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A split binding site for TFIIIC on the *Xenopus* 5S gene

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We have previously shown that of the two functional domains which constitute the *Xenopus* 5S gene promoter the common, conserved box A element is directly involved in the binding of the common transcription factor IIIC. Here, we describe the investigation of the role of the 5S gene specific promoter element, box C, in transcription factor binding. Analysis of 22 different single site basepair changes reveals that mutations created within the 5'-region of this segment interfere with transcription due to a reduced affinity for TFIIIA, whereas sequence alterations introduced into the 3'-region of the same element similarly inhibit transcription, but do not result in a measurable defect in TFIIIA binding. Instead, they clearly reduce the affinity for TFIIIC. DNase I protection experiments with TFIIIA on 5S mutants which have an increased spacing of box A and box C demonstrate that TFIIIA recognizes a specific box A sequence element and that the factor has to be properly aligned on the DNA template in order to allow stable complex formation with TFIIIC to proceed. The structural and functional organization of protein binding signals on the 5S gene internal control region will be discussed in regard to these results.

Key words: 5S gene/*Xenopus*/transcription/transcription factor binding/TFIIIC

Introduction

The initiation of transcription by RNA polymerase III occurs on preassembled complexes of DNA and specific transcription factors. Chromatographic separation of crude cellular extracts allowed at least three different protein fractions which are involved in the transcription of class III genes (Segall *et al.*, 1980) to be distinguished. One of these, TFIIIA, is specifically required for 5S gene expression (Honda and Roeder, 1980; Pelham and Brown, 1980) and it binds to the internal control region of the 5S gene in a sequence specific way and independent of other proteins (Engelke *et al.*, 1980). The other two activities, TFIIIB and TFIIIC, are involved in the formation of the stable preinitiation complex on 5S genes and other class III genes (Lassar *et al.*, 1983; Setzer and Brown, 1985; Bieker *et al.*, 1985).

TFIIIC binding to tRNA and VA genes has been characterized by DNase I protection experiments in a number of systems (Fuhrman *et al.*, 1984; Stillman and Geiduschek, 1984; Camier *et al.*, 1985; Carey *et al.*, 1986; Van Dyke and Roeder, 1987). Of the two conserved promoter elements in the coding region of these genes, the 3'-element (box B) appears to dominate over the 5'-element (box A) as binding determinant, although both sequences are clearly involved in the interaction with the factor (Stillman *et al.*, 1985; Baker *et al.*, 1986; Camier *et al.*, 1985).

Attempts to analyze TFIIIC interaction with the 5S gene, which requires prior binding of TFIIIA to the DNA, by DNase I protection have been unsuccessful; the footprint produced in the presence of the TFIIIC activity, stably sequestered together with TFIIIA, is indistinguishable from the footprint obtained with TFIIIA alone (Carey *et al.*, 1986). To circumvent this problem, we have previously established a functional assay for TFIIIC activity, which allows one to measure the specific affinity of a given 5S DNA template for TFIIIC quantitatively (Pieler *et al.*, 1987).

The fine structure of the *Xenopus* 5S gene internal control region has been characterized in detail. Ciliberto *et al.* (1983) were able to demonstrate that the 5'-element of tRNA genes (box A) is structurally and functionally interchangeable with the 5'-region of the 5S gene internal control region. Analysis of the binding of TFIIIC to a series of point mutants created within the 5S gene box A revealed that this common, conserved promoter element is directly involved in the binding of the common transcription factor IIIC (Pieler *et al.*, 1987). In contrast, the 3'-control segment of the 5S gene promoter (box C) appeared to be 5S gene specific; it does not match the tRNA box B consensus sequence (Galli *et al.*, 1981) and it is obviously not sufficient for binding of TFIIIC in the absence of TFIIIA (Lassar *et al.*, 1983). The same region provides one of the two major binding determinants for the 5S gene specific TFIIIA (Sakonju *et al.*, 1981; Sakonju and Brown, 1982; Bogenhagen, 1985; Pieler *et al.*, 1985a,b, 1987).

On the basis of the analysis of point mutants created within the box C element we have previously suggested that this sequence element might nevertheless as well be directly involved in the binding of TFIIIC (Pieler *et al.*, 1987). In order to obtain a more detailed insight into the role of specific nucleotides in the 5S gene 3'-promoter element in the binding of TFIIIA and TFIIIC, we have now permutated nucleotides 80 to 90 within the *Xenopus laevis* somatic 5S gene and determined the effect on transcription and factor binding. The results obtained indeed demonstrate a bi-functional role of the box C element in the formation of the stable preinitiation complex; it provides direct structural information for the binding of both TFIIIA and TFIIIC. Thus, the TFIIIC recognition site on the 5S gene has to be viewed as bipartite, with the common, conserved box A providing one and the 5S gene specific box C providing the other binding signal.

