The EMBO Journal vol.4 no.7 pp.1847-1853, 1985

Point mutational analysis of the *Xenopus laevis* 5S gene promoter

Tomas Pieler, Bernd Appel[1], Shiao Li Oei, Helga Mentzel and Volker A. Erdmann

Institut für Biochemie der Freien Universität Berlin, Thielallee 69-73, and [1]Max-Planck-Institut für Molekulare Genetik Berlin, Ihnestrasse 63-73, D-1000 Berlin 33, FRG

Communicated by V.A. Erdmann

We have introduced C to T transitions into GC and CG base pairs of the *Xenopus laevis* somatic 5S gene coding region and its 5′ flank in order to analyse their effects on transcription activity and regulation. These studies allow us to differentiate between the two promoter elements and their spacer, within the internal control region. Mutations within the 5′ element which dissent from the corresponding tRNA consensus sequence reduce transcription activity substantially without significantly affecting transcription factor (TF) III A binding. Mutations in the spacer region have no pronounced effect on transcription. The 3′ promoter element is found to extend to position 97, since mutations in this region interfere with transcription activity. This may be, at least partially, attributable to a reduced competition strength for TF III A.

Key words: RNA polymerase III promoter/5S gene transcription/transcription factor III A/*Xenopus laevis*

Introduction

Transcription of eukaryotic genes by RNA polymerase III is regulated by promoter elements which are located within the transcribed part of the gene. Analysis of *in vitro* transcription with *Xenopus* 5S rRNA deletion mutants has led to the determination of those 34 nucleotides of the coding region which are essential for proper transcription initiation (Bogenhagen *et al.*, 1980; Sakonju *et al.*, 1980). In the case of tRNA genes, two conserved sequence blocks could be defined as the major constituents of the internal control region (ICR), (Hofstetter *et al.*, 1981; Sharp *et al.*, 1981; Galli *et al.*, 1981), one of which (box A) has its equivalent structure in the 5′ region of the 5S ICR (Ciliberto *et al.*, 1983).

Apart from RNA polymerase III, at least two commonly required factors have been separated (Segall *et al.*, 1980; Shastry *et al.*, 1982); transcription of 5S genes requires one additional, specific factor (TF III A), which binds stably to the ICR in the absence of other factors (Engelke *et al.*, 1980). The same protein appears to play a crucial role in the developmental regulation of 5S gene expression (Brown, 1984), most likely by preventing histone H1-mediated formation of stably repressed chromatin structures (Schlissel and Brown, 1984; Gargiulo *et al.*, 1984).

Studies using deletion mutants of the *Xenopus* 5S gene in transcription competition assays (Wormington *et al.*, 1981) have revealed important changes in promoter strength. However, it is problematical, if not impossible, to attribute these observations to a loss of sequence alone, since an inevitable consequence of these deletions was the juxtaposition of different DNA elements, which might by chance have a regulatory effect themselves. One prominent example of a negative regulatory effect on tRNA transcription by flanking DNA elements has been described by Hipskind and Clarkson (1983). Wormington *et al.* (1981) could also demonstrate that just a few nucleotide changes result in a 4-fold difference in competition strength for TF III A, which was one of the crucial observations in the understanding of the mechanism which probably underlies the differential regulation of the oocyte and somatic 5S gene families in the course of *Xenopus* development (Brown, 1984).

To obtain a more detailed insight into the sequence requirements for the promotion of 5S gene transcription, and to complement the above experiments, we have performed a detailed analysis of the effect of point mutations on the promoter strength of the *X. laevis* somatic 5S gene.

Results

Mutagenesis of the X. laevis somatic 5S gene

About 300 individual clones from several bisulfite modification experiments on the 200 nucleotide long deletion mutant of the *X. laevis* somatic gene, cloned in both orientations into the appropriate M13 vectors (as detailed in Materials and methods) were raised and sequenced. Out of the 71 G and C residues in the 5S coding sequence, 35 were mutated resulting in A to G or C to T transitions. Mutations occur in a non-random manner, probably due to the formation of stable secondary structure elements in the single-stranded DNA. Similar observations have been described in the bisulfite study of a *Xenopus* tRNA gene (Folk and Hofstetter, 1983).

Table I. Analysis of mutant transcription

Mutant	Mutated position		Relative	Relative stability
	intragenic	extragenic	activity (%)	($t_{1/2}$M 5S/$t_{1/2}$WT 5S)[a]
GA51	/	−26	100	ND
GA28	/	−23,−4	100	ND
GA10	/	−21,−15	100	ND
CT49	12	/	100	1
CT23	17	/	100	ND
GA73	27	/	100	1.3
GA34	37	/	70	0.4
GA80	51	−21	10	0.9
CT11	52	/	100	0.8
GA53	56	/	50	1
GA78	60	/	30	0.4
GA91	61	/	30	0.4
GA40	65	/	80	1
GA20	66	/	80	ND
GA84	87	/	10	0.8
GA94	89	−4	20	ND
CT18	92	/	50	0.7
GA35	97	/	50	0.7
GA66	113	/	20	ND
GA60	117	/	20	ND

