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Specific Interaction of a Purified Transcription Factor with an Internal Control Region of 5S RNA Genes

David R. Engelke,
Sun-Yu Ng, B. S. Shastry and Robert G. Roeder
Departments of Biological Chemistry
and Genetics
Division of Biology
and Biomedical Sciences
Washington University School of Medicine
St. Louis, Missouri 63110

Summary

A factor necessary for the accurate transcription of cloned Xenopus 5S genes in vitro has been isolated from soluble extracts of X. laevis ovaries. The activity of the factor was monitored by its ability to facilitate transcription of exogenous 5S genes in unfertilized egg extracts which are otherwise incompetent for 5S gene transcription. The factor was purified via ion exchange chromatography, and apparently consists of a 37,000 dalton polypeptide. This factor is necessary for the transcription of both the oocyte-type and somatic-type 5S genes of Xenopus, but is not required for, and has no detectable effect upon, the transcription of a cloned Xenopus $tRNA_1^{Met}$ gene. The site of action of the factor has been investigated using the "footprinting" method of Galas and Schmitz (1978). The factor binds specifically to intragenic regions extending, approximately, from nucleotide positions 45 to 96 on both somatic and oocyte-type 5S genes. Additionally, this binding occurs independently of, and is not altered by, the presence of purified RNA polymerase III or unfertilized egg extracts. The probable role of this factor in transcription initiation is discussed.

Introduction

The 5S RNA genes in Xenopus provide attractive systems for the analysis of factors involved in eucaryotic gene transcription. Somatic-type genes (expressed in most cell types) and oocyte-type genes (expressed only in oocytes) from X. laevis and X. borealis have been cloned and extensively characterized by Brown and co-workers (Korn and Brown, 1978; Brown et al., 1979). The RNA polymerase III which transcribes these genes in oocytes has also been purified and characterized (R. G. Roeder, manuscript submitted) but has failed to show any selective or accurate transcription of purified oocyte-type 5S genes (Parker, Ng, and Roeder, 1976; Parker and Roeder, 1977; Ng, Parker and Roeder, 1979). In contrast, the oocyte-type 5S genes in chromatin from immature oocytes are accurately transcribed by the same (purified) RNA polymerase, suggesting that factors other than those associated with the purified RNA polymerase are necessary for specific transcription.

The analysis of factors involved in the transcription of eucaryotic genes has been facilitated by the development of soluble cell-free extracts in which specific genes (including Xenopus 5S genes) in purified DNA templates are selectively and accurately transcribed (Wu, 1978; Birkenmeier, Brown and Jordan, 1978; Ng et al., 1979; Weil et al., 1979a, 1979b). These extracts contain at least those components (including RNA polymerase III) necessary for transcription of purified DNA templates, but not necessarily all those which are required for regulation within the cell (Roeder et al., 1979a; see Discussion in Weil et al., 1979b). That these factors do not copurify with the RNA polymerase III was again indicated by the isolation from immature oocytes of a soluble post-chromatin fraction (containing no detectable RNA polymerase III) which was necessary for the accurate transcription of purified 5S DNA by a purified RNA polymerase III (Ng et al., 1979). More recently, the fractionation of soluble extracts from mature oocytes and from cultured mammalian cells has led to the identification of at least two subcellular fractions which are jointly required, along with an exogenous RNA polymerase III, for transcription of purified 5S genes (Roeder et al., 1979b).

In the present report we describe the purification, from oocytes, of a factor which is required for transcription of purified 5S genes. This task was facilitated by the availability of a soluble unfertilized egg extract which contains all the components necessary for (purified) 5S gene transcription except this factor. We show that this factor binds selectively to an internal gene region recently shown (Bogenhagen, Sakonju and Brown, 1980; Sakonju, Bogenhagen and Brown, 1980) to be the only region essential for accurate initiation of transcription on the 5S gene in a soluble system. This is the first report of a purified factor which is necessary for the accurate transcription of a purified eucaryotic gene.

