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Domains of the Positive Transcription Factor Specific for the Xenopus 5S RNA Gene

Douglas R. Smith,* Ian J. Jackson,† and Donald D. Brown
Department of Embryology
Carnegie Institution of Washington
115 W. University Parkway
Baltimore, Maryland 21210

Summary

The 40 kd positive transcription factor that interacts with the 50 nucleotide internal control region of 5S ribosomal RNA genes in Xenopus can be subdivided into three functional domains by proteolytic cleavage. At one end of the protein is a 10 kd domain that is required for efficient RNA transcription but not for binding to the DNA. This adjoins a second domain that binds to the 5′ end of the internal control region, a region of the DNA known to contribute only weakly to binding the protein but one that is essential for RNA transcription. The removal of both of these protein domains leaves a half of the protein that binds only to the 3′ side of the control region and is inactive in promoting transcription. Quantitative DNA binding and in vitro transcription experiments show that only a single molecule of 40 kd factor binds to the internal control region, and that this stoichiometry is sufficient to give maximal stimulation of 5S RNA transcription in a reconstituted system. Consideration of the essential DNA contact points of the factor, taken together with the constraints imposed by a 1:1 protein to DNA stoichiometry permit the linear projection of the extended protein molecule onto its binding site along the internal control region of the 5S RNA gene.

Introduction

Accurate initiation of transcription of 5S ribosomal RNA genes requires the interaction of a 40,000 dalton protein (40 kd factor; factor A; TFIIIA) with a 50-nucleotide region in the center of the 5S ribosomal RNA gene (Bogenhagen et al., 1980; Engelke et al., 1980; Sakonju et al., 1980, 1981). At least two additional components are needed to form a functional transcription complex (Shastry et al., 1982; Lassar et al., 1983), which then directs RNA polymerase III to initiate at the correct start site of the gene. In contrast, termination of transcription occurs when the polymerase recognizes a simple consensus sequence at the end of the gene (Bogenhagen and Brown, 1981; Cozzarelli et al., 1983). The importance of stable transcription complexes for accurate initiation of transcription, as first described for 5S RNA genes by Bogenhagen et al. (1982), is becoming generally recognized for all three forms of eucaryotic RNA polymerase (Davidson et al., 1983; Wandelt and Grummt, 1983). The availability of large amounts of the 40 kd protein has encouraged us to study the details of its interaction with the gene. The factor binds specifically to 5S RNA genes, and this interaction is an essential step in the formation of a stable transcription complex. This follows from experiments where different components are added sequentially to the gene (Lassar et al., 1983; Setzer, 1983). In this paper, we show that a single molecule of 40 kd factor binds to each gene. In addition, limited proteolysis of the 40 kd protein reveals that one end of the protein is required for efficient transcription of the gene but not for binding, while the remainder of the protein consists of two domains that bind to specific parts of the internal control region of the 5S RNA gene.

Results

Purification and Characterization of Two Proteolytic Fragments of the 40 kd Factor

The 40 kd transcription factor was purified from 7S RNP particle (a 1:1 complex of 5S RNA and the 40 kd factor) isolated from immature X. laevis oocytes (Picard and Wegnez, 1979; Pelham and Brown, 1980). We observed that limited proteolysis of the 7S particle (using trypsin, chymotrypsin, papain, or thermolysin) gives rise to a metastable 30 kd breakdown product, which can then be converted into a 20 kd product. These protein fragments were also seen in digests of the purified 40 kd protein, but were generated simultaneously rather than sequentially, and were less stable. The breakdown fragments from the 7S particle remain bound to 5S RNA and can be purified by the same protocol as that used to purify the 40 kd factor. All three proteins were greater than 95% pure as judged by gel electrophoresis, and each was estimated to contain less than 1% contamination with the other species (Figure 1).

We aligned the proteolytic fragments with respect to the cyanogen bromide cleavage map of the protein (Figure 2). There are reported to be three methionines per 40 kd molecule (Picard and Wegnez, 1979), but only three cyanogen bromide cleavage fragments are detected. The two small ones (CB-2 and CB-3, estimated to be approximately 11 and 9 kd) are on either side of a central large fragment (CB-1), judging from the two partial digestion products CB_{PA} and CB_{PB}. The amino and carboxyl orientation of the protein has not yet been determined. Judging from their markedly different staining intensity with silver, the two small cyanogen bromide fragments are very different in amino acid composition. In the 30 kd species, the small cyanogen bromide fragment, CB-2, has been almost entirely lost except for a small piece that can be seen at the bottom of the gel. A new partially cleaved fragment runs coincidentally at a position that is indistinguishable from that of the 20 kd proteolytic fragment. In the 20 kd species,

* Present address: Imperial Cancer Research Fund Laboratories, St. Bartholomew's Hospital, Dominion House, Bartholomew Close, London EC1A 7BE, England.
† Present address: Medical Research Council, Mammalian Development Unit, Wolfson House, 4, Stephenson Way, London NW1 2HE, England.