[a]M = Mutant, WT = wild-type, ND = not determined

Table II. Analysis of mutant transcription

Mutant	Mutated position		Relative activity
	intragenic	extragenic	(%)
GA2	21,25	−11	100
CT57	39,118	/	100
CT58	46,53	/	100
GA49	37,59	/	30
GA41	27,37,64,66	/	80
GA57	56,65,66,70	/	10
GA18	48,82	−24	30
GA33	21,82,89	/	10
GA55	37,51,86	/	10
CT37	78,92	−10	40
CT67	46,78,92	/	60
GA85	65,98	/	80
GA61	56,116	−23,−24	10

It is our view that there are major advantages as a consequence of the experimental approach used in this study compared with the oligonucleotide directed method: (i) one obtains a large number of mutants covering the entire gene in a single experiment and (ii) more importantly, the target nucleotides have not been predetermined by the experimenters' expectations, which may lead to rather unexpected findings (as will be demonstrated below). However, on the other hand, there are also some major disadvantages; mutagenesis is restricted to G and C residues and it results in transitions, never transversions.

Analysis of the transcription activities of mutated 5S genes

All the mutants listed in Tables I and II were transcribed *in vitro* in the presence of a 2-fold molar excess of M13mp1o X1oB as an internal, competing standard. This assay was chosen, since it is independent of variations in the total polymerase III activity of the biological material employed and, furthermore, since the oocyte 5S DNA competes for all the factors required for somatic

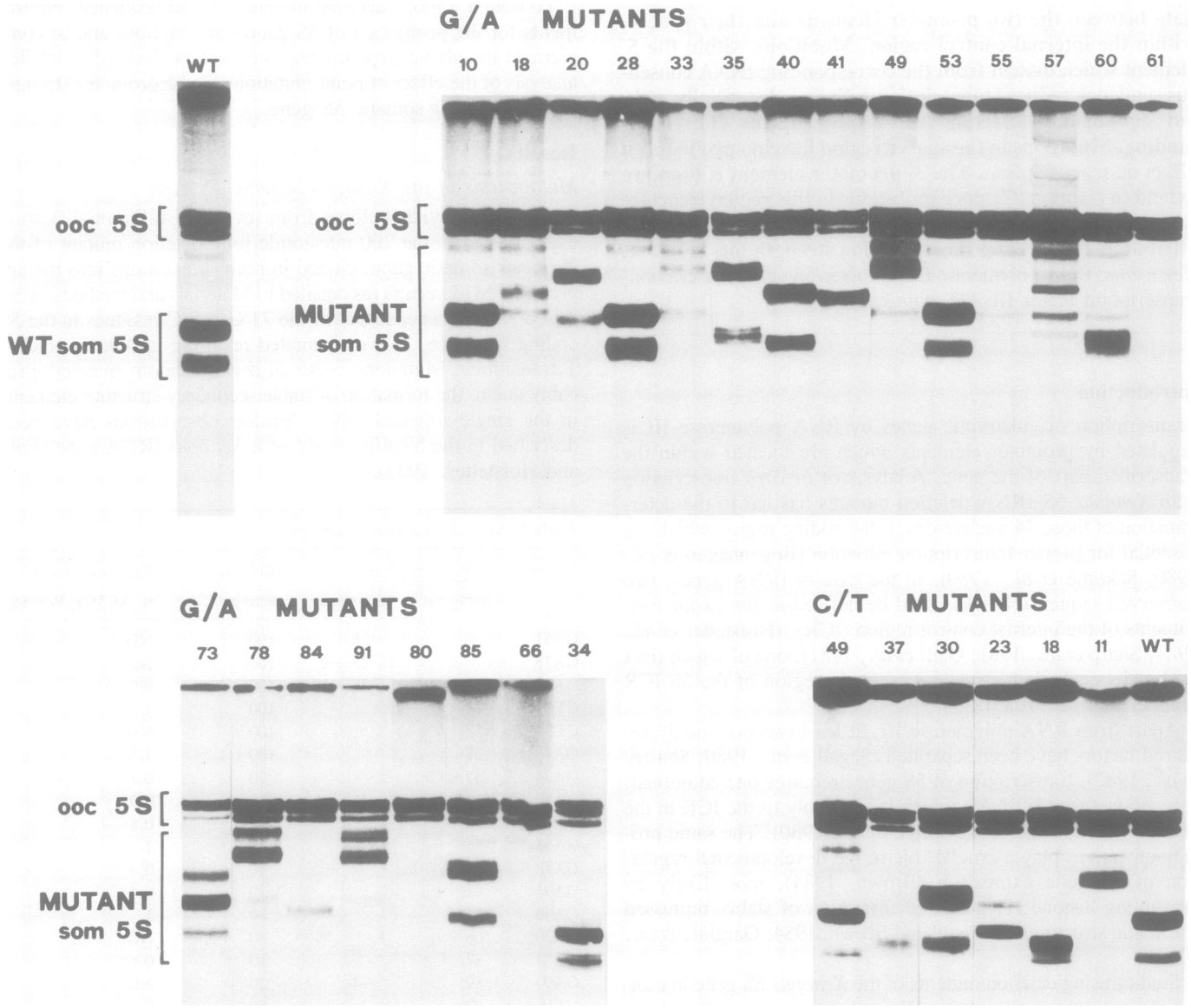

Fig. 1. Analysis of mutant transcription. Wild-type or mutant somatic 5S DNA (2.5 μg/ml) was co-transcribed with oocyte 5S DNA (5 μg/ml) which served as an internal standard. A quantitative estimate as well as mutant characteristics are listed in Tables I and II.