Results

Purification of a 5S Gene Transcription Factor

Fractionation of the Soluble Ovarian (Oocyte) Extract

We have previously described the preparation, from large Xenopus laevis oocytes, of an extract which accurately transcribes cloned X. laevis and X. borealis oocyte-type 5S genes (Ng et al., 1979). In the present experiments, an analogous extract was prepared from whole ovarian tissue. This extract was then subjected to ammonium sulfate precipitation and to chromatographic fractionation on DEAE-cellulose, DEAE-Sephadex and phosphocellulose, as described in Experimental Procedures, in order to purify the components required for selective 5S gene transcription. Figure 1 shows an analysis of the RNAs synthesized in the presence of the various fractions and pXbo3 DNA. As

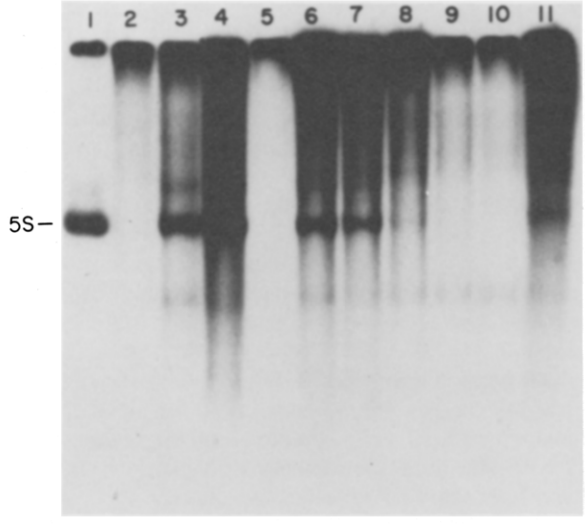

Figure 1. Fractionation of Oocyte Transcription Components

A soluble extract from X. laevis ovarian tissue was subjected to precipitation with ammonium sulfate and ion exchange chromatography on DEAE-cellulose, DEAE-Sephadex and phosphocellulose (step elution). Fractions were assayed for the ability to synthesize 5S RNA in the presence of pXbo3 DNA and in the presence or absence of purified X. laevis RNA polymerase III. Assays contained: (lane 1) 24 μl ammonium sulfate precipitate; (lane 2) 24 μl DEAE-cellulose flow-through + 0.1 μg RNA polymerase; (lane 3) 24 μl DEAE-cellulose eluate; (lane 4) 24 μl DEAE-cellulose eluate + 0.1 μg RNA polymerase; (lane 5) 24 μl DEAE-Sephadex flow-through; (lanes 6 and 7) 24 μl DEAE-Sephadex flow-through + 0.1 μg RNA polymerase; (lane 8) 24 μl phosphocellulose flow-through + 0.1 μg RNA polymerase; (lane 9) 24 μl phosphocellulose eluate; (lane 10) 24 μl phosphocellulose eluate + 0.1 μg RNA polymerase; and (lane 11) 12 μl phosphocellulose flow-through + 12 μl phosphocellulose eluate + RNA polymerase. The RNA polymerase III used in lane 7 was isolated by the method of D. R. Engelke and R. G. Roeder (manuscript submitted), while that in the remaining experiments was isolated by the method of R. G. Roeder (manuscript submitted). As indicated in the text, the recombined phosphocellulose fractions (compare lane 11) obtained as described here showed great variability with respect to their ability to synthesize 5S RNA. In some cases no 5S band was observed, while in others the selective synthesis of 5S RNA was much greater than that shown in lane 11.

observed with the unfractionated extract (Ng et al., 1979; see also below), the ammonium sulfate fraction (lane 1) and the DEAE-cellulose eluate (lane 3) effect the selective transcription of the 5S gene in the absence of exogenous RNA polymerase III. The addition of exogenous RNA polymerase III to the DEAE-cellulose eluate results primarily in additional non-specific transcription (lane 4). [The DEAE-cellulose flow-through fraction fails to elicit accurate transcription of the 5S gene, even in the presence of exogenous RNA polymerase III (lane 2).] When the DEAE-cellulose eluate is passed through DEAE-Sephadex to selectively adsorb the endogenous RNA polymerase III, the flow-through fraction directs the transcription of the 5S genes in the presence (lanes 6 and 7) but not in the absence (lane 5) of exogenous purified RNA polymerase III. When the RNA polymerase III-dependent DEAE-Sephadex fraction is subjected to chromatography on phosphocellulose, neither the unbound fraction (lane 8) nor the bound (step-eluted) fraction (lane 10) effects significant transcription of the 5S gene in the presence of exogenous RNA polymerase III. When these fractions are recombined in the presence of RNA polymerase III, however, a low level of 5S RNA synthesis is again observed (lane 11). This result, in combination with similar analyses with other cation exchangers (compare Roeder et al., 1979b), indicates the existence of two or more components required for 5S gene transcription. Since considerable variability in ability to effectively recombine the separated fractions was observed (see legend to Figure 1), however, a more reproducible assay was developed for one of the components indicated by the phosphocellulose fractionation.