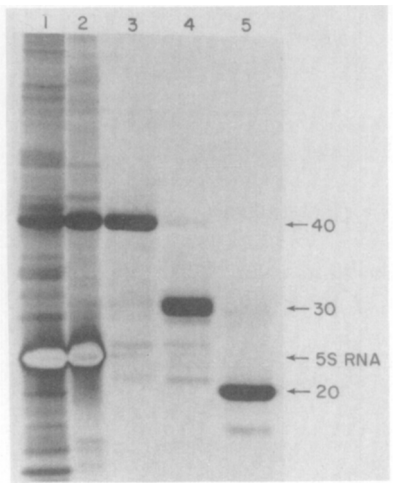

Figure 1. SDS Polyacrylamide Gel Showing Preparations of 7S Particle, 40 kd Factor, and the 20 and 30 kd Proteolytic Fragments at Various Stages of Purification

Lane 1: 7S particle (2.5 μg) after glycerol-gradient centrifugation. Lane 2: 7S particle (1.1 μg) after DEAE-cellulose chromatography. Lane 3: 40 kd factor (1 μg) after second BioRex-70 chromatography. Lane 4: 30 kd papain fragment (1 μg) after second BioRex-70 chromatography. Lane 5: 20 kd trypsin fragment (1 μg) after second BioRex-70 chromatography.

CB-2 is completely gone, and part of the large central fragment, CB-1, has also been removed (lane 4). In addition, the other small fragment, CB-3, is slightly smaller, indicating that there has been some digestion at the other end of the parent molecule as well. Approximate alignment of the 20 and 30 kd polypeptides with the cyanogen bromide cleavage sites of the 40 kd protein is shown in the diagram in Figure 2. The essential point is that proteolytic digestion occurs primarily from one end of the protein in a sequential manner, defining three structural domains.

Qualitative DNA Footprints

The 40 kd transcription factor protects 50 nucleotides in the center of the 5S RNA gene from digestion by DNAase I (Engelke et al., 1980; Sakonju et al., 1981). The coding strand is almost completely protected, while the noncoding strand exhibits characteristic hypersensitive sites. Although the protein protects the coding strand from DNAase, the critical nucleotides for binding of the protein to the DNA are on the noncoding strand and clustered at the 3′ end of the internal control region (Sakonju and Brown, 1982). We have compared the DNA-binding properties of the 30 kd and 20 kd proteolytic fragments with that of the intact transcription factor (Figure 3). The 30 kd fragment gives a footprint almost identical with that of the intact 40 kd factor. One subtle but reproducible difference is the reduction of hypersensitive cleavage sites at the 5′ end of the internal control region. In contrast, the 20 kd fragment only protects the 3′ side of the control region, giving about three-fifths of a footprint. This footprint is reminiscent of those generated when mutants deleted in the 5′ end of the control region are interacted with 40 kd factor (Sakonju et al., 1981).

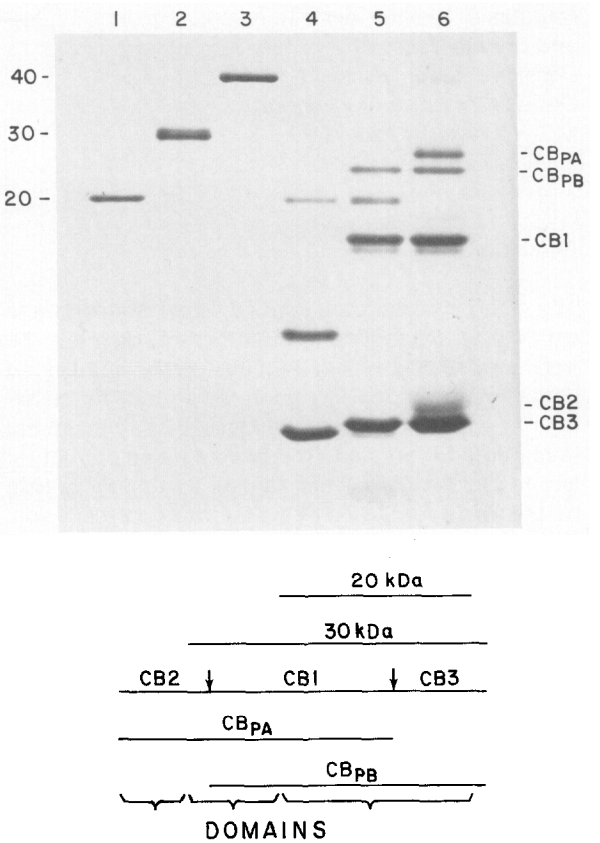

Figure 2. SDS Polyacrylamide Gradient Gel Showing the Cyanogen Bromide Cleavage Products of the 40 kd Factor and the 20 and 30 kd Proteolytic Fragments

Lane 1: 20 kd trypsin fragment, 0.3 μg; lane 2: 30 kd papain fragment, 0.5 μg; lane 3: 40 kd factor, 0.5 μg; lane 4: CNBr digestion products of the 20 kd trypsin fragment; lane 5: CNBr digestion products of the 30 kd papain fragment; lane 6: CNBr digestion of the 40 kd factor. Digestion is incomplete; CB_{PA} and CB_{PB} denote partial digestion products. Below is shown the approximate alignment of the 20 and 30 kd proteolytic fragments with the CNBr cleavage map of the 40 kd transcription factor. The amino and carboxyl termini of the protein have not been identifed.