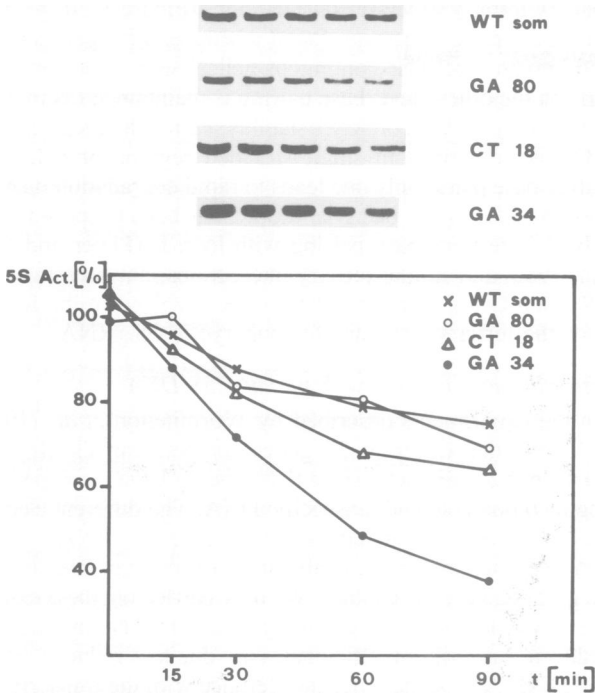

Fig. 2. Analysis of transcript stability. Wild-type and mutant 5S rRNAs were transcribed *in vitro* and re-incubated in *Xenopus* nuclear extracts (as detailed in Materials and methods). Several examples for the kinetics of degradation are shown (the complete data are listed in Table I).

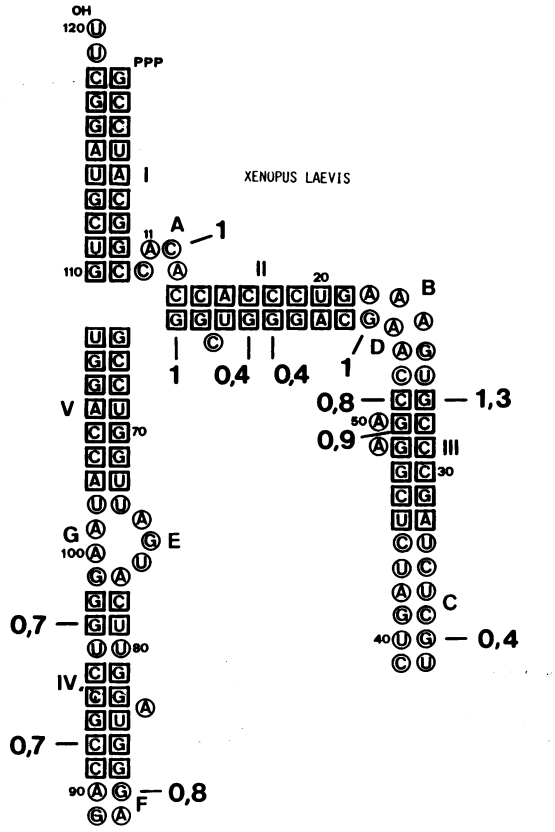

Fig. 3. Correlation of mutant RNA stability with the secondary structure of 5S rRNA. Altered relative stabilities as a consequence of single base transitions are indicated; the relative stability has been defined as $t_{1/2}$ mutant 5S rRNA/$t_{1/2}$ wild-type 5S rRNA.

5S DNA transcription, modulations in mutant transcription efficiencies will be amplified. Oocyte and somatic-type 5S transcripts were separated on 15% polyacrylamide gels as described by Wakefield and Gurdon (1983). Oocyte 5S transcripts are resolved into three distinct bands (Figure 1) probably due to their 3′-terminal length heterogeneity described earlier (Ford and Southern, 1973). Somatic-type 5S rRNA is often observed to split into two groups of bands, which is not the case under standard denaturing conditions (data not shown) and is the result of conformational variations.

Mutant 5S transcripts exhibit two striking characteristics; as a consequence of the introduction of mispairs in helices, or of sequence alterations in structurally important regions of the RNA molecule, their altered secondary/tertiary structure leads to a differing electrophoretic mobility for many of the mutated 5S rRNAs (Figure 1, see also Figure 3). In addition, the intensities of their transcription signals relative to the one observed for wild-type somatic 5S DNA varies substantially (Tables I and II). Every mutant has been analysed in at least two and maximally five independent assays. The sequences of most of the selected mutants have been reconfirmed by a second, independent round of sequence analysis and in some cases (GA60, GA61 and GA66) experiments were repeated with a second, independent preparation of double-stranded phage DNA in order to confirm the rather unexpected results.