Complementation of Soluble Unfertilized Egg Extracts

During the analysis described above, we studied the ability of unfertilized egg extracts to accurately transcribe purified 5S genes. Although unfertilized eggs are generally regarded as transcriptionally inactive, it has been shown that eggs and large oocytes contain comparable levels of RNA polymerase activity (Roeder, 1974). As shown in Figure 2, however, an egg extract shows no detectable transcription of exogenous 5S DNA (lane 1). In contrast, levels of 5S RNA synthesis comparable to those observed with oocyte extracts are observed in the egg extract with the addition of certain of the oocytic extract fractions described above. As shown in Figure 2, a highly selective transcription of the 5S gene is observed when egg extracts are mixed with the DEAE-Sephadex flow-through fraction (lane 2) or the bound phosphocellulose fraction (lane 4), but not with the unbound phosphocellulose fraction (lane 3). Hence a component(s) present in the bound phosphocellulose fraction (oocyte extract) is missing (or inactivated) in the egg extract. [Mixing experiments, in which oocytes and eggs were mixed prior to disruption, have indicated that an active transcription factor is not simply lost or inactivated by egg components during the extract preparation (data not shown)]. It is also apparent that the degree of selectivity (fractional level of 5S RNA synthesis) is much higher when the egg extract is complemented with the more purified oocyte fractions than when the oocyte DEAE-Sephadex fraction or the recombined (oocyte) phosphocellulose fractions are supplemented with purified RNA polymerase III (compare Figure 1, lanes 6 and 11). Thus the egg extracts appear to contain some component(s) which somehow restrict nonspecific transcription.

Purification of the 5S Transcription Factor

The egg extract complementation assay described above provided a convenient reproducible assay for the further purification of the 5S transcription factor(s) which binds to phosphocellulose. In subsequent experiments, phosphocellulose columns were eluted

