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Structural requirements for the interaction of 5S rRNA with the eukaryotic transcription factor IIIA

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ABSTRACT

In order to study the binding of the eukaryotic transcription factor IIIA to heterologous 5S rRNAs with a low degree of overall sequence conservation (<20%) we have utilized a transcription competition assay involving eubacterial, archaebacterial and eukaryotic 5S rRNAs. All the molecules inhibit Xenopus 5S rRNA transcription specifically, which suggests that only a small amount of specific conserved RNA sequences, if indeed any, are essential for the interaction of the transcription factor with the 5S rRNA molecule, whereas universal 5S rRNA secondary structure elements seem to be required. A fragment of Xenopus laevis oocyte 5S rRNA (nucleotides 41-120), which partially maintains the original 5S rRNA structure, also competes for TF III A. In vitro transcription of a naturally occurring mutant of the Xenopus laevis oocyte 5S rRNA gene, the pseudogene, which carries several point mutations within the TF III A binding domain is equally inhibited by exogenous Xenopus 5S rRNA.

INTRODUCTION

Xenopus 5S rRNA genes are characterized by internal promoter elements (1,2) like other RNA polymerase III genes. At least two separate elements within this internal transcription control region (ICR) have been characterized: "Box A" and "Box B" in the case of tRNA (3) and two homologous blocks for the adenovirus VA I gene (reviewed in 4). Eleven bases in the 5' half of the ICR in 5S rRNA genes were shown to be structurally and functionally homologous to the "Box A" element in tRNA genes, whereas the 3' half of the 5S ICR defines a distinct polymerase III promoter element (4) unique to 5S rRNA genes. Class III genes require multiple cellular factors for transcription by RNA polymerase III. At least two commonly required factors have been characterized (5,6) and a third one (TF III A), which forms a stable complex with isolated 5S DNA in the absence of other factors, is specifically required for 5S rRNA gene transcription (7-9).

The binding region of TF III A on the isolated 5S rRNA genes could be defined by footprinting analysis (7,8) and chemical modification studies (10). Protein-DNA interaction involves the ICR and several 3'- as well as 5'-flanking nucleotides. TF III A binding results in a small degree of unwinding in the DNA (11,12) and appears to require Zn^{++} as a cofactor (13).

In addition to its highly specific DNA binding capacity, TF III A forms a stable complex with 5S rRNA. In the course of Xenopus laevis oogenesis 5S rRNA is accumulated in the form of two distinct ribonucleoprotein (RNP) particles: in association with a single protein as a 7S RNP complex (14) and in association with two different proteins and tRNA as a 42S particle (15,16). The 7S protein is identical to TF III A (7) and, since individual cloned units of Xenopus 5S DNA support accurate transcription of 5S rRNA in crude extracts of Xenopus oocyte nuclei (17), it could be demonstrated that exogenous Xenopus 5S rRNA inhibits 5S gene transcription in vitro (7).

Here, we report on the analysis of the 5S rRNA primary and secondary structure requirements for the binding of TF III A by testing various 5S rRNA species with maximal sequence divergence as well as Xenopus laevis 5S rRNA fragments for their ability to inhibit 5S rRNA gene transcription in vitro.

The DNA structural requirements have been tested by employing a naturally occurring mutant of the Xenopus laevis oocyte 5S rRNA gene, the pseudogene, which lacks 20 nucleotides at the 3'-terminus of the coding sequence and which carries several point mutations within the ICR (18), in the TF III A dependant competition assay system.

MATERIALS AND METHODS

Isolation of 5S rRNA and DNA

5S DNA containing plasmids Xls 11 (provided by Dr. A. Krämer), Xlo B (+) and Xlo Δ 155 (provided by Dr. M. Birnstiel) were prepared according to standard procedures (28). E. coli, Saccharomyces carlsbergensis and Rattus rattus 5S rRNA were isolated from intact ribosomes (29) and Xenopus laevis 5S rRNA from 7S RNP particles (24) as described. Thermoplasma acidophilum and Equisetum arvense 5S rRNAs were kindly provided by Drs.