For those DNAs, which have only one nucleotide altered, a change in the intensity of the transcription signal may be directly attributed to the particular mutation. For the other DNAs, which have more than one mutated position within the coding or 5′-flanking sequence, we have tried to identify the nucleotide(s) responsible by comparing them with mutants which have only one base altered. While this may be justified for comparison of mutations which leave the transcription signal unaffected, the others, in particular those 5S DNAs with more than two mutations (Table II), can provide only supporting, albeit interesting, arguments.

Stability of transcripts

Since it was clear from the transcription analysis that many of the mutations result in a structurally disturbed transcript, it was important to establish whether the reduced transcription signal of several mutants could be due to a reduced rate of transcription, a higher rate of degradation or both. For this purpose, we transcribed wild-type or mutant somatic 5S DNA, extracted the 5S rRNA from denaturing polyacrylamide gels and incubated these RNAs again in nuclear extracts under transcription conditions but in the absence of radioactive label. At various times the RNA was extracted, analysed by gel electrophoresis and the 5S signal quantified (Figure 2).

It is obvious from these experiments that transcript stability varies substantially. Whereas some are as stable as the wild-type 5S rRNA, other mutant RNAs are much more readily degraded. We have calculated the half-lives of several mutant and wild-type 5S rRNAs from these data; their relative stabilities were estimated as $t_{1/2}$WT 5S/$t_{1/2}$M 5S (Table I). Three of 12 mutants tested (GA34, GA78 and GA91) exhibit a significantly reduced stability (0,4) and for one of them (GA34) this instability can partially or even completely account for the weakened transcription signal (70%). For the other two and additional mutants (CT18 and GA35) the rapid degradation will contribute significantly to the reduced transcription signal.

These findings also have interesting implications for 5S rRNA

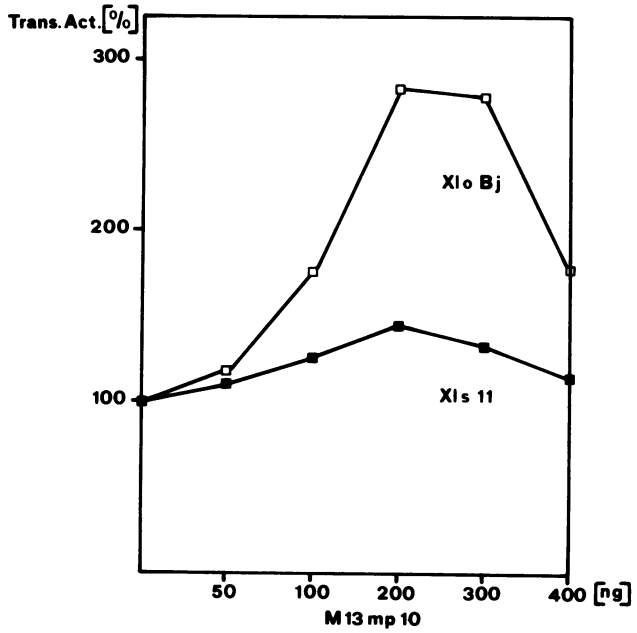

Fig. 4. Competition of *Xenopus* 5S transcription with unspecific vector DNA. *X. laevis* somatic 5S DNA (Xls11) and *X. laevis* oocyte 5S DNA (XloB) were transcribed in the presence of increasing amounts of M13mp10; the 5S DNA concentration was 5 μg/ml.

secondary/tertiary structure considerations (Figure 3). In general, the most dramatic effects are found whenever a mispair is introduced into an otherwise continuous helical segment (as in helix II). If, on the other hand, base pairing is maintained (as in helix IV), mutational effects on RNA stability are much less pronounced. Of the mutations in single-stranded regions or helix terminating base pairs, only one leads to rapid degradation (GA34, nucleotide 37). The eubacterial loop C has been proposed to be involved in tertiary base pairing with loop E (Pieler and Erdmann, 1982) and similar tertiary interactions, not necessarily of the Watson/Crick type, are likely to be involved in the formation of the tertiary structure for eukaryotic 5S rRNA.

Competition experiments with mutated 5S DNA

From the experiments described by Wormington *et al.* (1981) it is known that different 5S genes compete for TF III A in *Xenopus* nuclear extracts, since tRNA genes have a competition strength comparable with unspecific DNA. The different electrophoretic mobilities of mutant and wild-type 5S rRNAs, under the appropriate conditions, enable us to examine competition of these genes against one another. We have carried out these experiments with the aim of determining whether mutations leading to reduced transcription efficiencies are due to a reduced affinity for TF III A or some other interference with the transcription dynamics.