Quantitative DNA Footprinting—One Factor Molecule per Gene

We next carried out a series of titrations of DNA footprints with increasing amounts of the three proteins. These experiments were designed to compare the relative binding affinities of the three forms, and to determine the number of protein molecules that bind each gene. Figure 4 shows an example of a footprint titration where a constant amount of DNA was interacted with increasing amounts of the 40 kd transcription factor. Each concentration point was carried out in duplicate. A band in the control region was selected for quantitation, cut out, and counted along with a second band of constant intensity to normalize for differences in the DNAasel digestion. Similar experiments were conducted with the 20 and 30 kd fragments (not shown). Figure 5 summarizes the results. The binding curves of the 30 and 40 kd proteins were very similar; a nearly complete footprint was obtained when one molecule of protein was added for each gene. This result differs

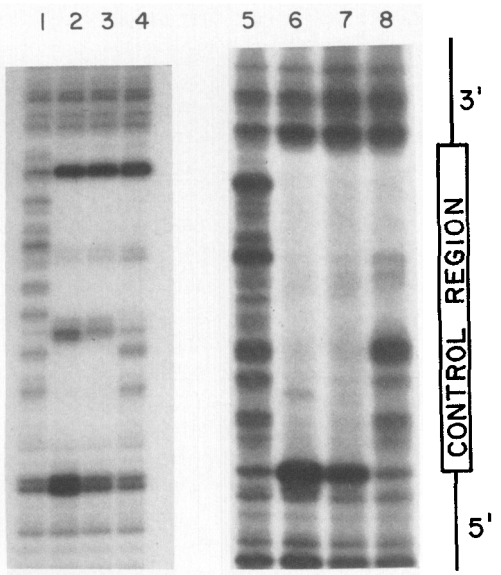

Figure 3. DNAase I Footprint Assay for Protection of 5S DNA by the 40 kd Factor and the 20 and 30 kd Proteolytic Fragments

Autoradiographs of sequencing gels are shown on which DNAase I footprint reactions were run using 5S DNA templates labeled on the noncoding strand (lanes 1–4) or on the coding strand (lanes 5–8). Lanes 1 and 5: control with no protein added; lanes 2 and 6: 40 kd factor; lanes 3 and 7: 30 kd papain fragment; lanes 4 and 8: 20 kd trypsin fragment.

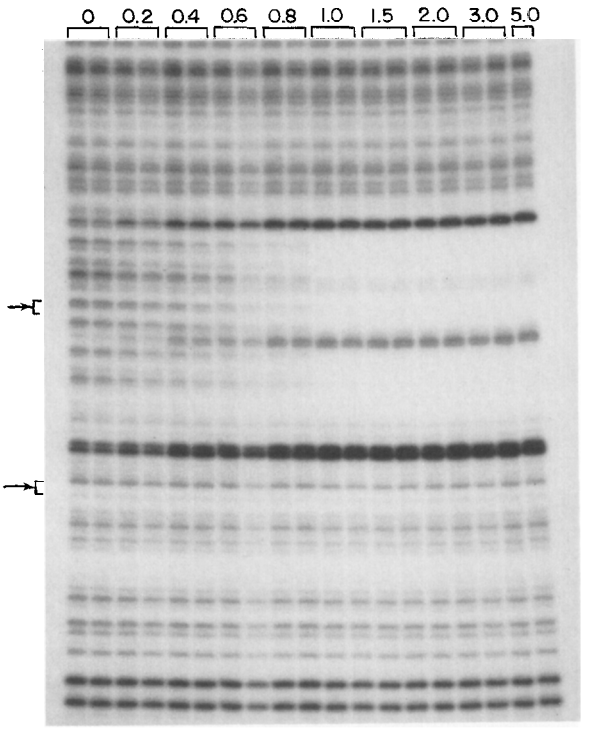

Figure 4. Quantitative DNAase I Footprint Assay for Protection of 5S DNA by Increasing Concentrations of the 40 kd Factor

An autoradiograph of a sequencing gel is shown on which DNAase I footprint reactions were run using a 5S DNA template labeled on the noncoding strand. Reactions were carried out in duplicate, and at the top of each set of lanes is indicated the molar protein:DNA ratio used in those samples. The bands that were cut out and quantitated by scintillation counting are indicated by arrows on the left. The upper doublet is part of the footprint; the lower band is unaffected by increasing protein and was used as reference.

from that of Hanas et al. (1983a), who concluded that two molecules of protein interact with each gene. Possible reasons for this discrepancy will be discussed later. The 20 kd fragment binds more weakly to the DNA requiring more than two molecules per gene to produce a complete footprint. However, based on experiments presented below, we believe that only one 20 kd molecule binds per gene, and that a weakened binding constant accounts for the higher concentration of factor required for saturation.

The Ability of the Proteolytic Fragments to Support Transcription

Transcriptionally competent somatic cell extracts can be fractionated so that 5S RNA transcription is completely dependent on the addition of exogenous 40 kd factor (Shastry et al., 1982; Lassar et al., 1983). The two proteolytic fragments were compared with the 40 kd factor in their ability to complement such a fractionated extract. The results (Figure 6) show that the 30 kd fragment has about 20% of the activity of the 40 kd protein while the 20 kd fragment is totally inactive. A titration of the extract with increasing amounts of the 30 and 40 kd proteins is shown in Figure 7. Saturation of the transcription response is achieved by both proteins at the same ratio of protein to DNA, namely one to one. We draw two conclusions from these experiments. First, the 10 kd domain that is missing in the 30 kd fragment is required for efficient 5S RNA transcription, although there is still some residual transcription-stimulation activity in the 30 kd fragment itself. Contamination of this fragment with the 40 kd protein, which might account for the residual activity, is too low to explain

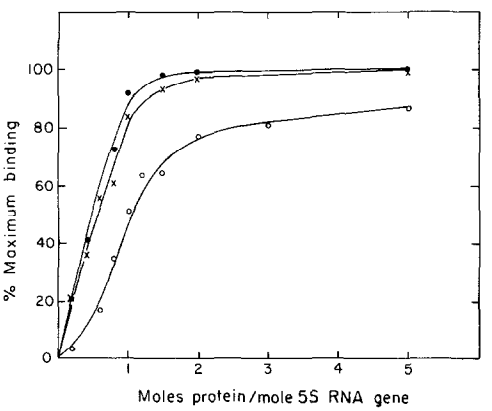

Figure 5. Saturation Curves for Binding of the 40 kd Factor and the 20 and 30 kd Proteolytic Fragments to 5S DNA

The data derived from the quantitative DNAase I protection experiment in Figure 4, and from similar experiments with the 20 and 30 kd fragments are plotted as the percent of maximum binding versus the molar protein:DNA ratio for each point. 40 kd factor —●—; 30 kd papain fragment —×—; 20 kd trypsin fragment —O—.

