understand the differential control of transcription of oocyte and somatic 5S RNA genes (for review, see Brown 1984). We have found that transcription in an S-150 extract derived from mature oocytes demonstrates some measure of this differential control in vitro. In this extract, the major oocyte 5S RNA gene is transcribed with only a small fraction of the efficiency of the somatic 5S RNA gene (Fig. 2). Peck et al. (1987) have recently reported results similar to those shown in Figure 2.

To determine whether the poor transcription efficiency of the major oocyte gene was due to a reduced

Figure 5. Comparison of TFIIIA binding to the somatic and major oocyte 5S RNA genes. Autoradiograms of primer extensions using the pOMSI construct as a template are shown. The pOMSI construct (1 nM) was incubated with 0 nM, 2 nM, 4 nM, 6 nM, 8 nM, 10 nM, and 12 nM TFIIIA in lanes 1–7, respectively. The reactions were treated with DNase, and TFIIIA-binding sites were visualized as described in Materials and methods. (Panel A) The products of annealing and extending the M13 sequencing primer, which hybridizes 5' to the Xbs 5S RNA gene, to the treated pOMSI template. (Panel B) The products of annealing and extending Xlo gene spacer-specific primer to the treated pOMSI template. The lanes labeled G are sequence ladders derived by extending the primers on pOMSI in the presence of dideoxy GTP.

affinity for TFIIIA, we used two adaptations of the DNase I protection assay to compare the affinity of TFIIIA binding to the somatic and major oocyte 5S RNA genes. The first of these methods was the same technique originally employed by Sakonju and Brown (1982) to compare the binding of TFIIIA to the somatic and trace oocyte genes: footprint competition. Figure 3 demonstrates that the major oocyte 5S RNA gene is as effective a competitor for TFIIIA binding as the somatic 5S RNA gene. As an additional control in these experiments, we reproduced the observation of Sakonju and Brown (1982) that the trace oocyte gene is a relatively poor competitor for TFIIIA (Fig. 3). One limitation of this competitive footprinting assay is that it simply shows that TFIIIA can be displaced from an indicator DNA by a competitor. The footprint competition technique does not directly determine equilibrium binding constants. Because a number of artifacts could impair binding of a protein to DNA, we have developed an additional variation of the footprinting technique to compare the binding of TFIIIA to two 5S RNA genes present in one binding reaction. This novel indirect footprinting technique has been presented in abstract form [McConkey and Bogenhagen 1985] and is similar to that

developed independently by Gralla (1985). The indirect footprinting method permits a simultaneous footprint titration for two binding sites. The occupancy of each binding site can be assessed independently when each site is adjacent to a unique primer hybridization site. Thus, the entire approach is different from that employed in footprint competition, where the protein concentration is held constant and the concentration of a competitor DNA is varied. This technique permitted us to confirm the observation that the major oocyte 5S RNA gene binds TFIIIA at least as tightly as the somatic 5S RNA gene.

The qualitative manner in which TFIIIA binds to a 5S RNA gene, reflected by the extent of DNase protection, may be an important determinant of the transcription efficiency. The regions protected from DNase by TFIIIA in the Xlo and Xbs 5S RNA genes are in fact slightly different (Engelke et al. 1980; Sakonju and Brown 1982; Bogenhagen et al. 1987). Figure 7 depicts the protected regions on both strands of the three 5S RNA genes used in this study. TFIIIA protects nucleotides 43–96 of the coding strand and nucleotides 45–98 of the noncoding strand of the Xbs 5S RNA gene from DNase I degradation. In contrast, the 5' boundary of the TFIIIA-protected region is foreshortened for both of the oocyte 5S RNA

Figure 6. Comparison of TFIIIA binding to the somatic and trace oocyte 5S RNA genes. Autoradiograms of primer extensions using the pTMSII construct as a template are shown. The pTMSII construct (1 nM) was incubated with increasing concentrations of TFIIIA in lanes 1–7, and the reactions were processed as described in Fig. 5. (Panel A) The products of annealing and extending the M13 sequencing primer, which hybridizes 5' to the Xbs 5S RNA gene, to the treated pTMSII template. (Panel B) The products of annealing and extending the pBR322 *EcoRI* site primer, which hybridizes 5' to the Xlt 5S RNA gene, to the treated pTMSII template. (Lanes G) Sequence ladders derived by extending the primers on pTMSII in the presence of dideoxy GTP.