Competition experiments were carried out essentially as des-

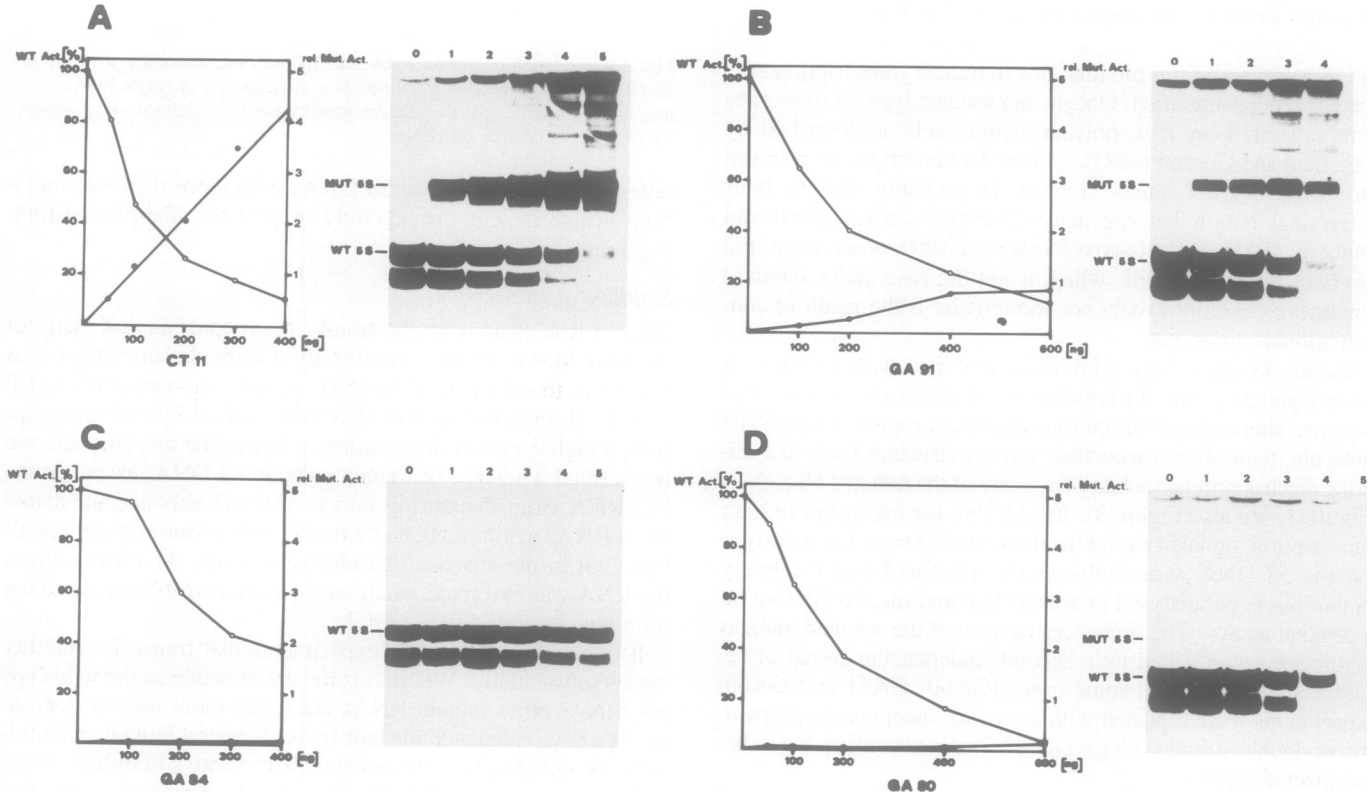

Fig. 5. Competition of wild-type somatic 5S DNA (5 μg/ml) with increasing amounts of several mutant somatic 5S DNAs. Open circles indicate the decreasing activity of wild-type somatic 5S transcription, filled circles the relative amount of mutant 5S transcription (mutant 5S rRNA activity/wild-type 5S rRNA activity). **Panel A** is mutant CT11 (C52 to T52), **panel B** mutant GA91 (G61 to A61), **panel C** mutant GA84 (G87 to A87) and **panel D** mutant GA80 (G51 to A51). Mutant DNA concentrations in the individual assays illustrated by the autoradiographs were in **A** and **C: (0)**, 0 ng, **(1)** 50 ng, **(2)** 100 ng, **(3)** 200 ng, **(4)** 300 ng and **(5)** 400 ng; in **B: (0)** 0 ng, **(1)** 100 ng, **(2)** 200 ng, **(3)** 400 ng and **(4)** 600 ng; in **D: (0)** 0 ng, **(1)** 50 ng, **(2)** 100 ng, **(3)** 200 ng, **(4)** 400 ng and **(5)** 600 ng.

Tabel III. Amount of mutant 5S DNA required to compete 50% of 100 ng wild-type somatic 5S DNA transcription

Mutant	Mutated position	50% Competition with (ng)
GA80	51,−21	150
CT11	52	100
GA91	61	160
GA84	87	260
GA94	89,−4	230
GA35	97	120