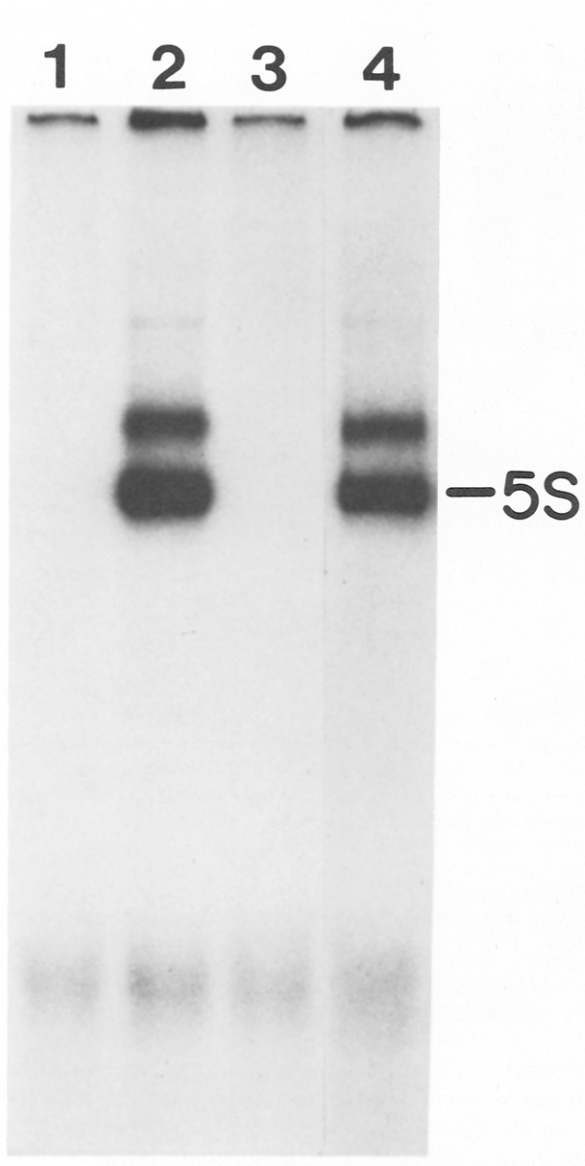

Figure 2. Complementation of 5S RNA Synthesis with Egg Extract and Oocyte Components

A soluble extract from unfertilized eggs was assayed for its ability to accurately transcribe 5S genes in pXbo3 DNA in the presence of oocyte extract fractions obtained as in Figure 1. All assays contained 20 μl egg extract, and the following additions: (lane 1) none; (lane 2) oocyte DEAE-Sephadex flow-through (3 μl); (lane 3) oocyte phosphocellulose flow-through (3 μl); (lane 4) oocyte phosphocellulose eluate (2 μl). The diffuse band lower than 5S in all lanes routinely appears in assays of crude extracts but is independent of the addition of a DNA template.

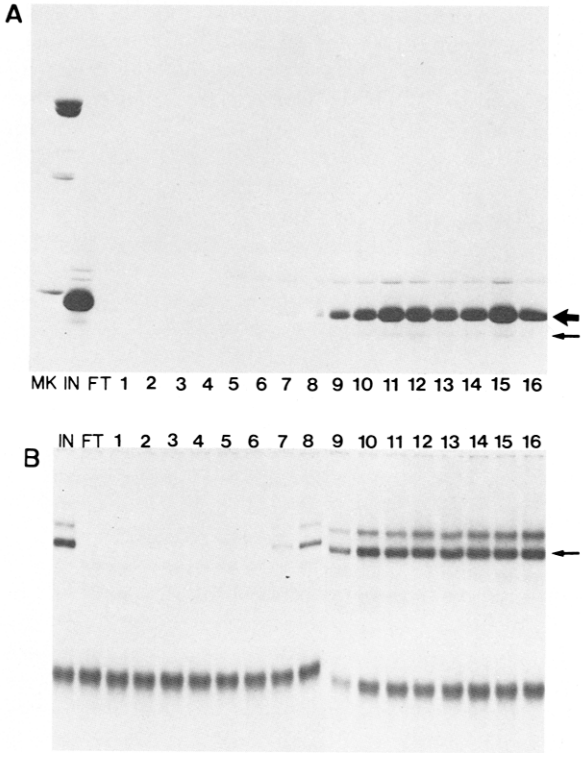

Figure 3. Chromatography of Ovarian Transcription Factor on Bio-Rex 70

Ovarian phosphocellulose gradient elution fractions containing 5S complementation activity were pooled and subjected to chromatography on Bio-Rex 70 as detailed in Experimental Procedures.

(A) Shows an analysis, by polyacrylamide gel electrophoresis under denaturing conditions, of the proteins present in 50 μl each of the input (IN), flow-through (FT) and gradient fractions (lanes 1–16) eluting between 0.05 and 0.4 M ammonium sulfate; E. coli RNA polymerase holoenzyme (lane MK) provided molecular weight standards (subunits of 165,000, 155,000, 93,000 and 39,000 daltons; Burgess, 1969). The putative 5S transcription factor in the gradient fractions (approximately 37,000 daltons) is indicated by the thick arrow, while an inseparable minor polypeptide (approximately 34,000 daltons) is indicated by the thin arrow.