An increase or decrease in the spacing of the two major functional domains in the *Xenopus* 5S gene promoter is deleterious for the ability of the 5S gene to form a stable complex, though a considerable rate of apparently correctly initiated transcription is maintained (Pieler *et al.*, 1985b, 1987). In this communication we show that TFIIIA looses contact with the A-box on these constructs, but maintains normal binding on the 3'-half of the internal control region. A model for the structural and functional organization of the 5S gene promoter will be discussed in the light of these results.

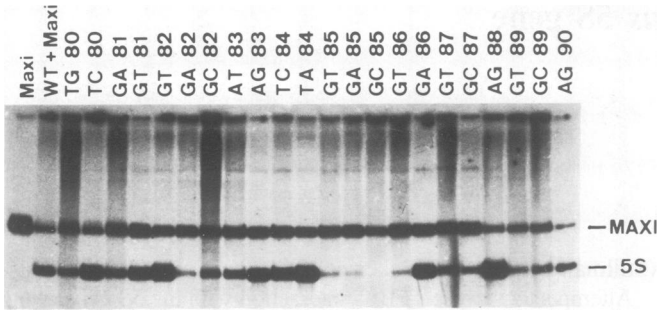

Fig. 1. Analysis of the effect of point mutations created within the box C element of the *X. laevis* somatic 5S gene internal control region on transcription. 5S DNA (12.5 μg/ml) was transcribed together with the 5S maxigene (p115/77; 12.5 μg/ml) in extracts reconstituted from partially purified, HeLa S100 derived transcription factors and RNA polymerase III. The reaction was started by the addition of rNTPs and radioactive label after a 30 min preincubation of DNA and proteins at room temperature; transcription was allowed to proceed for 80 min at 30°C and the reaction products analyzed on 8% sequencing gels. The first lane shows the transcription of the maxigene by itself, the other lanes transcription of the maxigene and WT or mutant 5S DNA as indicated above each lane.

Results

The transcription efficiency of the X. laevis somatic 5S gene promoter is severely affected by point mutations created within the box C sequence element

Twenty-two single site mutations were introduced into the *X. laevis* somatic 5S gene between nucleotides 80 and 90 directed by synthetic oligonucleotides, and the transcription efficiency of the resulting constructs analyzed as shown in Figure 1. Transcription reactions were carried out in the presence of the 5S maxigene, serving as an internal standard which competes for all the components of the transcription machinery utilized by the 5S mutant to be characterized. The relative activities as determined by this assay are summarized in Table I. In good agreement with our previous results (Pieler *et al.*, 1985a) sequence manipulations in basically every position between residue 80 and 90 of the 5S coding region affect transcription significantly. In several positions (T80, G82, T84, A88) one mutation led to an increase in transcription rate, whereas another resulted in a clear inhibition of transcription. The simplest mechanistic interpretation of these two types of observations is that in one case either contact sites for or a higher order structure recognized by one or more of the transcription factors on the DNA template is lost, whereas other sequence changes lead to a relative competitive advantage of mutant over maxigene in at least one of the steps in the initiation process. Other positions appear to play a key role in factor binding, since each of the three possible mutations interferes with transcription in a quantitatively significant way (G85, G87 and G89). There is no obvious general rule on a correlation of the type of mutation introduced and the biological effect observed to be deduced from these results, such as transversions, would have a greater effect than transitions, since there is an individual set of allowed and forbidden nucleotides for every position.

The 3′-border of the minimal structure required for the proper initiation of transcription on the *Xenopus* 5S gene has been mapped in the original deletion mutagenesis analysis (Sakonju *et al.*, 1980) to be located upstream of position +83. However, deletion beyond position +87 leads to a reduction in transcription rate and template exclusion strength (Wormington *et al.*, 1981); confirming and extending these results, we find that introduction of point mutations downstream of nucleotide +83 in many cases leads to a significant reduction in transcription rate (Pieler *et al.*, 1985a; this study). It is nevertheless surprising that deletion of several critical nucleotides as defined in our experiments (such as G85, G86 and G87) did not completely abolish the transcriptional competence; inspection of the substituted sequence in the +83 deletion mutant (as shown in Bogenhagen, 1985) reveals that several critical residues (such as nucleotides G86 to A88) are, by chance, maintained, which might contribute to the ability of +83 to transcribe.

In summary, transcription analysis of the complete set of point mutants between nucleotides 80 and 90 supports our earlier proposal that the 3′-border of the internal control region should be viewed as extending beyond nucleotide +83.