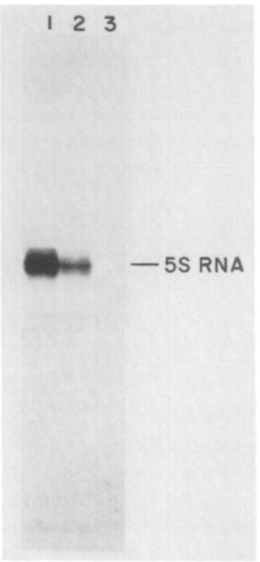

Figure 6. Transcription of 5S RNA in a Factor-Dependent Extract

A 5S DNA plasmid was preincubated with either the 40 kd factor (lane 1), the 30 kd papain fragment (lane 2), or the 20 kd trypsin fragment (lane 3), and then transcribed in a fractionated somatic cell extract that was dependent on exogenously added factor.

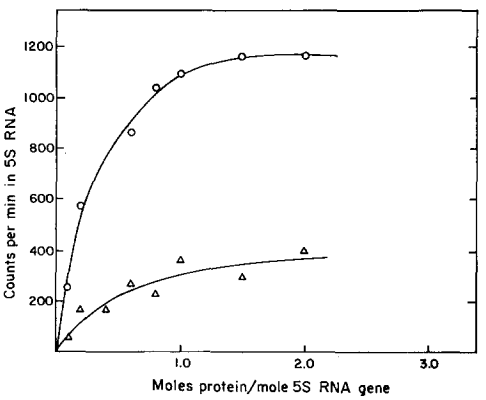

Figure 7. Saturation Curves for Transcription of 5S RNA in a Factor-Dependent Extract Supplemented with Either the 40 kd Factor or the 30 kd Papain Fragment

Transcription reactions were carried out at a number of protein:DNA ratios, and the 5S RNA bands were cut out and quantitated by scintillation counting. The results are plotted as counts per minute in 5S RNA versus the molar ratio of protein to DNA at each point. 40 kd factor —O—; 30 kd papain fragment —Δ—.

the results of the response curve. Secondly, the experiments give independent support for the conclusion that only a single molecule of the transcription factor binds to each gene.

Additional Evidence for One Factor Molecule Binding per Gene

Sakonju and Brown (1982) summarized the indirect data suggesting that only one molecule of protein interacts with each 5S RNA gene. The 3′ side of the control region is required for any binding to the 5′ side. In a model where two molecules bind cooperatively, one might expect the first protein molecule to bind the 3′ side, giving half of a footprint, and only with a higher concentration of the protein would there be a complete footprint. They showed that this is not the case for the 40 kd factor, as can also be seen in Figure 4. The same is true for the 30 kd fragment (data not shown). If two molecules of the factor bind the internal control region, one would expect that a deletion mutant having only the 5′ half of the control region would still have some residual binding affinity to a molecule of the protein. Nevertheless, even high concentrations of the 40 kd factor gave no detectable footprint when nucleotides at the 3′ end of the control region were deleted. Even so, these objections might be overcome if the 40 kd protein was present as a dimer in solution and always interacted with the gene in that form. However, sucrose gradient centrifugation experiments show that both the 40 kd protein and the 30 kd fragment sediment mainly as monomers in solution (data not shown). A minority of both proteins smear through the gradient without forming any band at the location expected of dimers.

A different kind of assay system gives independent confirmation that only one molecule of protein interacts with each gene. It was shown originally by S. Sakonju (unpublished) that a fraction of the complexes between 40 kd factor and a 5S RNA gene fragment is stable enough to separate from naked DNA by nondenaturing polyacrylamide gel electrophoresis. Figure 8 shows that the two proteolytic fragments also form stable complexes with a 5S DNA-containing fragment whose mobilities can be distinguished from the complex formed between the 40 kd factor and the gene. If two molecules of protein interacted with each gene, we would expect to obtain heterodimers when a mixture of 40 and 30, or 40 and 20 kd proteins are interacted with the DNA. A mixture of two different-sized proteins gives no complexes of intermediate mobility—only a mixture of the original sizes (Figure 8). Purification of the protein from each of the two bands and assay by gel electrophoresis shows, as expected, that the slower-moving band contains only the 40 kd protein, while the 30 kd protein is only present in the faster-moving band (data not shown). Since both the 40 kd protein and the 30 kd fragment are known to exist as monomers in solution, this lends strong support to the conclusion that only one protein molecule interacts with each gene.