(B) Shows an analysis of the same input (IN), flow-through (FT) and gradient (1–16) fractions in the egg extract complementation assay. In this case, 1 μl aliquots of the gradient fractions were assayed with 20 μl aliquots of the egg extract in the presence of pXbo3 DNA.

with a linear gradient of ammonium sulfate. Under these conditions the complementation activity was eluted in a broad peak between 0.30–0.60 M ammonium sulfate. The most active fractions were then subjected to chromatography on Bio-Rex 70. As the assays in Figure 3B show, the input sample (IN) complements the egg extract, resulting in highly selective 5S gene transcription. No complementation activity is detected in the flow-through (FT) fraction or the early gradient fractions (1–6). The bulk of the complementation activity is eluted from the column in a broad peak extending from 0.16 (fraction 7) to 0.40 M (fraction 16) ammonium sulfate. Beyond this point (data not shown) the activity diminishes slowly.

The proteins present in the input, flow-through and gradient fractions from the Bio-Rex 70 column are seen in the sodium dodecylsulfate-polyacrylamide gel analyses presented in Figure 3A. Several protein bands are visible in the input (IN) fraction. Of these, only the major 37,000 dalton polypeptide (thick arrow) and a minor 34,000 dalton polypeptide (thin arrow) consistently co-purify (fractions 7–16) with the complementation activity. The molar ratio of these two polypeptides is approximately 20:1. A polypeptide of

43,000 daltons was more widely distributed throughout the gradient (in fractions 5–16) and could sometimes be separated from the complementation activity and from the other two proteins by additional chromatographic steps (data not shown). It has not yet been possible to separate the 37,000 and 34,000 dalton polypeptides by additional purification steps, but other considerations favor the major polypeptide species as the 5S transcription component (see Discussion).

Approximately 5 μg of the 37,000 dalton protein can be isolated per gram of ovary, suggesting an approximate maximal purification (assuming quantitative yields) of about 40,000 fold over total ovarian protein. However, we have not yet accurately quantitated the yields of the complementation activity (or the 37,000 dalton protein) through the various steps.

Specificity of the Purified Factor for 5S Gene Transcription

The above experiments show that the purified oocyte factor is necessary for the transcription of Xbo 5S genes in the crude egg extract. We have also investigated the transcription of a cloned X. borealis somatic 5S gene and a cloned X. laevis $tRNA^{Met_1}$ gene in these systems. Transcription of the Xbs 5S gene in the oocyte extract results in the appearance of a single discrete 5S RNA (compare Brown et al., 1979). Transcription of the $tRNA^{Met_1}$ gene in the oocyte extract results in the synthesis of two RNA species, approximately 98 (pmet-t) and 80 (met-t) nucleotides long, respectively. These results are in agreement with the results of Kressman et al. (1978) and with kinetic and fingerprint studies of S. G. Clarkson and R. A. Koski (personal communication), who have shown that the larger RNA is a precursor to the smaller $tRNA^{Met_1}$ species.

Like the oocyte 5S genes, the X. borealis somatic 5S gene is not transcribed in the soluble egg extracts (Figure 4, lane 3); however, the addition of the purified 5S transcription factor results in a level of 5S RNA synthesis equal to or greater than that observed with the oocyte-type 5S genes (Figure 4, lane 4). In contrast to these results, the $tRNA^{Met_1}$ gene is transcribed in the egg extract in the absence of other factors (Figure 4, lane 5). Significantly, the purified oocyte factor has no noticeable effect on the transcription of this gene (Figure 4, lane 6). We conclude that the purified oocyte transcription factor is not essential for tRNA gene transcription in vitro but that it is essential for transcription of both somatic and oocyte-type 5S genes.

Site and Mechanism of Action of the 5S Transcription Factor

Although the purified factor appeared to be only one of at least two or more factors necessary for the accurate transcription of the 5S gene (see Figure 1;