Figure 7. Regions protected from DNase I by TFIIIA binding to three 5S RNA genes. The nucleotide sequences of residues 40–100 of the Xbs, Xlo, and Xlt 5S RNA genes are shown. Nucleotides protected from DNase digestion by bound TFIIIA are underlined (coding strand) or overlined (noncoding strand). The nucleotide residues differing from the Xbs 5S RNA gene are represented by lowercase letters. Sites of nuclease hypersensitivity are indicated by narrow triangles. This summary of the TFIIIA protection pattern is derived from data in Figs. 5 and 6 and published data (Sakonju and Brown 1982; Bogenhagen et al. 1987).

genes. TFIIIA protects only residues 51–93 of the noncoding strand for Xlo 5S RNA gene, and the footprint contains an interesting DNase I hypersensitive site on the coding strand near residue 51. This hypersensitive site has been observed previously (Reynolds and Gottesfeld 1985). The protected region of the Xlt 5S RNA gene is considerably smaller than that of either the Xbs or Xlo 5S RNA gene. TFIIIA only protects nucleotides 53–98 on the noncoding strand and 53–96 on the coding strand (Sakonju and Brown 1982; G.A. McConkey, unpubl.; Fig. 6). The lack of protection of base pairs in the 5' end of the Xlt 5S RNA gene ICR may be related to the poor competition of the Xlt gene with the Xbs gene for TFIIIA binding.

The differences in the extent of DNase I protection for the three 5S RNA gene ICRs may reflect differences in the binding of particular domains within TFIIIA, as predicted by a recent model for the structure of TFIIIA (Miller et al. 1985). This model postulates that TFIIIA consists of nine zinc-binding domains, or 'fingers,' each of which binds approximately 5.5 base pairs of DNA (Rhodes and Klug 1986). Thus, consecutive fingers are thought to bind along alternating faces of the DNA helix. Two kilodaltons of the carboxy-terminal end of TFIIIA, termed the 'interaction domain,' are not immediately involved in DNA binding, but are required for transcription (Smith et al. 1984). The orientation of this carboxy-terminal domain with respect to the DNA might be expected to vary for the three 5S RNA gene types in this study due to the different patterns of binding of the TFIIIA fingers to each ICR. The lack of binding of TFIIIA to 5–6 bp at the 5' end of the ICR of the Xlo 5S RNA gene might involve an altered binding of the most carboxy-terminal finger that is thought to contact these base pairs in the Xbs 5S RNA gene. Further analysis of the contact points for TFIIIA binding to all three 5S RNA gene types [similar to those recently published for the Xbs 5S RNA gene (Fairall et al. 1986)], is required to support this model.

Our observation that the major oocyte 5S RNA gene binds TFIIIA tightly is not inconsistent with the well-known inactivation of these genes in somatic cells. The reduced concentration of TFIIIA in somatic cells (Shastri et al. 1984; Andrews and Brown 1987) almost certainly plays a role in selective transcription of somatic-type genes. However, even when the concentration of TFIIIA in embryos is raised by microinjection of the TFIIIA protein (Brown and Schlissel 1984) or its mRNA (Andrews and Brown 1987), the derepression of oocyte gene transcription that results is only transient.

The binding of TFIIIA is only the first in a series of events required for transcription of 5S RNA genes. At least two additional factors, common polymerase III factors designated TFIIIB and TFIIIC, are required for assembly of 5S RNA genes into stable complexes (Segall et al. 1980; Shastri et al. 1982; Setzer and Brown 1985). The ability or inability of a gene to be assembled into a stable active complex can determine its transcriptional activity for multiple rounds of transcription (Bogenhagen et al. 1982; Lassar et al. 1983; Brown 1984). A great deal remains to be learned regarding the quantitative and kinetic blocks to stable complex formation.

Stable complex formation can be considered a stepwise process in which the first step, binding of TFIIIA, is reversible. Several lines of evidence suggest that binding of TFIIIC may be the second step in stable complex assembly, the step required for template commitment (Lassar et al. 1983; Bieker et al. 1985; Setzer and Brown 1985; Segal 1986). Thus, TFIIIC must recognize and bind to a preexisting TFIIIA-5S DNA complex in order to stabilize the complex. We suggest that TFIIIC may be able to distinguish between complexes of TFIIIA bound to either the oocyte or somatic 5S RNA genes, just as DNase I footprinting visualizes different protection patterns (Fig. 7). The ability of the major oocyte 5S RNA genes to bind TFIIIA tightly to form a binary complex is not sufficient to insure that the genes will be actively transcribed. We would suggest that impaired binding of TFIIIC to the TFIIIA : oocyte gene complex limits transcription of these genes in the S-150 extract and possibly within somatic cell chromatin. Experiments to test this model are in progress.