Discussion

One Molecule of the 40 kd Transcription Factor Binds Each 5S RNA Gene

It is necessary to discuss some of the technical problems of the measurement of protein/DNA stoichiometry to try to account for the differences between our findings and those of Hanas et al. (1983a). They concluded that two molecules of the protein bind per gene, exhibiting a sigmoidal binding curve at low protein concentrations suggestive of cooperativity. Some of our earlier experiments also revealed a

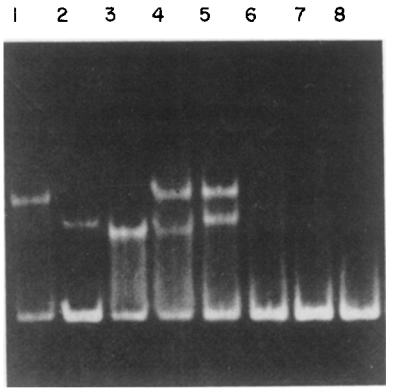

Figure 8. Electrophoresis of DNA–Protein Complexes on a Nondenaturing Low Ionic Strength Polyacrylamide Gel

Proteins were incubated with either a 5S DNA fragment (lanes 1–5) or a similarly sized pBR322 DNA fragment as a control (lanes 6–8). Lanes 1 and 6: 40 kd factor; lanes 2 and 7, 30 kd papain fragment; lanes 3 and 8: 20 kd trypsin fragment; lane 4, mixture of 40 and 20 kd proteins; lane 5, mixture of 40 and 30 kd proteins. The exact conditions are given in Experimental Procedures.

sigmoid curve. However, special care in handling the protein below concentrations of 1 μg/ml (including careful cleaning of microtubes and the addition of nonionic detergent and serum albumin) reduced or eliminated this effect. Even pipetting of dilute protein solutions can inactivate the 40 kd factor. We therefore conclude that apparent cooperativity is due to inactivation of the protein at low concentrations. This explanation cannot account for the results of Hanas et al. when using higher concentrations of the protein. But there are some other considerations that we believe were not taken into account in their experiments. First, the factor has considerable affinity for the sides of ultracentrifuge tubes, even in particle form, so their estimation of the binding capacity of the factor by quantitation of free protein after sucrose-gradient centrifugation with plasmid DNA is likely to be an overestimate. Second, divalent zinc ions, known to be required for DNA binding of the factor (Hanas et al., 1983b), were not included in their buffers. Finally, after RNAase treatment of 7S particle, oligoribonucleotides remain bound to the protein (as is evident from the requirement for urea treatment to get full retention of the protein on Bio Rex-70 resin) and may compete for DNA binding, thus leading to an overestimate of the factor to DNA ratio.

The value of one 40 kd protein molecule for each 5S RNA gene was arrived at by three independent methods. First, footprint titrations gave this value (Figures 4 and 5). Second, a fractionated extract dependent upon added 40 kd factor was used to demonstrate that one molecule of factor per gene stimulates transcription maximally (Figure 7) under conditions where the DNA concentration limits the reaction. A 30 kd proteolytic fragment of the factor showed binding to the 5S RNA gene identical with that of the 40 kd factor, and also stimulated transcription maximally at one molecule of the protein per gene (albeit to only 20% the level of the 40 kd protein). The fact that the 40 and 30 kd proteins bind the gene with the same apparent affinity led us to do mixing experiments. If two molecules of the protein bind each gene, then a mixture of 40 and 30 kd proteins with DNA should produce heterodimers. Using a gel assay (Figure 8), no evidence of heterodimers was ever seen, giving further evidence of one protein molecule bound to each gene.

Functional Domains of the 40 kd Transcription Factor

The ability to purify two proteolytic digestion fragments that are cleaved from one end of the factor molecule enables us to demonstrate, through progressive loss of function and binding, a linear alignment of the protein domains with the 5S gene internal control region (Figure 9). The factor has been shown to consist of three structural domains: a large 20 kd domain at one end, and two smaller 10 kd domains at the other (Figures 2 and 9). The 20 kd domain contains the sequence-specific DNA binding site that recognizes key nucleotides on the 3′ side of the control region, but is not by itself sufficient to activate the gene for transcription. An adjacent domain of 10 kd, in conjunction with the 20 kd domain, extends binding to the 5′ end of the control region. The resultant 30 kd protein supports transcription very weakly. The third domain, of 10 kd, does not bind directly to the DNA, although it induces DNAase I-hypersensitive sites at the 5′ end of the control region. It is responsible for most of the transcription-enhancing activity of the intact protein.

From these studies, along with previously published analyses of contact points between the 40 kd factor and the internal control region (Sakonju and Brown, 1982; also shown in Figure 9), we can begin to assign some details to the protein–DNA interaction. The 20 kd domain (about half of the protein) extends over about three-fifths of the internal control region from residue +63 to +97. There is one region of the DNA (+79 to +89) that has so many adjacent essential contact points with the factor that it appears that the protein must be intertwined along the major groove for at least one complete turn of the helix in close association with the noncoding strand. This is clearly the region of tightest binding. In the absence of this 3′ region, the protein cannot bind to the rest of the internal control region (Sakonju et al., 1981). The 5′ end of the internal control region binds the factor less tightly and probably in a qualitatively different way than does the 3′ end. Perhaps the protein lies along one surface of the DNA from residues +45 to +79 rather than wrapping around it. If so, the protein–DNA interaction at the 5′ end would more closely resemble those proposed for procaryotic proteins with their cognate DNA-binding sites (Anderson et al., 1981; Pabo and Lewis, 1982). On the other hand, the factor is clearly very extended in its binding configuration, with 300 amino acids or less interacting with 50 nucleotides. The necessarily small diameter of the protein (about half that of the DNA) required to extend over this region makes it difficult to reconcile such a model with the