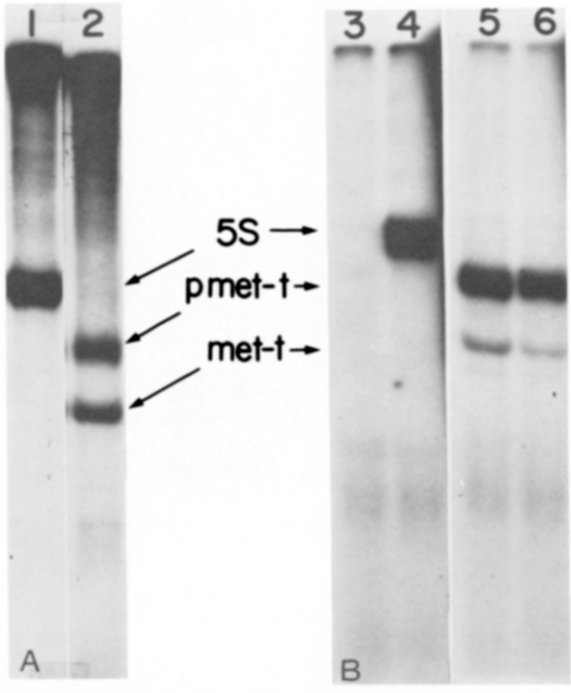

Figure 4. Transcription of Somatic 5S RNA and $tRNA^{Met}$ Genes in Egg Extracts in the Presence of a Purified Transcription Factor

(A) Shows a control transcription experiment in which 20 μl of soluble extract from ovarian tissue were incubated with the following templates: (lane 1) pXbs1; (lane 2) $pXlmet_1$.

(B) Shows the results of transcription experiments in which 20 μl of soluble extract from unfertilized eggs were incubated with the following: (lane 3) pXbs1; (lane 4) pXbs1 plus 0.15 μg purified transcription factor; (lane 5) $pXltmet_1$; (lane 6) $pXltmet_1$ plus 0.15 μg purified transcription factor. The positions of the 5S RNA and the two $tRNA^{Met_1}$ transcripts are indicated. The pmet-t band is a precursor to the met-t ($tRNA^{Met_1}$) band (see text).

Roeder et al., 1979b; our unpublished observations), it seemed plausible that this factor might act via site-specific binding to the 5S gene. To investigate this possibility, a modification of the "footprint" technique of Galas and Schmitz (1978) was used. In the reported method, a discrete fragment of double-stranded DNA, radiolabeled at the 5′ end of only one strand, is partially digested (approximately one cut per molecule) with DNAase I. When the DNA is subsequently denatured and analyzed on sequencing gels, a "ladder" of DNA fragments is observed. If a protein is firmly bound to a discrete site on the DNA, that site is protected from DNAase digestion, and no fragments ("rungs") appear which are equal in length to the number of nucleotides between the labeled terminus and the protected site.

In the experiments described below, we have used fragments derived from Xbs1 (somatic) 5S DNA and from Xbo1 (oocyte) 5S DNA. The former DNA contains a single somatic gene, while the latter contains three distinct oocyte-type 5S genes (Korn and Brown, 1978), two of which (types 1 and 3) are identical to those genes present in the Xbo3 DNA fragment used

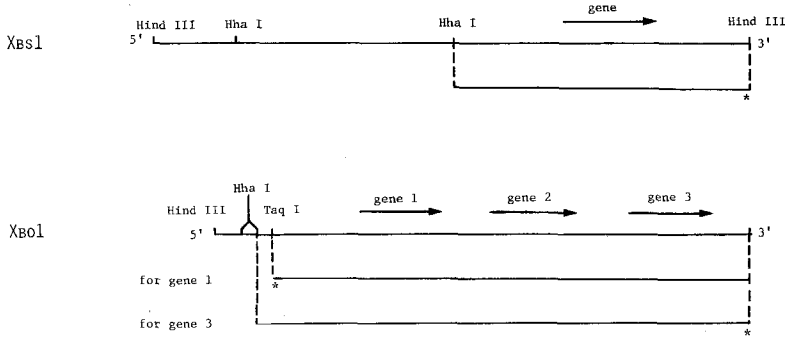

Figure 5. Generation of 5S DNA Fragments for DNAase Protection Studies

Xbs1 and Xbo1 fragments containing somatic and oocytic 5S genes from X. borealis were obtained from plasmids pXbs1 and pXbo1 as described in Experimental Procedures and prepared for DNAase protection studies as follows.

Xbs1. Purified Xbs1 fragment was digested with Hha I. The largest fragment containing the somatic 5S gene was separated by electrophoresis in 3.6% agarose and repurified as for the original Xbs1 fragment. Single-stranded ends were filled using α-^{32}P-dATP as the radiolabeled nucleotide, which selectively labeled the 3′ end of the noncoding strands. The end of each fragment which contains ^{32}P label is indicated with an asterisk (*).