R.T. Walker and N. Ulbrich, respectively. E. coli $tRNA^{Phe}$ was purchased from Boehringer, Mannheim.

Structural characterization of Xenopus laevis 5S rRNA and 5S fragments

Enzymatic sequencing reactions using 3'- and 5'-labelled RNA were carried out as published (30).Single-strand specific, limited digestions using ribonucleases T_1 (0.1 U, P-L Biochemicals), T_2 (0.05 U, Calbiochem) and A (5ng, Boehringer) were performed in 100µl 50mM Tris-HCl, pH 6.8, 5mM $MgCl_2$, 50mM NaCl with a total amount of 10µg RNA for 30 minutes at 25°C. The reactions were stopped by ethanol precipitation with subsequent identification of the fragments by sequencing gel analysis. The double-strand specific reaction, using CSV RNase isolated from the venom of Naja naja oxiana (provided by D. Kluwe) was carried out under the same conditions. Nuclease S_1 digestion was as described previously (31).

In vitro transcription of plasmid DNA

Xenopus laevis germinal vesicle extracts were prepared and transcription performed as described by Schmidt et al. (32). The extract of 10 germinal vesicles was used for one transcription reaction. The labelled nucleotide was (α-^{32}P)rGTP (400-600Ci/mmol).

Fingerprint analysis of RNAs

RNAs were eluted from gel slices by incubation for 5h at 37°C in 10mM Tris, 500mM NaCl, 1mM EDTA, pH 7.5. The RNA was collected by repeated ethanol precipitation. Transcripts were analyzed by the fingerprinting method of Silberklang et al. (33).

RESULTS

5S rRNA gene and pseudogene transcription in vitro

Double stranded DNAs containing a single unit of the Xenopus laevis somatic 5S rRNA (pXls 11), a single oocyte 5S rRNA repeat unit (pXlo B (+)), containing a 5S rRNA gene and a 5S rRNA pseudogene, and a subclone of pXlo B (+) containing the pseudogene sequence only (pXlo Δ 155) were transcribed in vitro as described (17).

The somatic 5S DNA clone produces one major product (Figure

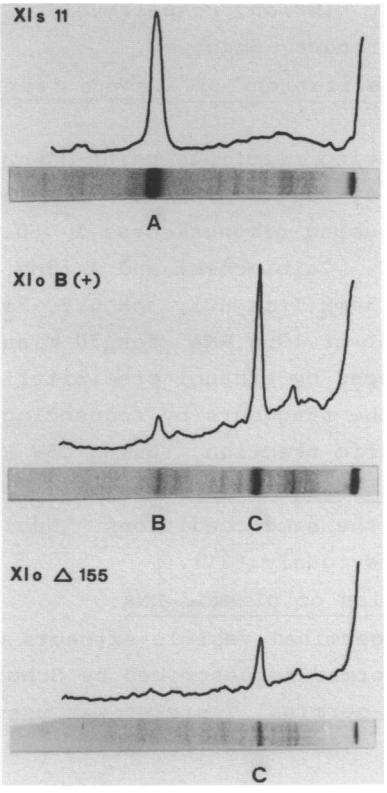

Figure 1: Densitometric and electrophoretic analysis of the major products in in vitro transcription assays with various Xenopus laevis 5S DNA clones using Xenopus nuclear extracts. pXls 11 contains a single copy of the somatic 5S rRNA gene, pXlo B (+) a single oocyte repeat unit, consisting of the oocyte 5S rRNA gene and the 5S rRNA pseudogene, which is a duplication of the first 101 nucleotides of the oocyte 5S rRNA gene, and pXlo Δ 155, which is a deletion mutant of pXlo B (+), containing the pseudogene sequence only. The major products, as revealed by fingerprint analysis (Figure 2) are:
A) somatic 5S rRNA, B) oocyte 5S rRNA and C) the pseudogene transcript.