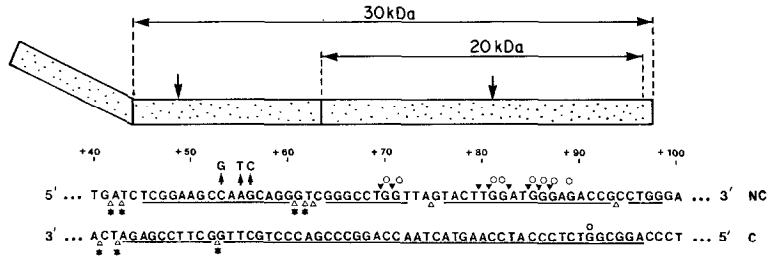

Figure 9. Projection of the 40 kd Transcription Factor onto the Internal Control Region of the 5S RNA Gene

The essential contact points (Sakonju and Brown, 1982) for guanine (○) and phosphate (▼) residues, as well as the DNAase I-hypersensitive sites (△), are indicated along the DNA sequence either on the coding (C) or noncoding (NC) strand. The numbering is from the start of the 5S RNA gene. The underlined part of the sequence indicates the region protected from DNAase I cleavage. The hypersensitive sites marked with stars are those that are either reduced or eliminated in the footprint of the 30 kd fragment. The three nucleotide differences that account for the weaker binding of the factor to the oocyte-type 5S RNA genes are indicated by vertical arrows near the 5′ end of the control region above the sequence. The approximate locations of the CnBr cleavages (two vertical arrows) and the sites of cleavage yielding the 20 and 30 kd fragments are shown above on the schematic representation of the protein where they would project onto the DNA according to the footprint data presented here.

extensive DNAase I protection in this region. Thus it is also possible that the protein wraps around the DNA in this part of the control region as well, and simply associates less tightly and specifically at the 5′ end than it does at the 3′ end.

One end of the protein is required for specific initiation of transcription but is not involved in binding to the DNA. It is not surprising that this "transcription" domain of the protein is adjacent to the part of the protein that binds to the 5′ part of the control region. The 5′ part of the control region is essential for accurate initiation of transcription (Sakonju et al., 1980). This region of the protein may interact with the other factors that are required to form a competent transcription complex or with RNA polymerase III. It is interesting to note in this regard that this 5′ end of the 5S gene control region contains a sequence with weak homology to the 3′ part of the tRNA and VA RNA internal control regions (Fowlkes and Shenk, 1980). This sequence has been proposed by Lassar et al. (1983) as the site of the interaction of another as yet uncharacterized component of the transcription complex, factor C.

The linear projection of the protein over its DNA-binding site provides us with testable predictions. For example, the three base changes that distinguish oocyte from somatic 5S RNA genes are clustered near the 5′ end of the internal control region (Figure 9). These changes cause a somatic 5S RNA gene to bind the 40 kd protein about four times more tightly than an oocyte 5S RNA gene (Sakonju and Brown, 1982; Wormington et al., 1981). Therefore, the part of the protein responsible for this differential recognition is predicted to be within the 10 kd domain that binds the 5′ end of the internal control region.

Experimental Procedures

Preparation of 7S Particle

The 40 kd factor was initially purified from Xenopus laevis ovaries in the form of 7S RNP particle essentially as described by Hanas et al. (1983a). The procedure was scaled up about four-fold; HEPES buffer was used in place of Tris, and all buffers contained 10 μm $ZnCl_2$. Glycerol gradients were centrifuged in an SW27 rotor at 22,000 rpm for 48 hr at 2°C, and contained 0.05 mM phenylmethylsulfonyl-fluoride. After elution from DEAE cellulose, about 50 mg of the 7S particle was diluted carefully with an equal volume of autoclaved glycerol at 0°C and stored at −70°C without dialysis. This preparation could be stored for over six months without any loss of DNA-binding activity. At this stage of purification the protein was 80%–90% pure (see Figure 1).

40 kd Factor Purification and Preparation of the Two Proteolytic Fragments

The 40 kd factor was purified from 7S particle as follows. All steps were carried out at room temperature unless otherwise noted. One to ten milligrams (protein) of 7S particle were adjusted to 0.1 M KCl concentration with a buffer containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 10 μM $ZnCl_2$, and 20% glycerol (buffer A), the volume of solution being kept to a minimum. This material was passed through a small BioRex-70 (BioRad) column (0.1 ml of packed resin per mg protein), equilibrated in buffer A with 0.1 M KCl added, and the column was rinsed with an additional column volume of this buffer. RNAase A (PL Biochemicals) was added to the eluate containing the 7S particle (50 μg RNAase A per mg protein starting material), incubated for 5 min, and an equal volume (to the total) of buffer A containing 0.1 M KCl and 10 M urea (warmed slightly to dissolve the urea) was gently added and mixed by inverting the tube several times. The tube was placed on ice and slowly loaded onto a second BioRex-70 column (same resin to protein ratio). The column was rinsed with two bed volumes of buffer A containing 0.1 M KCl, and then slowly with 20 bed volumes of buffer A containing 0.5 M KCl. The factor was then eluted with buffer A containing 1 M KCl, the flow rate being kept to a minimum to maximize the concentration of protein in the peak fractions. For 2 mg of starting material, single-drop fractions were collected and the protein eluted between drops 4 and 7. Small aliquots were frozen at −70°C. The protein was greater than 95% pure and stable for at least two months at −70°C.

The 30 kd fragment was prepared by digesting the 7S particle that had been purified through the first BioRex-70 column step (before adding RNAase A) with papain at 20 μg per mg protein for 30 min at room temperature. The fragment was then further purified in the same manner as the 40 kd factor.

The 20 kd fragment was prepared in a similar way, except the 7S particle was treated with trypsin (TPCK treated, PL Biochemicals, 60 μg per mg protein starting material) for 45 min at room temperature. Before adding RNAase A, an excess of trasylol (FBA pharmaceuticals) was added at 200 μg per mg of starting material. The flow rate for the 0.5 M KCl wash step was kept to a minimum to avoid excessive losses (the 20 kd fragment leaks slowly off the column in 0.5 M KCl).