If the function of the box C promoter element would be qualitatively as well as quantitatively defined by the sum of potential contact sites provided by every individual nucleotide, one should be able to deduce the effect of multiple sequence alterations from the data obtained on the single site mutations listed in Table I. If, on the other hand, the function of an individual nucleotide is context-dependent, the results for the multiple mutations should be different from what one would predict on the basis of the assumption made above. In order to analyze this aspect of the structure/function relationship, we have created a

Table I. Transcription and factor binding analysis of box C point mutants

Mutant	Relative transcription activity (% of WT)	Relative template exclusion strength (% of WT).
TC80	200	120
TG80	40	n.d.
GA81	40	70
GC81[a]	10	30
GT81	60	80
GA82	10	70
GC82	30	n.d.
GT82	180	110
AG83	70	60
AC83[a]	40	70
AT83	30	n.d.
TC84	60	60
TA84	190	110
TG84[a]	90	100
GA85	10	10
GC85	10	70
GT85	10	10
GA86	80	80
GC86[a]	20	50
GT86	10	80
GA87[a]	10	100
GC87	30	110
GT87	30	60
AG88	270	130
AC88[a]	50	100
GA89[a]	20	100
GC89	30	120
GT89	30	110
AG90	100	110
AC90[a]	50	100

[a]Data taken from Pieler *et al.* (1985b).
n.d. = not determined.

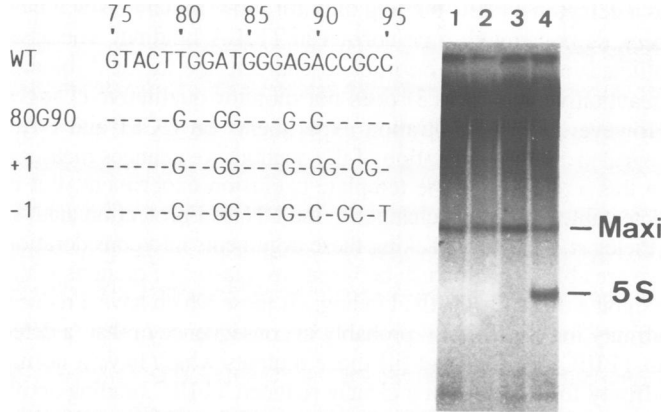

Fig. 2. Analysis of the effect of multiple point mutations introduced into box C of the *X. laevis* somatic 5S gene on transcription. Three different 5S mutants with an oligo GC-base pair element in place of the box C promoter element were analyzed on their ability to be transcribed following the procedure outlined in Figure 1. The sequence of the three mutants analyzed (**lane 2**: 80G90, **lane 3**: +1, **lane 4**: −1) is indicated together with the WT box C sequence (**lane 1**: WT).

promoter mutation which has a continuous stretch of eleven GC base pairs in the place of the box C. Out of the five base changes thus introduced, by itself only one of them (TG80) led to a considerable decrease in transcription rate (to 40%, Table I). The other mutations (AG83, TG84 and AG90) had only a slight or no effect at all on transcription and one of them (AG88) even results in a significant increase in promoter strength (to 270%, Table I). Thus, one would predict that these mutations when introduced altogether should constitute a 5S promoter structure which functions at about wildtype level. However, transcription analysis of this and two related constructs (Figure 2) reveals that they are transcriptionally inactive. This result indicates that not only the base pair change *per se* is relevant on the level of protein/DNA interaction, but also the structural context into which it is embedded and which is itself dependent on interactions of neighbouring base pairs (i.e. stacking). We cannot rule out the possibility that a positive mutation introduced into the 3′-half of box C (such as AG88) cannot compensate for the effect of a negative mutation in the 5′-half of the same region (such as TG80), because the two parts appear to be involved in the binding of different proteins (as discussed below).

The affinity of the 5S gene internal control region of TFIIIA is primarily affected by mutations into the 5′-half of the box C element

The template exclusion assay measures the ability of the 5S gene to stably sequester TFIIIA and TFIIIC in the fast step (Lassar *et al.*, 1983) and TFIIIB in the rate-limiting step of preinitiation complex formation (Bieker *et al.*, 1985). The use of *in vitro* transcription systems, reconstituted from the individual, partially purified components required, allows one to manipulate the relative concentrations of transcription factors involved in the initiation events. With the intention of establishing a fast, quantitative assay for TFIIIA binding, we have employed the 5S gene specific factor as the limiting component, otherwise following the classical template exclusion scheme (Figure 3). Since TFIIIB and TFIIIC are in excess over TFIIIA, quantitative sequestration of factor A in the stable preinitiation complex with factor C precludes transcription of the 5S maxigene, but it does not interfere with tDNA transcription (Figure 3A). In order to be in the most sensitive range of DNA concentrations, we next