The actual mechanism that leads to differential activation of 5S RNA genes may be more complicated than indicated in this model. Until the factors TFIIIB and TFIIIC are purified and characterized, it remains possible that the identity or properties of these factors may vary in somatic cells and oocytes.

Materials and methods

Cloned 5S RNA genes

The three 5S RNA gene types were originally inserted in pBR322. These genes and their flanking regions are diagrammed in Figure 1A. The *X. borealis* somatic-type gene is XbsΔ3' + 132 (Bogenhagen et al. 1980), which contains 48 bp of 5'-flanking DNA and extends to nucleotide 132, 12 nucleotides beyond the transcription termination site. This gene is also

cloned between the *Hind*III and *Bam*HI restriction sites of pUC8 (Vieira and Messing 1982) and named XbsΔ3' + 132/pUC. Note that the coding region of the Xbs 5S RNA gene differs only at positions 12 and 34 with respect to the *Xenopus laevis* somatic 5S RNA gene (Peterson et al. 1980). The *X. laevis* major oocyte gene is XloΔ3' + 158, which contains 355-bp of natural spacer DNA 5' to the coding region and extends to approximately 38 nucleotides beyond the gene (Bogenhagen and Brown 1981). A *Fnu*4HI/*Eco*RI fragment of XloΔ3' + 158 containing only 12 bp of 5' spacer DNA cloned in pUC8, was used for TFIIIA binding experiments. The *X. laevis* trace oocyte-type gene, Xlt191, is a 191-bp insert derived from the first of three tandem repeats in Xlt 1 (Peterson et al. 1980; Sakonju and Brown 1982).

Dual gene constructs were generated by inserting the *Pvu*II fragment of XbsΔ3' + 132/pUC containing the somatic 5S RNA gene into pBR322 clones containing either the Xlo or Xlt 5S RNA gene. The *Pvu*II fragment containing the Xbs 5S RNA gene includes the multiple cloning site of pUC8 and the flanking sequences to which the M13 commercial primers (N.E. Biolabs, Inc., Beverly, MA) anneal. The *Pvu*II fragment was inserted in both orientations into the unique *Pvu*II site in XloΔ3' + 158/pBR. These constructs are termed pOMSI or pOMSII, depending on their orientation (see Fig. 1B). The *Pvu*II fragment was also inserted in both orientations into the unique *Pvu*II site in Xlt191/pBR, and named pTMSI and pTMSII (see Fig. 1C).

Transcription assays

Various templates were transcribed in an S-150 extract of mature *X. laevis* oocytes prepared as described by Glikin et al. (1984). The templates, at a saturating DNA concentration of 4 nM, were incubated with 16 μl of extract for 60 min at 22°C in a 24-μl reaction containing buffer [10 mM KCl, 6 mM MgCl₂, 10 mM HEPES (pH 7.6), 1 mM dithiothreitol (DTT)], 1 mM ATP, 200 μM CTP and UTP, 10 μM GTP (5 μCi [α -³²P]GTP), and 2 μg/ml α -amanitin. The reactions were stopped with 276 μl of stop solution [0.3 M NaCl, 10 mM EDTA, 100 mM Tris-HCl at pH 7.6, 0.5% sodium dodecyl sulfate, 10 μg/ml yeast tRNA]. The transcripts were purified by two rounds of phenol extraction, followed by ethanol precipitation. The pellets were suspended in loading solution [4 M urea, 1 mM EDTA, 10 mM Tris-HCl at pH 7.6, 0.125% bromophenol blue and xylene cyanol]. The RNA was separated by electrophoresis under partial denaturing conditions according to the method of Wakefield and Gurdon (1983).

DNase I protection assay

TFIIIA binding and DNase I treatment were described by McConkey and Bogenhagen (1987). TFIIIA was purified from immature *Xenopus* ovaries according to methods published previously (Smith et al. 1984). XbsΔ3' + 132/pBR was end labeled following linearization with *Eco*RI by filling in with AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) and [α -³²P]dATP (ICN, Irvine, CA). The labeled DNA was purified on a 1-ml Sephadex G-100 (Sigma Chemical Co., St. Louis, MO) column and digested with *Bam*HI. The singly end-labeled fragment was purified by polyacrylamide gel electrophoresis and squash elution (Maxam and Gilbert 1977).