Protein concentration was determined using the Coomassie blue-binding assay of Bradford (1976) with bovine serum albumin (BSA) as a standard (Sigma, crystallized and lyophilized, globulin-free). The concentration of 40 kd factor measured by this assay was checked against that obtained by other protein assays. Both the Lowry assay (Lowry et al., 1951) using BSA as a standard and a measurement of interference fringes obtained in a

Beckman model E ultracentrifuge on a concentrated solution of factor in a synthetic boundary cell (Babul and Stellwagen, 1969) agreed within 15% of the value obtained by the Bradford assay (being somewhat lower in each case). Moreover, Hanas et al. (1983) have reported that the Bradford assay agrees to within 20% with the Lowry assay and with amino acid analysis.

Analysis of Cyanogen Bromide Peptides

The 40 kd factor (5 μg), 30 kd fragment (3.5 μg), and 20 kd fragment (2.5 μg) were treated separately with cyanogen bromide in a reaction containing 2 μl of protein (in buffer A + 1 M KCl), 9 μl of 50 mg/ml cyanogen bromide, and 43 μl of 88% formic acid. The reaction was carried out for 24 hr at 25°C in a sealed 500 μl conical plastic tube, lyophilized to dryness with a trap containing sodium hydroxide pellets, redissolved in 100 μl H_2O, and lyophilized again two times. The samples were dissolved in 20 μl SDS-sample buffer, boiled 2 min, treated with 6 μl of 1 M iodoacetamide, and one-half of each sample loaded onto a polyacrylamide gradient gel and electrophoresed as described in the following section.

SDS Polyacrylamide Gel Electrophoresis

A modified SDS gel system was employed, which gave better resolution of protein bands than the standard Laemmli system (Laemmli, 1970). For routine analysis of proteins, the separation gel contained 12% acrylamide, 0.3% bis-acrylamide, 0.6 M Tris-phosphate, pH 8.8, 0.1% SDS 0.06% TEMED, and 0.06% ammonium persulfate. The stacking gel contained 5% acrylamide and 0.025% bis-acrylamide in the same buffer. A gel 18 × 20 × 0.075 cm thick was electrophoresed at 150 V for 6–7 hr in buffer containing 0.1 M Tris, 0.75 M glycine, and 0.1% SDS. For analysis of bands cut from DNA–protein gels (see below), the gel system was the same except that the separating gel contained 16% acrylamide and 0.09% bis-acrylamide. For analysis of cyanogen bromide peptides, the same buffer system and stacking gel were also employed, but the separating gel consisted of a linear polyacrylamide gradient containing from 12% acrylamide, 0.3% bis-acrylamide to 28% acrylamide, 2.8% bis-acrylamide. Electrophoresis was carried out for 18 hr at 150 V.

All gel solutions were filtered through a prerinsed 0.2 μ Millipore filter to eliminate silver-staining artifacts. Sample buffer consisted of 0.6 M Tris-phosphate, pH 8.8, 1% SDS, 0.1 M DTT, 1 mM EDTA, and 0.01% bromophenol blue. In most cases, samples were dissolved in 10 μl sample buffer, boiled for 2 min, and treated for 30–40 min at 37°C with 3 μl of 1 M iodoacetamide (warmed slightly to dissolve). The iodoacetamide treatment was required to obtain sharp protein bands (the 40 kd factor is cysteine-rich) and to eliminate silver-staining artifacts from the DTT in the sample buffer (Hashimoto et al., 1983). The gels were fixed and stained with silver nitrate essentially as described by Giulian et al. (1983).

DNA Preparation and Radiolabeling Procedures

Plasmid pXbs201 (4262 bp; containing a 249 bp Hind III/Bam HI fragment with a somatic 5S gene from X. borealis ligated into plasmid pBR322; Bogenhagen et al., 1980) was prepared using published procedures (Smith and Calvo, 1980) from E. coli K12 strain SF8 (F^-, thr, leu, thi, supE, lacY, tonA, gal, rec B, rec C, lop II, hsd S_k) into which the plasmid had been transferred by $CaCl_2$-induced transformation (Smith and Calvo, 1979). This plasmid preparation, containing a small amount of E. coli RNA, was efficiently transcribed in X. laevis oocyte nuclear extracts (Birkenmeier et al., 1978) and was used without further purification in transcription experiments. The 249 bp fragment containing a somatic 5S gene was prepared from 500 μg of the plasmid following digestion with restriction endonucleases Hind III and Bam HI (Bethesda Research Laboratories). The fragment was purified by polyacrylamide gel electrophoresis and DEAE cellulose chromatography as described (Smith and Calvo, 1980). A 267 bp Hae III fragment from plasmid pBR322 was prepared in a similar manner.

Template for DNAase I protection experiments consisted essentially of linearized pXbs201 that was end-labeled at the Hind III site located about 50 nucleotides upstream from the start of the 5S gene. Template labeled on the coding strand was prepared by digestion of 60 μg pXbs201 with endonuclease Hind III followed by end-labeling with α^{32}P-dATP (Amersham, 400 Ci/mmole) and reverse transcriptase (Smith and Calvo, 1980), and cleavage with endonuclease Eco RI to liberate a 29 bp fragment from the end of the plasmid containing only pBR322 sequences. DNA concentration was measured on a small aliquot by absorbance at 260 nm.