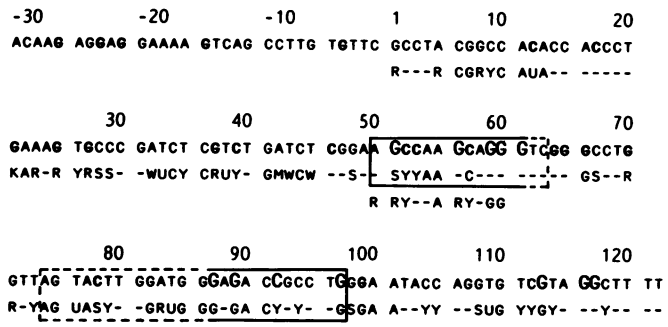

Fig. 6. Alignment of the *X. laevis* 5S DNA sequence with the eukaryotic 5S rRNA consensus sequence (Erdmann *et al.*, 1984) and the consensus structure of the tRNA box A element (nucleotides 50−60 in the 5S DNA sequence) (Ciliberto *et al.*, 1983). Bold letters indicate all C and G residues which have been mutated and large, bold letters those which, when substituted, interfere with the transcription activity. The spans of the two internal promoter elements are indicated (as detailed in the Discussion).

cribed by Wormington *et al.* (1981). Since we have used a different vector system, we found it necessary to analyse unspecific competition with the vector molecule alone (Figure 4). Oocyte and somatic 5S DNA were competed with M13mp8 double-stranded DNA and it turned out that there is no competition effect but, instead, an unspecific stimulation of transcription activity in the range of DNA concentrations employed. This effect is most pronounced for ooctye 5S DNA (up to 2.8-fold) but detectable also for somatic 5S DNA (up to 1.4-fold). Stimulation of 5S transcription by unspecific DNA has also been reported by Gargiulo *et al.* (1984). These effects have been taken into consideration in the calculation of the amount of uncompeted 5S transcription in the competition of wild-type somatic 5S DNA with the mutated genes (Figure 5).

Mutant CT11 was found to be unaltered in its transcription efficiency (Table I) although it exhibited a drastically different electrophoretic mobility. When employed in the competition experiment with wild-type somatic 5S DNA (Figure 5A), it competes effectively for TF III A, as anticipated. As one would expect for genes with equal competition strengths, the wild-type signal is reduced to 50% when equimolar amounts of the two plasmids are used. In perfect agreement with the theoretical expectations, there is a linear correlation between the amount of mutant DNA employed and the ratio of wild-type to mutant signal, which equals one when equimolar amounts of template DNA are used.

The transcription signal of mutant GA91 is reduced, but nevertheless measurable (Figure 5B). This DNA still competes for TF III A, albeit with a slightly reduced strength (Table III) but here a >6-fold molar excess of mutant over wild-type somatic 5S DNA is required to produce a signal of equal strength. Similar results (data not shown) were obtained for mutant GA35 (Table III).

Mutant GA80 has a barely measurable signal (Figure 5D) but, as before, competition strength is only slightly reduced. The most pronounced examples of weak competitors are provided by mutants GA84 (Figure 5C) and GA94 (Table III). The reduced affinity of these two 5S DNA molecules, which can be attributed to mutations in position 87 and 89 of the coding region, will contribute significantly to the nearly complete loss of transcription signal.

Discussion

There are several important and elegant studies on *Xenopus* 5S promoter characterisation relevant to our data. The 3′ and 5′ borders of the internal control region (nucleotides 50 and 83, respectively) have been determined by *in vitro* transcription experiments using deletion mutants of the *Xenopus* 5S gene (Bogenhagen *et al.*, 1980; Sakonju *et al.*, 1980). The ICR can be split into two components, one of which is structurally and functionally equivalent to the first element of the bipartite eukaryotic tRNA promoter (Ciliberto *et al.*, 1983).

In agreement with these studies, we did not detect any influence on transcription activities exhibited as a consequence of mutations upstream from nucleotide 50. The modulation of competition strength as observed for deletions in the 5′-flanking region (Wormington *et al.*, 1981) could not be attributed to any of the mutations we have produced in this part of the gene.

The 5′ promoter element

Mutations within the region homologous to the tRNA box A element which coincides exactly with the first 11 nucleotides of the ICR 5′ border, lead to a drastic decrease of transcription efficiency whenever they deviate from the consensus sequence for tRNA box A, even if the corresponding positions are not conserved in the consensus sequence for eukaryotic 5S rRNA (Figure 6). This observation argues in favour of the hypothesis that the concept of transcription by RNA polymerase III, under the direction of an internal promoter, is valid only in higher eukayrotes (Ciliberto *et al.*, 1983).

As revealed by the competition experiments (Table III), TF III A binding is not substantially influenced by these mutations. Although protection against DNase I digestion spans nucleotides 43−98 of the coding region (Engelke *et al.*, 1980; Sakonju and Brown, 1982), the main contact points of the protein with the 5S gene have been found to be located in the extreme 3′ region of the DNase I-protected part of the gene (Sakonju and Brown, 1982). In addition, deletion mutants extending beyond the 5′ border of the ICR (up to position +74) exhibit partial protection of the remainder of the control region against DNase I digestion in the presence of TF III A (Sakonju *et al.*, 1981). In conjunction with our observations, these data allow the conclusion that the box A element is not essential for the specific recognition of the 5S gene by TF III A. It is likely that the 3′ element serves as a specific binding site for one (or more) of the other common factors involved in the transcription of class III genes.