Xbo1-Gene 1. The single-stranded ends of the Xbo1 fragment left by Hind III were filled with cold deoxynucleotide triphosphates under the same conditions used for end-labeling. The blunt-ended fragment was separated from unincorporated deoxyribonucleoside triphosphates by gel filtration on Sephadex G-100 and then cleaved with restriction endonuclease Taq I, leaving free pG-pC ends which were filled using α-^{32}P-dCTP. The small Taq I fragment (85 nucleotides) was allowed to remain in the DNAase protection reaction, since fragments of that length and shorter migrated completely through the analytical gel slabs. Only cleavage products of the large Taq I fragment, labeled at the 3′ end of the coding strand, are visible in Figures 6–8.

Xbo1-Gene 3. For DNAase protection studies on gene 3, both 3′ ends of the Xbo1 and Hind III fragment were filled with α-^{32}P-dATP, followed by complete cleavage with Hha I. Only cleavage products of the large fragment containing the 5S gene sequences and labeled on the 3′ end of the noncoding strand are visible in Figure 7.

in the factor purification (above) and in preceding studies from this laboratory (Ng et al., 1979; Weil et al., 1979b). The Xbo1 DNA was used for the binding studies because its complete sequence is known (Korn and Brown, 1978) and because the terminal locations of genes 1 and 3 make them convenient for footprinting. All the genes on this DNA were previously shown to be transcribed in soluble nuclear extracts (Korn and Brown, 1978), and at least one or more are also transcribed in our whole oocyte extracts (data not shown). The generation of end-labeled DNA fragments from the Xbo1 and the Xbs1 DNAs is detailed in Experimental Procedures and in Figure 5. To estimate the sizes of the various DNAase-generated fragments, these same end-labeled fragments were partially digested with the restriction endonuclease Hae III, and the resulting fragments were subjected to electrophoresis (in adjacent lanes) with the other digestion products. As observed by other investigators (Galas and Schmitz, 1978; Ross et al., 1979), DNAase cleavages do not occur with equal frequency between each base pair, even in the absence of proteins other than DNAase, resulting in an incomplete ladder containing bands of variable intensities. Nonetheless, because of the rather constant spacing (in a given region) of the bands, and because Hae III cuts within the 5S genes (for example, at positions 8 and 61 in Xbs1 5S gene) the length of fragments terminating in and around the genes could be estimated with a confidence of ±1 nucleotide.

Nucleotide positions are numbered positively proceeding from the first base of each gene sequence toward the distal (3′) end of the gene (see Korn and Brown, 1978), and a specific cleavage position refers to the first phosphodiester bond following the corresponding base (in the direction of transcription).

Factor Interactions with 5S DNA in the Absence of Other Components

The data in Figure 6 show footprint analyses for Xbs1 and Xbo1 DNA fragments. In each case lane 5 shows the DNAase digestion pattern in the absence of factor and lanes 1–4 show the digestion patterns in the presence of increasing amounts of factor. The bands which result from cleavage within and adjacent to the Xbs1 gene and Xbo1 gene 1 are readily visualized (the vertical bars indicate the gene regions). For these genes, differences in the digestion pattern become visible at intermediate levels and are most obvious at the highest protein:DNA level shown in Figure 6. (No further changes are observed at higher protein:DNA ratios; data not shown). Significantly, the only visible differences in the digestion patterns occur in intragenic regions, the boundaries of which are indicated by numbered hatch marks. Moreover, the data fail to indicate a sequential appearance of protection within the binding regions. [Although the Xbo1 gene 2 has not been analyzed in any detail, it is apparent from Figure 5 that there are some significant perturbations of the digestion pattern in the presence of the factor.]