1 A), which is identical to Xenopus laevis somatic 5S rRNA, as demonstrated by fingerprint analysis (Figure 2 A).

The oocyte 5S DNA repeat unit produces two major products, oocyte 5S rRNA (Figures 1 B and 2 B) and a transcript of about 240 bases (Figures 1 C and 2 C). Fingerprint analysis of the oocyte 5S rRNA reveals the characteristic spots in comparison to somatic 5S rRNA (Figure 2 A and B). The second, longer

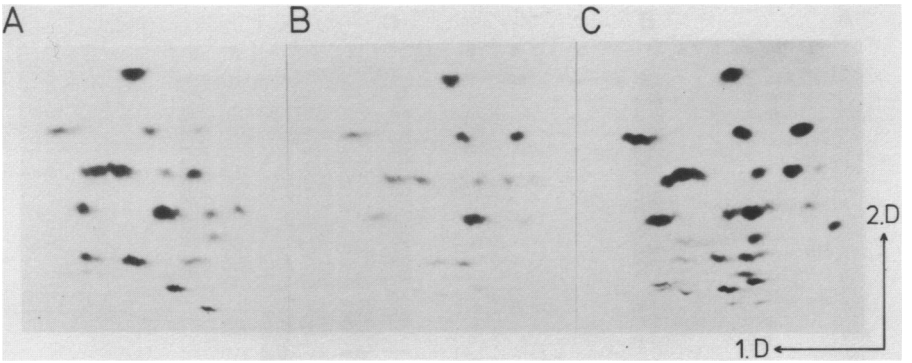

Figure 2: RNase T_1 fingerprint analysis of Xenopus laevis oocyte nuclear extract transcription products. Oligonucleotide separation was on cellulose acetate strips (first dimension) and by homochromatography on PEI thin layer plates (second dimension). Transcription product of pXls 11: Xenopus somatic 5S rRNA (A); transcription products of pXlo B (+): Xenopus oocyte 5S rRNA (B) and oocyte pseudo 5S RNA (C).

product is a transcript originating from the pseudogene promoter and terminating in the vector DNA; it contains all the T_1 oligonucleotides one would expect from the pseudogene sequence (Figure 2 C). This finding is in line with earlier studies on the transcription of 5S RNA pseudogenes using microinjection techniques (19,20).

An estimation of transcriptional efficiency from the densitometric analysis (Figure 1) reveals a twofold dominance of the pseudogene promoter over the authentic one in our in vitro studies.

The plasmid Xlo Δ 155 shows one major product (Figure 1 C), which is identical to the pseudogene transcript, and no detectable oocyte 5S rRNA.

Xenopus laevis somatic 5S rRNA gene transcription is inhibited by heterologous 5S rRNAs in vitro

The presence of increasing amounts of Xenopus laevis oocyte 5S rRNA in in vitro transcription assays of plasmid Xls 11 inhibits the synthesis of 5S rRNA (Figures 3 A and 4 A) whereas only a low level of inhibition was observed even at a high concentration of tRNA (Figures 3 C and 4 A); this observation