The ICR spacer element

Those nucleotides 3′-flanking to the tRNA box A equivalent may be viewed as the spacer element in the bipartite promoter. The mutant transcription data allow its 5′ border to be determined as either nucleotide 62 or 63, since several nucleotide substitutions in these positions do not affect transcription significantly. Deletion of nucleotides 72 and 73, or insertion of a synthetic linker in these positions does not result in the loss of transcrip-

tion activity (Ciliberto *et al.*, 1983) and specific binding of TF III A is still observed after deletion of the first 74 nucleotides of the coding region.

This leads us to conclude that the 3′ border of the ICR spacer element must be located beyond position 73. Only oligonucleotide-directed mutagenesis (which we are currently undertaking) will allow a more precise definition, since bisulfite mutagenesis provided us with only very few mutations in this particular region of the gene.

The 3′ promoter element

More profound information on the extent of the second promoter element within the ICR becomes available from our mutant studies. Transitions in positions 87 and 89 lead to a dramatic reduction in transcription activities as well as the most pronounced reduction in competition strength for TF III A. This was surprising since none of these nucleotides was thought to be essential for the initiation of 5S transcription, as shown again by deletion mutagenesis (Bogenhagen *et al.*, 1980), or required for TF III A binding (Sakonju *et al.*, 1981). However, they are located within the protected part of the gene from footprinting with DNase I (Engelke *et al.*, 1980) and the same nucleotides have been shown to be among those which constitute the primary contact sites for TF III A (Sakonju and Brown, 1982). In addition, 3′ deletions beyond nucleotide 97 lead to a dramatic decrease in competition strength (Wormington *et al.*, 1981). Additional evidence comes from a recent report on the isolation of an inactive *Drosophila* 5S gene (Sharp *et al.*, 1984), which differs from its active counterpart in only a single position (G to A transition of nucleotide 86).

We are left with the paradox that two different, independent single site mutations within a region which is not essential for 5S transcription result in a near to complete loss of transcription activity, coupled with a significant reduction in competition strength. It is thus our opinion that the span of the 5S ICR should be viewed as extending beyond position 83 and including nucleotides 84 – 97.

Other mutations within the DNase I-protected region (C92, G97) have less pronounced, but nevertheless clearly negative effects on transcription.

The most surprising effect was observed with mutations in the extreme 3′ region of the 5S gene. These mutations are located outside the TF III A-protected region and inside a portion of the gene which can be deleted without any detectable effect on competition strength (Wormington *et al.*, 1981), indicating that they will probably have no influence on the initiation of 5S transcription, but might interfere with either the termination (Bogenhagen and Brown, 1981) or the release of the transcript from the template DNA. Experiments which address these questions are currently being carried out.

Materials and methods

Enzymes and substrates

Restriction enzymes, nuclease *Bal*31, DNA polymerase (large fragment) and T4 DNA ligase were purchased from Boehringer, Mannheim. rNTPs, dNTPs and ddNTPs were from Pharmacia P-L Biochemicals, the M13 universal primer (17-mer) as well as [α-^{35}S]dATP and [α-^{32}P]GTP were from Amersham. Readysolve scintillation cocktail was from Beckman.

Plasmid constructions

The pXls11 (Peterson *et al.*, 1980) subclones M13mp10 Xls 560-764 and M13mp11 Xls 560-764, containing the entire 5S coding region and 46 and 36 nucleotides of the 5′- and 3′-flanking regions, respectively, were constructed using *Bal*31 resection (Maniatis *et al.*, 1982).

Deletions in the 3′-flanking region were started from the unique *Eco*RV site in pXls11 with exonuclease *Bal*31; the gene-containing portion of the resected DNA molecule was liberated from the vector by *Hind*III endonuclease and cloned directly into *Hind*III/*Sma*I-treated M13mp8. The extent of deletions in individual clones was mapped physically and determined exactly by dideoxy sequencing (Sanger *et al.*, 1977). Subclone M13mp8 Xls 764 was then used for the deletion of 5′-flanking sequences; the 5S DNA cleaved with *Ava*II (at positions 276 and 409) was subjected to *Bal*31 resection and subsequently cleaved at the unique *Sst*I site in the polylinker region. The resected genes were cloned directly into *Sma*I/*Sst*I-cut M13mp10. The extent of the deletions was determined as described above. The orientation of the gene in deletion mutant M13mp10 Xls 560-764 was reversed by cloning it directly into *Hind*III/*Eco*RI-treated M13mp11. The plasmid M13mp10 Xlo B was obtained by cloning the oocyte 5S transcription unit from pXlo B(+) into *Hind*III-treated M13mp10.

Chemical mutagenesis

The strategy used for mutagenesis was similar to that used by Folk and Hofstetter (1983), employing chemical modification of cytidines with sodium bisulfite (Shortle and Nathans, 1978).