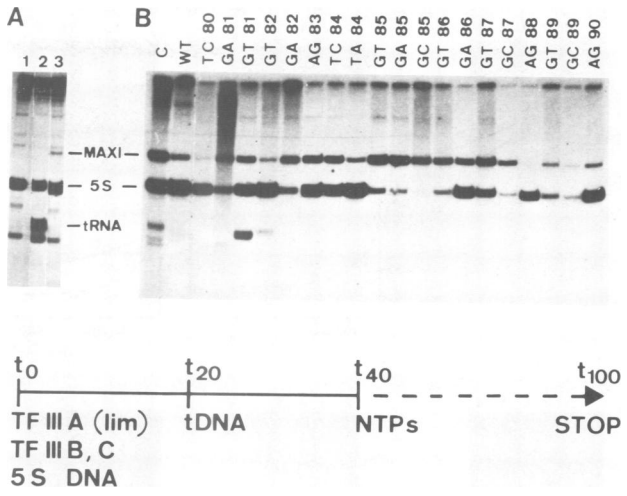

Fig. 3. Analysis of the binding of TFIIIA to 5S DNA mutants by the template exclusion assay. (**A**) TFIIIA is the limiting component in the preincubation mixture. WT 5S DNA (20 μg/ml) was preincubated with TFIIIA, B and C for 20 min at room temperature; then either vector DNA (**lane 1**: M13mp10RF; 10 μg/ml), a tRNA gene (**lane 2**: pXItmet1; 2.5 μg/ml) or the 5S maxigene (**lane 3**: 10 μg/ml) were added and after a second preincubation step of 20 min at room temperature the reaction started and processed as outlined in Figure 1. (**B**) Template exclusion assay with limiting amounts of TFIIIA and non-saturating template concentrations. WT or mutant 5S DNA (12.5 μg/ml) were used in the first preincubation step to sequester limiting amounts of TFIIIA; the assay was then performed with the 5S maxigene as second template (12.5 μg/ml) as described in (**A**) and outlined schematically at the bottom of the figure.

established conditions of non-saturating template concentrations, leading to complexation of about 80% of the available TFIIIA when one uses the wildtype 5S DNA template (Figure 3B). The results from the analysis of our box C mutants under these conditions are shown in Figure 3B and summarized in Table I. We find a rather good correlation of transcription activity and factor A exclusion strength for mutations created in the 5′-region of box C, whereas beyond nucleotide G86, an inhibition of transcription activity no longer correlates with a reduced affinity of TFIIIA. These findings agree with and extend our previous results obtained by footprint titration analysis of factor A binding to two different point mutants; GC81, transcribed at a low rate, was clearly defective in TFIIIA binding, whereas GA87, also inhibited in transcription, was apparently normal in factor A interaction (Pieler *et al.*, 1987). The simplest interpretation of the data obtained is that the 5′-region of box C serves as TFIIIA binding signal, whereas the 3′-region of the same element appears to be directly involved in the interaction with another protein.

Previous studies on the interaction of TFIIIA with the 5S gene have revealed that methylation of any of the six guanines between nucleotides 80 and 90 interferes with the binding of factor A (Sakonju and Brown, 1982); these results may appear to contradict our finding that several base changes introduced into position G87 and G89 (GA87, GC87, GA89, GC89, GT89) do not interfere with TFIIIA binding, though reducing transcription activity (Table I). However, we feel that both results are nevertheless compatible, since the stereochemical situation in consequence of the introduction of a methyl group into the major groove of the DNA double helix is not identical to the effect of any of the sequence changes which we have produced. Thus, the methylation interference experiments detect nucleotides in close contact with the protein, but they do not necessarily identify specific structural elements required for the binding of fac-