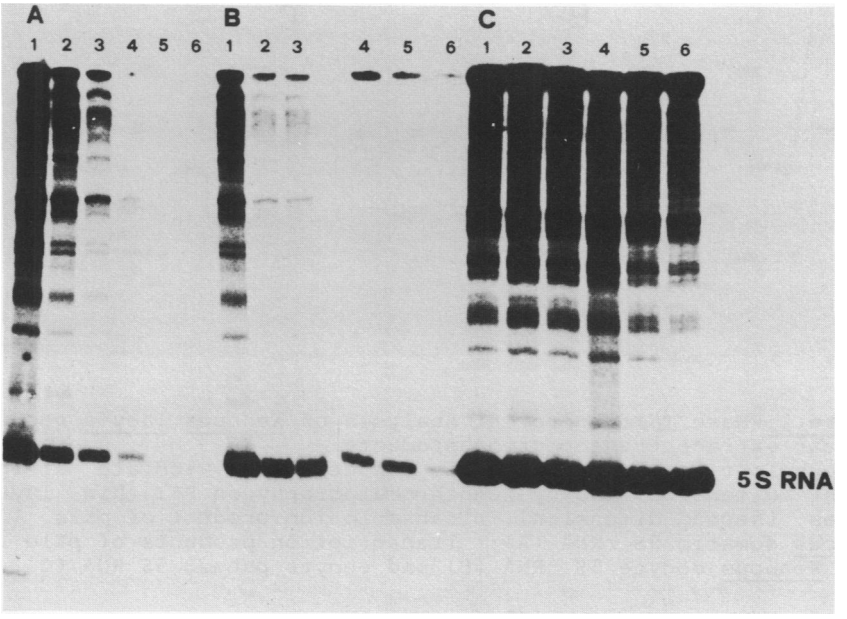

Figure 3: In vitro transcription of pXls 11; A) 1-6: in the presence of increasing amounts of Xenopus laevis oocyte 5S rRNA, B) 1-6: increasing amounts of E. coli 5S rRNA and C) 1-6: increasing amounts of E. coli $tRNA^{Phe}$. The individual amounts of exogenous RNA present in each assay are as shown in Figure 4 A.

is well in line with previous studies (7,21). In control experiments, no end-labelling of the exogenous 5S rRNA in nuclear extracts was observed.

Other 5S rRNAs, such as E. coli 5S rRNA (Figures 3 B and 4 A), Rattus rattus 5S rRNA, Thermoplasma acidophilum 5S rRNA and Equisetum arvense 5S rRNA (Figure 4 B) were also able to inhibit 5S rRNA gene transcription specifically, albeit with differing efficiencies.

In particular, an eighteenfold molar excess of Xenopus laevis 5S rRNA and a twentythreefold molar excess of E. coli 5S rRNA over the 5S plasmid DNA were required to result in a 50% reduction of transcriptional activity in vitro.

A fragment of Xenopus laevis 5S rRNA is capable of inhibiting 5S gene transcription specifically

We have isolated two 5S rRNA fragments, arising from nicks

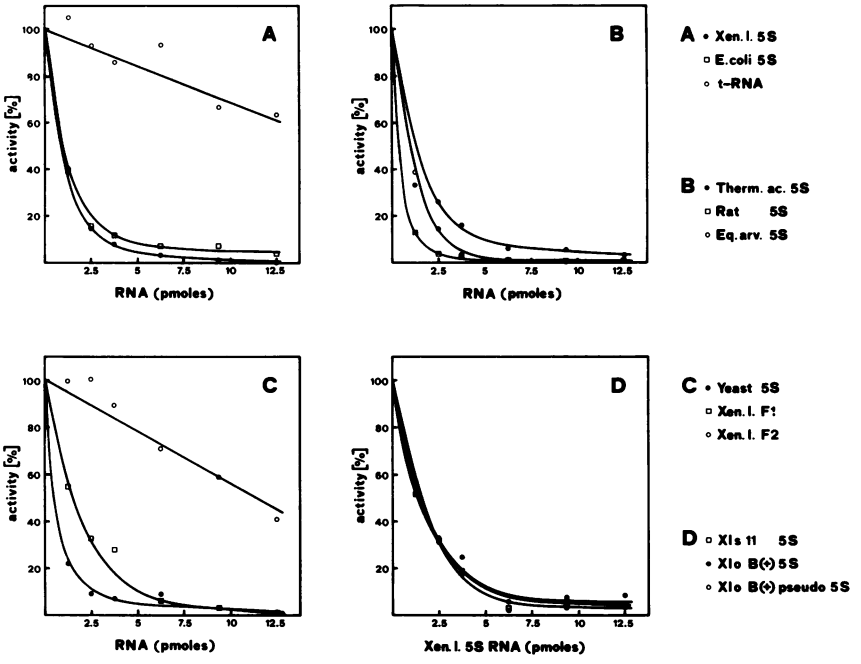

Figure 4: Quantitative analysis of in vitro transcription competition assays. Total amount of Xenopus laevis somatic 5S rRNA produced with pXls 11: A) in the presence of increasing amounts of Xenopus laevis oocyte 5S rRNA (o), E. coli 5S rRNA (□) and E. coli tRNA^{phe} (●), B) increasing amounts of Thermoplasma acidophilum (●). Equisetum arvense (o) and Rattus rattus (□) 5S rRNA, and C) increasing amounts of Saccharomyces carlsbergensis (●) 5S rRNA, Xenopus laevis 5S rRNA fragment F1 (nucleotides 41-120), (□), and fragment F2 (nucleotides 1-40), (o). The quantitative analysis of the major products of in vitro transcription with various Xenopus 5S clones in the presence of increasing amounts of Xenopus laevis 5S rRNA is depicted in D): (□) Xenopus laevis somatic 5S rRNA from pXls 11, (●) Xenopus laevis oocyte 5S rRNA from pXlo B (+) and (o) Xenopus laevis pseudo 5S RNA from pXlo B (+).