Footprint competitions were conducted by mixing increasing concentrations of unlabeled competitor DNA with 2 nM of the end-labeled XbsΔ3' + 132 DNA and the minimal amount of

TFIIIA determined to give full protection in the absence of competitor, usually 5 nM. The intact plasmids used for competition were pUC9, Xlt191 inserted in pBR322, XbsΔ3' + 132 or XloΔ3' + 158 inserted in pUC8. The autoradiograms were quantitated by densitometry with an LKB Ultrosan scanning densitometer. To permit calculations of the extent of DNase I cleavage, data for two bands inside the control region and two bands outside the control region were analyzed. The band intensities were normalized relative to the controls observed for DNase I cleavage of free DNA. An index termed 'ICR accessibility' was calculated as the ratio of the sums of relative intensities inside the control region divided by the ratio of the sums of relative intensities outside the control region.

The indirect DNase protection assay

This assay consisted of two halves: DNase footprinting (similar to the standard DNase protection assay) and primer extension (see Fig. 4A). Template DNAs, as supercoiled plasmids, were incubated with increasing concentrations of TFIIIA in J buffer (Birkenmeier et al. 1978), lacking glycerol but containing 0.1% NP-40. The DNA-protein complexes were treated with 0.3 μg/ml DNase I for 60 sec. Sufficient DNase to nick the majority of templates was required to minimize the background of the primer extensions. The DNase was stopped with 5 volumes of a solution containing 0.3 M NaCl, 10 mM EDTA, 100 mM Tris-HCl (pH 7.6), and 3 μg/ml yeast tRNA. The reactions were limited to 40 μl to allow easy mixing of the DNase and the DNase stop solution. Rapid, complete inhibition of DNase is mandatory. The stopped reactions were organically extracted and precipitated with ethanol at -20°C overnight. The reactions may be stored at this point indefinitely.

The footprints were selectively visualized by annealing a ³²P-labeled primer and performing primer extension. Primers that anneal 5' to the protein binding site were used. The purified templates were isolated by centrifugation in an Eppendorf microfuge at 4°C for 30 min. The pellets were dried and the DNA resuspended to a concentration of 10 nM in 1 mM Tris-HCl (pH 7.6), and 0.1 mM EDTA. The DNase-treated template was mixed with kinase-labeled single-stranded primer at final concentrations of 8 nM and 20 nM, respectively. The primer: template ratio was crucial. The DNA was denatured by incubating the primer/template mix at 100°C for 2 min, followed by quick freezing in a -50°C ethanol bath. Then 0.2 volumes of reaction buffer (375 mM KCl, 200 mM Tris-HCl at pH 8.0, 75 mM MgCl₂) was added to the reaction. The mix was incubated at 37°C for 15 min to allow annealing. Incubation at 25-37°C was empirically determined to yield the optimal hybridization of various 12-17 nucleotide primers. Following hybridization, the mix was restored to room temperature and supplemented with 2 mM DTT, 0.5 mM of the nucleotides dGTP, dATP, dTTP, and dCTP, and 3 units of AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL). The samples were incubated for 10 min at 22°C, followed by 45 min at 37°C. The reactions were terminated by adding EDTA, tRNA, and sodium acetate to final concentrations at 20 mM, 10 μg/ml, and 0.3 M, respectively. The mix was precipitated with ethanol, dried, and suspended in 5 μl of 95.75% formamide, 10 mM EDTA, 0.125% bromophenol blue, and 0.125% xylene cyanol. The DNA was denatured at 100°C and separated on a 6% polyacrylamide: 8 M urea gel.

pOMSI and pTMSII were incubated with a limited titration of TFIIIA and treated with DNase according to the procedure for the indirect DNase protection assay. The somatic 5S RNA gene

in both pOMS and pTMS constructs was visualized by annealing the M13 17-mer sequencing primer (N.E. Biolabs, Inc., Beverly, MA) which hybridizes 5' to the gene. A commercial primer which anneals 5' to the *EcoRI* restriction sites of pBR322 (and, hence, 5' to the trace oocyte gene) was used to visualize the trace oocyte 5S RNA gene in pTMS. To visualize the major oocyte gene in pOMS, an 18-mer primer was synthesized which anneals to the Xlo 5S RNA gene spacer DNA, 43–25 bp 5' to the coding region. All primers were tested for their ability to hybridize uniquely to the appropriate template.

G sequencing ladders were generated by the chain termination method (Sanger et al. 1977) and separated by electrophoresis adjacent to the indirect footprints. The sequence was generated by annealing the labeled primers to the templates (linearized) using the same ratio as the indirect footprints. The annealing and extension conditions were identical to those described for the indirect footprints except that dideoxyguanosine triphosphate was included at 0.8 μ M and only 15 μ M of the dGTP was added. The extensions were chased with 1 mM of dGTP for 15 min at 37°C.

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