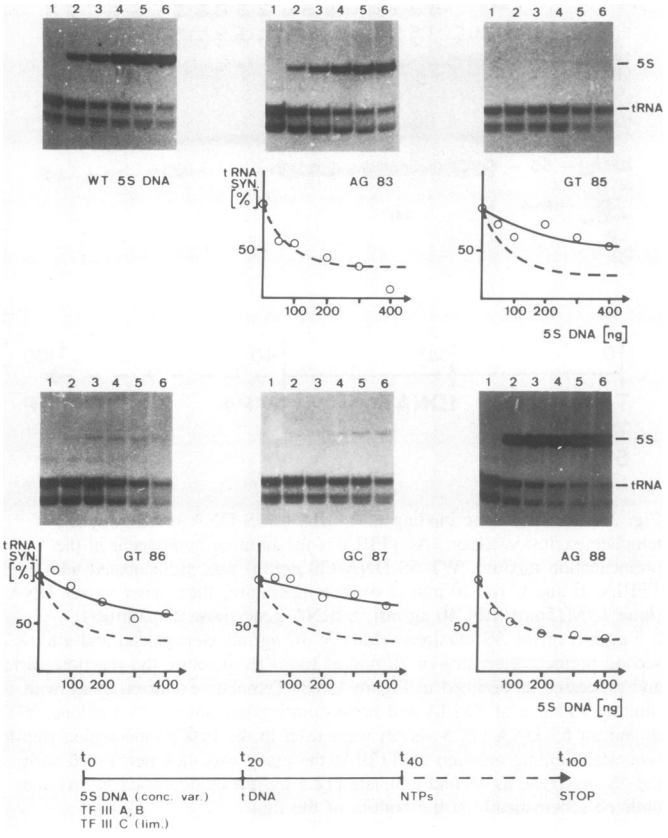

Fig. 4. Quantitative TFIIIC binding assay on mutant 5S DNA. Increasing amounts of 5S DNA or derived mutants were incubated in the first preincubation step at room temperature with TFIIIA, TFIIIB and limiting amounts of TFIIIC; the final DNA concentration was adjusted to 20 μg/ml with M13mp10RF phage DNA. After 20 min a constant amount of tDNA (pXItmet1; 50 ng) was added and a second preincubation step performed for 20 min at the same temperature. Transcription was then started by the addition of rNTPs and radioactive label and the reaction allowed to proceed for 60 min at 30°C. The amount of 5S DNA template was: **lane 1** – 0 ng, **lane 2** – 50 ng, **lane 3** – 100 ng, **lane 4** – 200 ng, **lane 5** – 300 ng, **lane 6** – 400 ng. The assay is outlined schematically at the bottom of the figure.

tor A. Interestingly, the only mutation introduced into position G87 which interferes with transcription and TFIIIA binding is the G to T transversion, which best mimics the methylation of G87 in the N7 position.

Results from the analysis of the interaction of TFIIIA with 5S gene deletion mutants are in good agreement with the data reported in this study; 3′-deletions up to nucleotide +87 maintain a normal pattern of protection, further deletion up to nucleotide +83 reduces the affinity for TFIIIA and leads to the loss of protected residues at the 3′-side of box C; deletion of another three nucleotides to position +80 totally abolishes the ability of the 5S gene to bind to TFIIIA (Sakonju *et al.*, 1981).

Binding of TFIIIC to the internal control region of the Xenopus 5S gene is dependent upon 3′-sequence elements in box C

We have previously established an assay which measures the stable binding of the TFIIIC activity to the 5S DNA/TFIIIA complex (Pieler *et al.*, 1987). Briefly, limiting amounts of TFIIIC are stably sequestered on increasing amounts of the 5S gene and the quantity of free TFIIIC after the preincubation step is estimated by transcription of a tDNA template (Figure 4). Negative effects measured in this way can be directly attributed to a defect in TFIIIC binding only for those mutants which have been demonstrated to be normal in TFIIIA binding. The assay utilized in this study in order to characterize factor A binding quantitatively (Figure 3) does not monitor qualitative changes. However, footprint titration experiments on GC81 and GA87 revealed a good correlation of the quantitative changes measured in this assay and in the template exclusion experiment, but no detectable qualitative changes in the DNase I protection analysis (Pieler *et al.*, 1987). Taking these arguments into consideration, we are able to distinguish between two classes of mutants which exhibit a defect in TFIIIC binding: (i) those which have a reduced affinity for TFIIIA and, probably in consequence of that, a defect in TFIIIC binding, and (ii) those mutants which have a normal affinity for TFIIIA but a clearly reduced TFIIIC binding activity. Only the second group defines positions which are likely to be directly involved in TFIIIC binding. Inspection of the results obtained reveals that they are all located in the 3′-region of box C: GC86, CG87 (Figure 4), GA87 (Pieler *et al.*, 1987) and GT89 (not shown). Those mutants with a defect in the binding of TFIIIA and, probably as a consequence of that, with a reduced affinity for TFIIIC (such as GT85, Figure 4) are located in the 5′-region of box C (Table I). Four sequence alterations led to a major increase in transcription activity (TC80, GT82, TA84 and AG88); none of these mutations were found to result in a significantly higher affinity for either TFIIIA (Table I) or TFIIIC (Figure 4, AG88). Finally, one mutant with a reduced TFIIIA binding activity was normal in TFIIIC binding (AG83, Figure 4). This last observation strengthens our earlier suggestion on the basis of a similar result for GC81 that interaction of TFIIIC with a weak binary TFIIIA/5S DNA complex might compensate for the defect in TFIIIA binding via protein/protein interactions.

In conclusion, these data on TFIIIA and TFIIIC binding suggest that nucleotides in the 5′-region of box C (81 to 86) are primarily involved in the binding of factor A, whereas, in addition to base pairs located within the common, conserved box A element and identified previously (Pieler *et al.*, 1987), the nucleotides at the 3′-side of box C (87 to 89) serve as an affinity site for TFIIIC.