at nucleotide positions 40 and 41, which have been identified by sequencing gel analysis. The 3'-fragment (F1), containing nucleotides 40/41-120, specifically inhibits 5S rRNA gene transcription, whereas the complementary 5'-fragment (F2) does not (Figure 4 C). The low degree of inhibition, observed at high concentrations of the smaller RNA fragment, resembles unspecific inhibition, as observed in the case of tRNA (Figure 4 A).

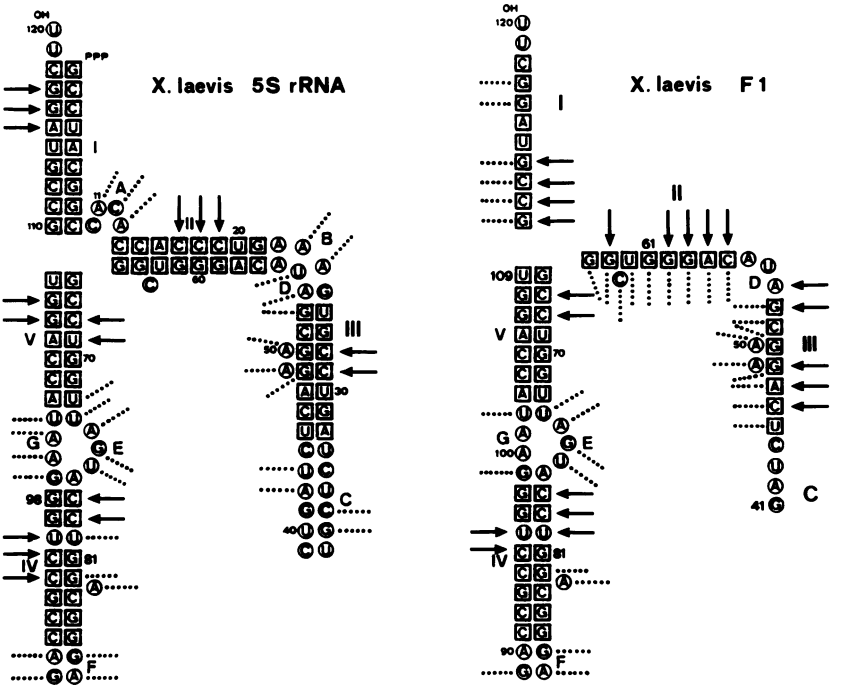

Figure 5: Structural analysis of Xenopus laevis oocyte 5S rRNA and of a fragment from the same molecule (F1). Nucleotides, which were found to be accessible in single-strand specific nuclease digestion reactions using nuclease S_1 and ribonucleases A, T_1 and T_2 are indicated in broken lines, whereas nucleotides hit by the double-strand specific CSV ribonuclease are marked by arrows. Nucleotides, which are thought to be involved in basepairing are enclosed by squares, those in single-stranded regions by circles.

The structure of intact Xenopus laevis oocyte 5S rRNA and of the 3'-fragment have been analyzed by single- and double-strand specific nuclease digestions (Figure 5). The experimental data are in support of the general secondary structure model for eukaryotic 5S rRNA (22).

The 3'-fragment appears to maintain the structure of helices IV and V, as well as of loops E, F and G, whereas the rest of the molecule does not seem to adopt any preferential configuration.