Template labeled on the noncoding strand for quantitative footprint experiments was prepared in a similar way. The plasmid was first treated for 20 min with RNAase A (50 μg per 300 μg plasmid), phenol-extracted, passed through a 0.8 × 22 cm BioGel-A 1.5 M column (BioRad) equilibrated with 0.3 M sodium acetate, 1 mM EDTA, and ethanol-precipitated. RNA-free plasmid (60 μg) was then digested with endonuclease Hind III followed by treatment for 1 hr with 30 U calf intestinal phosphatase (Efstratiadis et al., 1977) in the same reaction buffer. The plasmid was then phenol-extracted, ethanol-precipitated, and labeled in a 10 μl reaction with 0.5 mCi ^{32}P-ATP (Amersham, 3000 Ci/mmole) and 12 U polynucleotide kinase (Bethesda Research Laboratories; Maxam and Gilbert, 1977). The labeled DNA was then digested with endonuclease Eco RI, phenol-extracted, and passed again through the BioGel column to remove the 29 bp Eco RI/Hind III fragment. After ethanol-precipitating and redissolving the plasmid in footprint buffer without NP-40 and BSA (see below), the concentration was carefully measured as before by absorption at 260 nm.

Transcription Reactions

Transcriptions were carried out using fractionated X. laevis cell extracts and purified RNA polymerase III. Extracts were prepared from an X. laevis kidney cell line and fractionated into A, B, and C fractions on a phosphocellulose column as described by Shastry et al. (1982). RNA polymerase III was purified from X. laevis oocytes as described by Cozzarelli et al. (1983). Transcription reactions were carried out as follows. Varying amounts of 40, 30, and 20 kd proteins were incubated with 0.1 μg of pXbs201 DNA, in 7.5 μl of buffer containing 70 mM NH_4Cl, 20 mM HEPES, pH 7.5, 7 mM $MgCl_2$, 2.5 mM DTT, 0.1 mM EDTA, 6% glycerol, and 0.1% NP-40 for 30 min at room temperature (protein dilutions were carried out in the same buffer at 0°C). Following this, 2.5 μl of fraction B, 3 μl of fraction C, 2 μl of RNA polymerase III (14 U), and 3 μl of nucleoside triphosphates (to give final concentrations of 500 μM ATP, CTP, and TTP) 25 μM GTP and 0.5 mCi/ml α-^{32}P-GTP (Amersham, 3000 Ci/mmole) were added simultaneously and the reation was allowed to proceed for 1 hr at room temperature. The ^{32}P-RNA was purified and electrophoresed. The band of 5S RNA was excised and counted.

DNAase I Protection Experiments

DNAase I footprints at saturating levels of the 40, 30, and 20 kd proteins were done on 3′- and 5′-end-labeled templates as follows. The proteins were incubated at room temperature for 15–30 min with 0.1 μg DNA in a 20 μl reaction (1.8 nM concentration) in a buffer containing 70 mM NH_4Cl, 20 mM HEPES, pH 7.5, 7 mM $MgCl_2$, 2.5 mM DTT, 6% glycerol, 10 μM $ZnCl_2$, 0.1% NP-40, and 100 μg/ml BSA. Proteins were diluted in the same buffer at 0°C. Pancreatic DNAase I (Sigma; 12.5 ng) was added and after 70 sec the reactions were stopped and the DNA fragments purified. The samples were boiled for 1 min before electrophoresis on 8% sequencing gels at 1200 V for 6 hr.

Quantitative footprints using the 5′-end-labeled template were performed in essentially the same manner with incubation for only 15 min before adding DNAasel. The conical plastic centrifuge tubes in which the dilutions and footprints were carried out were extensively washed in a jet of H_2O to remove foaming material adhering to the sides of the tubes as received from the manufacturer (BioRad). The concentration of protein was carefully measured in duplicate from an aliquot of the same tube used to prepare the dilutions at the start of each experiment. The same size aliquot was then used to prepare the first dilution in the series. The footprints were carried out in duplicate immediately after preparing the dilutions, and special care was taken to mix the solutions gently without any foaming or bubble formation that could result in denaturation of the protein.

Following electrophoresis of the samples, the gel autoradiographed and specific bands (Figure 4) were excised and counted.

Electrophoresis of DNA–Protein Complexes

DNA–protein complexes were separated on low ionic strength polyacrylamide gels by a modification of techniques published by Fried and Crothers (1981). The gels contained 4.5% acrylamide, 0.675% bis-acrylamide, 20 mM HEPES, pH 8.3, 50 μM spermine, 0.1 mM EDTA, 6% glycerol, 0.1% TEMED, and 0.05% ammonium persulfate. The gel plates (18 × 20 cm), were siliconized and the 0.75 mm thick gel was allowed to polymerize for at least 1 hr before setting up in the apparatus. The electrophoresis buffer was the same as above but also contained 1 mM DTT. The gel was allowed

to stand overnight in contact with the buffer, and was then pre-electrophoresed for 4 hr at 150 V with recirculation of the buffer. Samples of 10 μl were prepared in buffer containing 50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10 μM $ZnCl_2$, and 20% glycerol and contained 0.2 μg of DNA fragment plus either 0.2 μg of 40 kd factor, 0.05 μg of 30 kd fragment, 0.1 μg of 20 kd fragment, a mixture of 0.225 μg 40 kd factor and 0.075 μg 30 kd fragment, or a mixture of 0.22 μg 40 kd factor and 0.12 μg 20 kd fragment. Samples were incubated for 30 min at room temperature and electrophoresed for 8 hr at 75 V. The gel was stained with ethidium bromide and photographed under UV light.

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