Binding of TFIIIA to 5S gene mutants with an altered spacing of box A and box C

An increased spacing between the two major functional domains of the 5S gene promoter, box A and box C, impedes stable complex formation with TFIIIA and TFIIIC, though maintaining a considerable degree of transcription (Pieler *et al.*, 1987). Since the results discussed above suggested that the domain structure of the internal control region partly reflects the bipartite functional organization of the TFIIIC binding site, we wanted to know whether the inability to form a stable complex on the spacer mutants is directly attributable to an altered mode of TFIIIC/DNA interaction, or whether it might be a consequence of a structurally altered TFIIIA/5S DNA complex which does not allow stable binding of TFIIIC.

DNase I protection experiments on two spacer mutants with either 5 or 10 nucleotides inserted between residues +66 and +67 reveals that in both cases a normal pattern of protection and hypersensitivity is maintained in the 3′-region of the promoter, whereas protection is lost in the box A region and for the inserted sequence elements which are now in place of the box A relative to box C. In addition, new hypersensitive sites are induced at the 5′-border of protection (Figure 5). These findings confirm the demonstrated dominance of the intermediate segment together with box C in the binding of TFIIIA as pointed

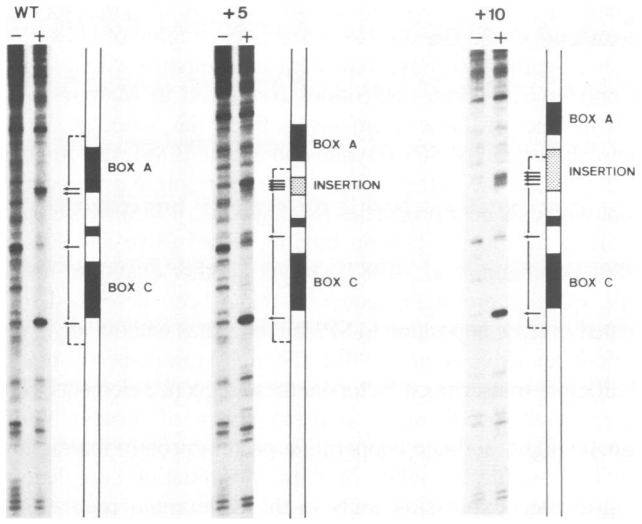

Fig. 5. DNase I protection of 5S genes with an increased spacing of box A and box C in the presence of TFIIIA. The non-coding strand of WT or mutant 5S DNA with either 5 (+5) or 10 (+10) nucleotides inserted between residues 66 and 67 of the *X. laevis* somatic 5S gene was labeled at the 3′-end, incubated with saturating amounts of TFIIIA and subjected to DNase I digestion as described in Materials and methods. Protected (I) and induced (-) cleavage sites are indicated.

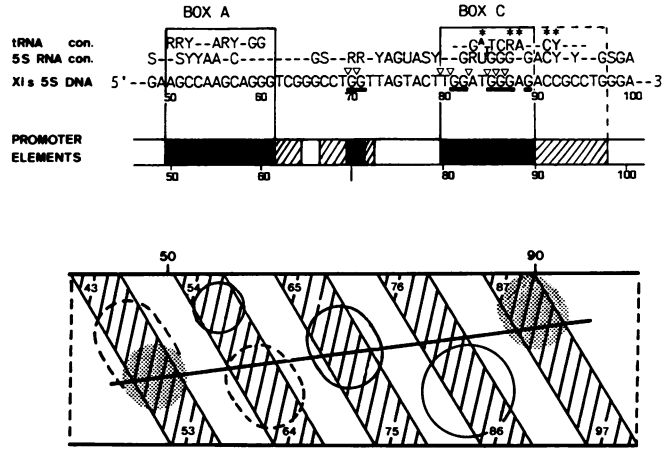

Fig. 6. Structural organization of protein binding signals on the *X. laevis* 5S gene internal control region. The internal sequence of the 5S gene is aligned with the eukaryotic 5S rRNA consensus sequence (Erdmann *et al.*, 1985), the tDNA box A consensus sequence (Ciliberto *et al.*, 1983) and a refined version of the tDNA box B consensus sequence based on the comparison of 97 vertebrate, nuclear encoded tRNAs (sequence compilation in Sprinzl *et al.*, 1985a,b) have been aligned with the 5S gene box C element. Guanine residues that interfere, when methylated, with the binding of TFIIIA are underlined, and phosphate residues which make contact with factor A are indicated by open triangles (Sakonju and Brown, 1982). Extensions of the thus defined promoter elements as detected by site directed mutagenesis and discussed before (Pieler *et al.*, 1987) are represented by cross-hatched regions. Below is a cylindrical projection of the schematic double helix with eleven base pairs per turn (Rhodes and Klug, 1986). Dotted areas which indicate contact sites for TFIIIC, solid circles indicating contact regions for TFIIIA and the significance of areas marked by broken lines are discussed in the text.

out previously. Moreover, the loss of protection in the box A region clearly indicates that this promoter element plays a role in the proper orientation of TFIIIA on the 5S DNA, though not being essential for binding *per se*. A quantitative analysis of TFIIIA binding by the footprint titration assay reveals a two- to four-fold reduction in the overall relative affinity for TFIIIA caused by the insertions.