5S rRNA pseudogene transcription *in vitro* is inhibited by 5S rRNA

Inhibition of *Xenopus laevis* oocyte 5S rRNA and pseudo 5S rRNA transcription from plasmid Xlo B (+) in the presence of increasing amounts of *Xenopus laevis* 5S rRNA has been studied in parallel. The inhibitory effect on both genes is identical, as judged from the quantitative analysis (Figure 4 D). Moreover, it is also identical to the behaviour of plasmid Xls 11, analyzed in the same assay.

DISCUSSION

5S rRNA primary and secondary structure requirements for the binding of TF III A

Xenopus laevis 5S rRNA inhibits *in vitro* transcription of cloned *Xenopus* 5S DNA in *Xenopus* oocyte nuclear extracts (7) as does Hela 5S rRNA in Hela cell extracts (21). The use of heterologous 5S rRNA molecules in this transcription competition assay should contribute to the identification of specific RNA sequences required for the interaction with TF III A.

Heterologous eukaryotic (mammal, plant and yeast), eubacterial and archaebacterial 5S rRNAs are, without exception, specific transcriptional inhibitors, albeit with differing efficiencies (Figure 4 A and B). This finding is well in line with RNA exchange experiments on the 7S RNP particle, which demonstrate the possibility of heterologous eukaryotic and eubacterial 5S rRNA binding (Andersen et al., in preparation). On the other hand, a recent report on footprint competition assays, carried out in order to study the binding of TF III A to a variety of single stranded nucleic acids, postulates the inability of *E. coli* 5S rRNA to compete for factor A, whereas heterologous eukaryotic 5S rRNAs exhibit the same inhibition capacities as in our transcription competition assay (23).

This apparent difference may be a consequence of the use of isolated *Xenopus* TF III A in their study. Nuclear extracts might contain additional cofactors which favour RNA/protein interaction and thus stabilize the interaction of *E. coli* 5S rRNA with the transcription factor.

However, the universal finding of specific binding of he-

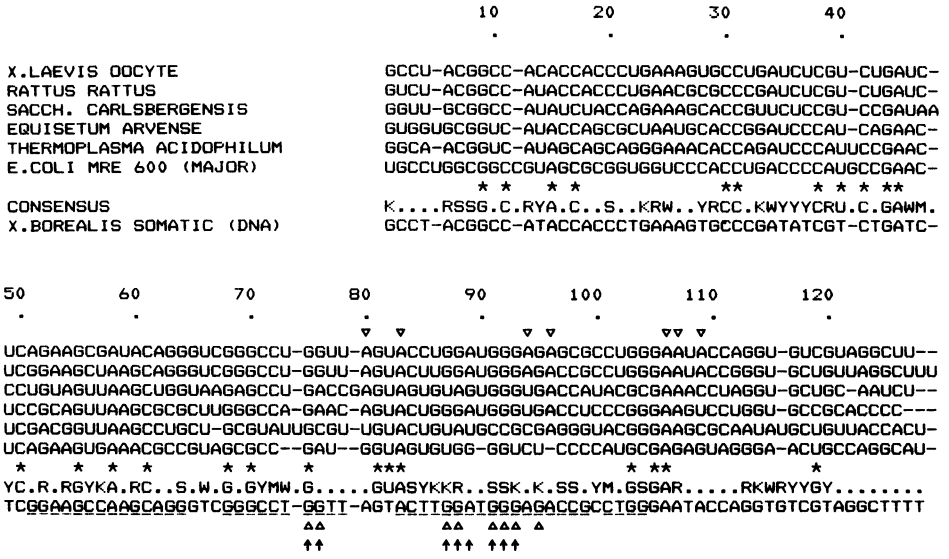

Figure 6: Sequence alignment of Xenopus laevis oocyte 5S rRNA (34), Rattus rattus 5S rRNA (35), Saccharomyces carlsbergensis 5S rRNA (36), Equisetum arvense 5S rRNA (37), Thermoplasma acidophilum 5S rRNA (38), E. coli 5S rRNA (39) and Xenopus borealis 5S DNA (40). The following symbols were used for the consensus sequence: R-purine, Y-pyrimidine, W-A or U, S-G or C, K-G or T, M-A or C. An asterisk (*) stands for absolutely conserved nucleotides.
Open triangles (∇) above the Xenopus laevis oocyte 5S rRNA sequence indicate those adenines, which are protected against chemical modification upon binding of TF III A (24). Open triangles (Δ) underneath the 5S DNA sequence mark guanines and arrows phosphate groups, whose chemical modification interferes with the binding of TF III A (10).The underlined sequence represents protection against DNase I digestion in the presence of the transcription factor (10).