Single-stranded phage DNA was prepared from the two deletion mutants described in the preceding paragraph (Messing, 1983) and, in order to protect phage DNA sequences from bisulfite modification, annealed to a 3- to 5-fold molar excess of *Hind*III/*Eco*RI-treated M13mp10 double-stranded DNA by boiling for 3 min in 0.2 M $NaPO_4$ (pH 6.8), followed by slow cooling. The sample was then treated with sodium bisulfite as described (Shortle and Nathans, 1978) for 20 min at 37°C. Termination of the reaction was by immediately loading the sample onto a small column filled with Sephadex G50 in 10 mM Tris (pH 7.6), 1 mM EDTA, 100 mM NaCl. Fractions containing DNA (monitored directly at 260 nm) were incubated with 0.1 M Tris base at 37°C for 16 h. The DNA was then precipitated with ethanol, the gapped region filled using DNA polymerase (large fragment) (Maniatis *et al.*, 1982) and transfected into competent *Escherichia coli* JM 103 (Messing, 1983). Single, colourless plaques were picked and subjeted directly to dideoxy sequencing (Sanger *et al.*, 1977).

In vitro transcription and analysis of mutant 5S genes

Double-stranded phage DNA was isolated by caesium chloride density gradient centrifugation according to standard procedures (Maniatis *et al.*, 1982). *In vitro* transcription assays with *X. laevis* nuclear extracts were performed as described by Birkenmeier *et al.* (1978). Separation of oocyte and somatic-type 5S rRNA was achieved using electrophoresis conditions introduced by Wakefield and Gurdon (1983). In order to quantify the transcription products, radioactive bands were excised from the gel and the radioactivity measured in a Beckman scintillation counter.

The stability of wild-type and mutant 5S rRNAs was analysed by incubation of the radioactive RNA under transcription conditions for increasing time intervals, followed by phenol extraction, gel electrophoresis under denaturing conditions and quantification of intact 5S rRNA as described above.

Transcription competition assays were carried out essentially as published (Wormington *et al.*, 1981).

Acknowledgements

We would like to thank Dr. A. Krämer and Professor M. Birnstiel for providing recombinant DNA molecules, Dr. M. Digweed for critical reading of the manuscript and Mrs. A. Schreiber for the careful preparation of the figures. This work was supported by funds of the Deutsche Forschungsgemeinschaft (SFB 9/B5) and the Fonds der Chemischen Industrie e.V.

References

Birkenmeier,E.H., Brown,D.D. and Jordan,E. (1978) *Cell*, **15**, 1077-1086.
Bogenhagen,D.F. and Brown,D.D. (1981) *Cell*, **24**, 261-270.
Bogenhagen,D.F., Sakonju,S. and Brown,D.D. (1980) *Cell*, **19**, 27-35.
Brown,D.D. (1984) *Cell*, **37**, 359-365.
Ciliberto,G., Raugei,G., Costanzo,F., Dente,L. and Cortese,R. (1983) *Cell*, **32**, 725-733.
Engelke,D.R., Ng,S.-Y., Shastry,B.S. and Roeder,R.G. (1980) *Cell*, **19**, 717-728.
Erdmann,V.A., Wolters,J., Huysmans,E., Vandenberghe,A. and DeWachter,R. (1984) *Nucleic Acids Res.*, **12**, r133-r166.
Folk,W.R. and Hofstetter,H. (1983) *Cell*, **33**, 585-593.
Ford,P.J. and Southern,E.M. (1973) *Nature*, **241**, 7-12.
Galli,G., Hofstetter,H. and Birnstiel,M.L. (1982) *Nature*, **294**, 626-630.
Gargiulo,G., Razvi,F. and Worcel,A. (1984) *Cell*, **38**, 511-521.
Hipskind,R.A. and Clarkson,S.G. (1983) *Cell*, **34**, 881-890.
Hofstetter,H., Kressmann,A. and Birnsteil,M.L. (1981) *Cell*, **24**, 573-583.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
Messing,J. (1983) *Methods Enzymol.*, **101**, 20-78.
Peterson,R.C., Doering,J.L. and Brown,D.D. (1980) *Cell*, **20**, 131-141.
Pieler,T. and Erdmann,V.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4599-4603.
Sakonju,S. and Brown,D.D. (1982) *Cell*, **31**, 395-405.
Sakonju,S., Bogenhagen,D.F. and Brown,D.D. (1980) *Cell*, **19**, 13-25.

Sakonju,S., Brown,D.D., Engelke,D., Ng,S.-Y., Shastry,B.S. and Roeder,R.G. (1981) *Cell*, **23**, 665-669.
Sanger,F., Nickler,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
Schlissel,M.S. and Brown,D.D. (1984) *Cell*, **37**, 903-913.
Segall,J., Matsui,T. and Roeder,R.G. (1980) *J. Biol. Chem.*, **255**, 11986-11991.
Sharp,S., DeFranco,D., Dingermann,T., Farrell,P. and Söll,D. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6657-6661.
Sharp,S., Garcia,A., Cooley,L. and Söll,D. (1984) *Nucleic Acids Res.*, **12**, 7617-7623.
Shastry,B.S., Ng.S.-Y. and Roeder,R.G. (1982) *J. Biol. Chem.*, **257**, 12979-12986.
Shortle,D. and Nathans,D. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2170-2174.
Wakefield,L. and Gurdon,J.B. (1983) *EMBO J.*, **2**, 1613-1619.
Wormington,W.M., Bogenhagen,D.F., Jordan,E. and Brown,D.D. (1981) *Cell*, **24**, 809-817.

Received on 3 May 1985