These data suggest that TFIIIA has to be properly aligned on the 5S DNA template in order to allow stable complex formation with TFIIIC to take place.

Discussion

Analysis of the effect of 30 different point mutations introduced into nucleotides 80 to 90 of the *X. laevis* somatic 5S gene box C region on transcription and factor binding reveals that this promoter element serves a dual function: (i) it provides essential recognition sequences for TFIIIA binding in its 5′-portion and (ii) it is directly involved in TFIIIC binding via the 3′-region. Footprinting of 5S genes with an altered spacing between the two major functional domains provides evidence for the involvement of box A sequence elements in the specific binding of TFIIIA which is critical for stable complex formation with TFIIIC. The functional organization of the *Xenopus* 5S gene internal control region is schematically outlined in Figure 6.

TFIIIA binding to the Xenopus 5S gene

Three distinct sequence elements have been identified to be responsible for the binding of transcription factor IIIA; the 5′-region of box C together with the intermediate promoter segment define the essential minimal structure which allows specific interaction with TFIIIA, and box A sequence elements are required in order to obtain the complete set of contacts between protein and DNA. The dominance of the ICR 5′-domain in factor A binding is well established by footprinting experiments using 3′- and 5′-deletion mutants (Sakonju *et al.*, 1981) or linker scanning mutants with inexact spacing (Bogenhagen, 1985), by the definition of contact sites for TFIIIA with modification interference studies (Sakonju and Brown, 1982) and also by the analysis of the effect of point mutations on factor binding (Pieler *et al.*, 1985a,b, 1987). Nevertheless, apart from DNase I protection data a role for the box A element in factor A binding was also indicated by the finding that the oocyte 5S gene specific base substitutions in positions 53, 55 and 56 leads to a four-fold decrease in competition strength (Wormington *et al.*, 1981) and, in addition, methylation protection was observed for most of the guanines between nucleotides 51 and 60 in the presence of the protein (Fairall *et al.*, 1986). Similarly, base substitutions in positions 56 and 57 lead to a considerable decrease in transcription activity, whereas transitions of C52 and C53 had no inhibitory effect (Pieler *et al.*, 1985a; McConkey and Bogenhagen, 1987). Taking these results into consideration, we have tentatively indicated nucleotides 55 to 57 as a third binding site for TFIIIA (Figure 6).

Since mutations 3′ to the conserved portion of the A box have also been found to interfere with transcription and stable complex formation in crude extracts (Pieler *et al.*, 1985b, 1987) an additional critical sequence element has to be viewed to be defined by nucleotides 60 to 64; a detailed analysis of transcription factor binding to these residues has still to be performed and thus, these nucleotides might play a role in either TFIIIA or TFIIIC binding.

Klug and coworkers (Miller *et al.*, 1985; Rhodes and Klug, 1986; Fairall *et al.*, 1986) have proposed an interesting model for the interaction of TFIIIA with the internal control region of the 5S gene which is based on the assumption that each element of a repeat structure in the protein ('Zn-fingers') interacts with an exactly spaced DNA sequence repeat (GG motif) in the 5S promoter. Several nucleotides proposed to interact with TFIIIA

according to this model are in agreement with the results from our mutagenesis studies. However, other nucleotides which we propose to be critical for protein binding are located outside of the GG repeats and, in addition, some of the repeated guanines can be mutated without an effect on transcription. These findings do not necessarily contradict the MRC model, since we cannot exclude the possibility that the effects we measure are indirect and a reflection of the demonstrated context dependence of the individual nucleotides, rather than implying a direct involvement of the mutated base-pair in protein binding. Nevertheless, we believe that the three sequence elements defined in our work and discussed above highlight the essential DNA sequences required for the binding and proper functional orientation of TFIIIA on the 5S gene. This does not contradict the existence of additional contact regions with minor functional importance, possibly including those nucleotides which have been defined by Rhodes and Klug (1986).