terologous 5S rRNAs to TF III A allows one to ask about the RNA sequence requirements for the specificity of this interaction. Primary structure comparison (Figure 6) reveals a relatively low degree of absolute sequence conservation (<20%), although the secondary structure of these molecules is highly conserved (22).

Our previous chemical modification studies on the 7S RNP particle (24, Figure 6) have established that the structural domain of the Xenopus 5S rRNA comprising helices IV and V as well as loops E, F and G (Figures 5 and 6) is primarily in-

volved in factor A binding. According to the generalized secondary structure of 5S ribosomal RNA (22) the only conserved nucleotide stretches within this domain are located in loops E and G: Pu U A and Pu Pu Pu, respectively. All three adenines located in the corresponding positions of Xenopus laevis 5S rRNA (nucleotides 75-77 and 99-101) are protected by TF III A against diethylpyrocarbonate modification (24) suggesting a function of these particular conserved sequences in the binding of the transcription factor.

Furthermore, a fragment of Xenopus laevis 5S rRNA, containing the intact TF III A binding domain and structurally disturbed 3'- as well as 5'-flanking sequences (Figure 5) specifically inhibits 5S rRNA transcription in vitro (Figure 4 C). Thus, the primary and secondary structural features of helices IV and V and of the adjacent loops appear to be sufficient for recognition by the protein.

On the basis of studies on the interaction of TF III A with 5S DNA, Sakonju and Brown (10) have suggested that a common structural feature between 5S rRNA and the unwound, noncoding strand of the 5S DNA might be recognized by factor A. Their results are depicted in Figure 6, and a first correlation with the data on 5S rRNA binding reveals that the corresponding regions overlap, but that they are not identical to each other. On the basis of this observation, it still appears possible that there is indeed a common structural element in this overlapping region, but there is no experimental evidence for the extensive formation of a RNA-like stem and loop structure in 5S DNA upon binding of TF III A. However, binding was shown to result in: i) a small degree of helix unwinding (11,12) and ii) major effects on the chemical accessibility of the 3'-half of the nonconding 5S DNA strand which is homologous to the 5S rRNA TF III A binding sequence (10). Further experiments, which allow a more direct correlation of DNA- and RNA-binding data, will have to be carried out in order to satisfactorily answer this question.

5S pseudogene trancription is under control of TF III A

The Xenopus laevis 5S RNA gene and pseudogene diverge completey beyond nucleotide 101; furthermore, the pseudogene

carries several point mutations, resulting in a fragmentary 5S rRNA structure with helices I and V completely disrupted and mispairs introduced into the remaining helices (25). Our competition experiments demonstrate (Figure 4 D) that none of these base changes alters the affinity to TF III A, which again argues against the formation of a 5S rRNA-like stem and loop structure in the 5S DNA as a recognition/binding site for the transcription factor. No mutations occur in the conserved sequences of loops E and G, which we suggest serve as RNA recognition signals (as discussed above).

Wormington et al. (26) have shown that the competition strength of the Xenopus laevis oocyte 5S RNA gene and pseudogene with a 5S RNA maxigene is only one fourth of that of the somatic 5S RNA gene, although all three are equally efficient in in vitro transcription assays. Thus, the different competition strengths must be due to factors other than TF III A. Moreover, assembly of transcriptionally active 5S rRNA gene chromatin in vitro reduces the level of pseudogene transcription drastically (27). Again, additional factors, apart from TF III A, appear to play a vital role in the regulation of 5S gene activity in vivo.

Experiments, which make use of a variety of fragments in the transcription competition assay and in vitro mutagenesis studies with the Xenopus 5S rRNA gene are currently under investigation and will allow a more detailed understanding of the 5S DNA/rRNA interactions with TF III A.

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