TFIIIC binding to the 5S DNA/TFIIIA complex

The specific and stable interaction of the TFIIIC activity with the 5S gene requires the presence of TFIIIA (Lassar *et al.*, 1983). Thus, cooperative protein/protein interactions appear to be essential for the formation of the stable preinitiation complex. We have provided evidence for the direct involvement of two distinct sequence elements located within the internal control region of the *Xenopus* 5S gene in the binding of TFIIIC; mutations introduced into either box A (Pieler *et al.*, 1987) or box C (this study) interfere with transcription, have no detectable effect on TFIIIA binding but clearly reduce the affinity for TFIIIC. The at least bipartite structural organization of the DNA binding signal for TFIIIC resembles the situation described for the binding of the same activity to tRNA genes (Stillman and Geiduschek, 1984; Camier *et al.*, 1985). The tRNA box B region is clearly the dominant structure in the binding reaction (Baker *et al.*, 1986), though the A-box by itself is sufficient for specific TFIIIC protein recognition (Stillman and Geiduschek, 1984). The 5S gene 5′-binding signal has possibly to be viewed as extending into the non-conserved sequence elements flanking box A, since McConkey and Bogenhagen (1987) described mutations of residues 46, 47 and 48 which inhibit transcription but have no detectable effect on the affinity for TFIIIA.

Alignment of the tRNA box B consensus sequence derived from a comparison of 97 different vertebrate, nuclear encoded tRNAs (sequence compilation from Sprinzl *et al.*, 1985a,b) with the *Xenopus* 5S gene box C segment (Figure 6) reveals a considerable degree of homology for the nucleotides located in the 3′-region of this element (Figure 6). Interestingly, they include all those residues in the 5S gene which we have found to be involved in factor C binding (nucleotides 87 to 89). The simplest interpretation of these observations is that those nucleotides which correlate with the tDNA box B consensus sequence provide direct and specific contact sites for TFIIIC, whereas the absence of critical sequence elements in the 5′-portion of the 3′-promoter element is compensated for by the presence of TFIIIA as affinity site. In such a way, protein/protein contacts together with direct TFIIIC/DNA interactions would help in the formation of the ternary preinitiation complex.

General implications

A surprising aspect of the structural organization of protein binding signals on the 5S gene promoter is the observed narrow spacing of individual sequence elements involved in the binding of different proteins, such as in box C, described here. A similarly tight arrangement of two different transcription factor binding signals has been described for different RNA polymerase II dependent genes. The upstream regulatory region of U2 snRNA genes contains two conserved sequence elements with a spacing of only two to three nucleotides (reviewed in Mangin *et al.*, 1986). One of these elements defines the binding site for transcription factor SP1 (Dynan and Tjian, 1985), the other one is identical to the octamer motif characterizing a promoter element with enhancer properties (Mattaj *et al.*, 1985) which serves similarly as specific protein binding signal (Sive and Roeder, 1986; Bohmann *et al.*, 1987). In yet another example, histone gene specific promoter sequences have been described to be located in close apposition to SP1 binding sites without any spacer nucleotides (Sive *et al.*, 1986). Though stimultaneous binding of different transcription factors to these sequence elements awaits experimental proof, such an arrangement of protein binding signals might facilitate cooperative protein/protein interactions, which allow the formation of stable preinitiation complexes as programmed expression units in the differential regulation of eukaryotic genes (Brown, 1984).

Materials and methods

Enzymes and reagents

All enzymes were from Boehringer, Mannheim. rNTPs, dNTPs and ddNTPs were from Pharmacia P-L Biochemicals and the universal M13 sequencing primer (17mer) as well as [α-^{32}P]GTP and [α-^{35}S]dATP from Amersham. Chemicals for DNA synthesis were obtained from Applied Biosystems; all other reagents were from Merck. Mutagenic primers (18mers; different mixed probes for every position) were produced on an automated DNA synthesizer (Applied Biosystems, model 380a) and purified by FPLC (Pharmacia).

Site directed mutagenesis

Mutagenesis was performed as described before (Pieler *et al.*, 1987) using the protocol developed by Kramer *et al.* (1984). Plasmid DNA was purified by CsCl gradient centrifugation.

DNase I footprinting experiments

5S gene containing DNA fragments from M13mp9 XIs 560-764 (Pieler *et al.*, 1985a) or spacer mutants +5 and +10 (Pieler *et al.*, 1987) were 3′-end labeled at the *Eco*RI site (non-coding strand), bound to TFIIIA liberated from 7S particles by RNase treatment (Hanas *et al.*, 1984) and processed as described before (Pieler *et al.*, 1987).

Extracts and factor preparations for in vitro transcription

Transcription factors and RNA polymerase III from HeLa S100 extracts were fractionated in principle as described before (Segall *et al.*, 1980) with minor modifications as outlined in Pieler *et al.* (1987). Transcription reactions were carried out as specified in the legends to the individual figures.

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Note added in proof

After submission of this mansucript, Yoshinaga *et al.* (*Proc. Natl. Acad. Sci. USA*, **84**, 3585–3589, 1987) published experiments revealing that the TFIIIC activity is composed of at least two different proteins, whose binding specificities are exactly those which would be predicted from our study.

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