The autoradiograms in Figure 7 show similar analyses for Xbo1 genes 1 and 3 and for the Xbs1 gene (lanes 1 and 2 in panels A–C) at a saturating factor: DNA ratio. For increased clarity, the figures show magnified views of regions in which altered DNAase digestion patterns are observed. In agreement with the results indicated above (Figure 6; see also Figure 8, below), no alterations were visible in the extragenic regions resolved in other portions of these gels. As shown in Figure 7A, the only visible differences in the Xbs1 digestion patterns in the absence (lane 2) and presence (lane 1) of the factor occur in the intragenic region extending from positions 47–96, which define

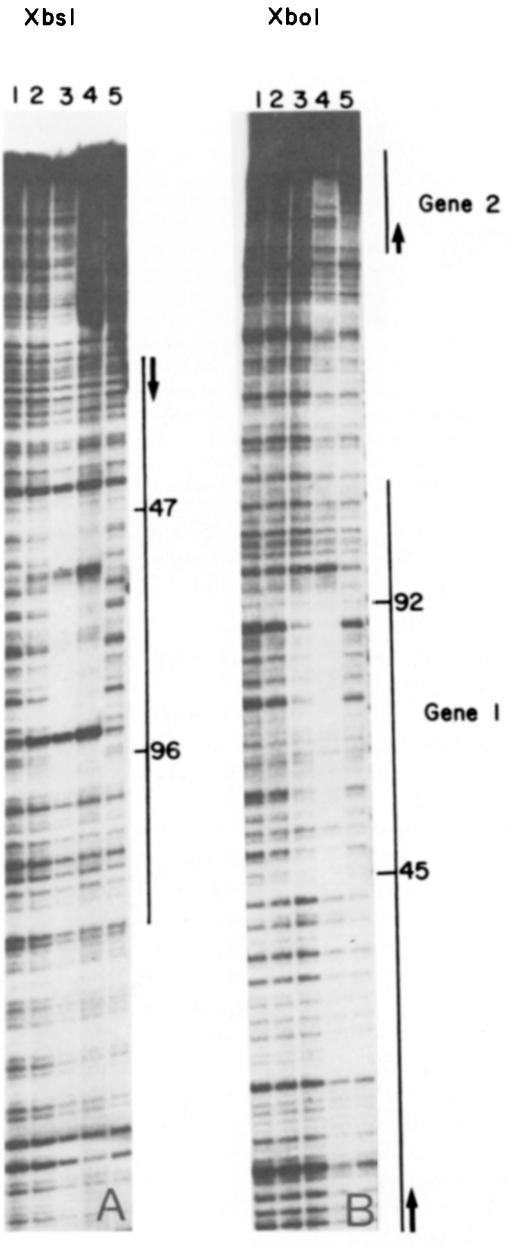

Figure 6. Xbs1 and Xbo1 Gene 1 Footprint Analyses with Purified Transcription Factor

The Xbs gene (A) and the Xbo1 gene 1 (B) footprints were performed with the terminal labeled fragments indicated in Figure 5. The analyses were performed with 15 ng of the Xbs1-derived fragment and 29 ng of the Xbo1-derived fragment, according to the procedures detailed in Experimental Procedures and in Figure 7. In the present cases, however, the analyses were performed with variable amounts of the purified factor (Bio-Rex 70 fraction) and the autoradiograms show the complete gel analyses. The amounts of factor employed were: (lane 1) 6 ng; (lane 2) 12 ng; (lane 3) 25 ng; (lane 4) 50 ng; (lane 5) none. Continuous lines indicate the positions of 5S gene sequences; arrows indicate the direction of transcription; and numbered bars alongside the gels indicate the minimum boundaries of the intragenic protected regions (see also text and Figure 7).

the limits of a protected region. The horizontal bars show the positions of all the potential fragments in this region, including those which are not visible because of the differential DNAase cleavage. The data also show short unprotected sequences (positions 60–63, 73–75 and 92–93) which include four highly reproducible, intensified cleavages (positions 60, 61, 74 and 92, indicated by small arrows). The exact significance of the intensified cleavages is unknown, but they have been observed in other systems (Schmitz and Galas, 1979; Ross et al., 1979). The protected or enhanced cleavage sites within the Xbs1 and the Xbo1 gene sequences (below) are summarized in Figure 9.

A similar analysis of the Xbo1 gene 1 region extending from about positions +36 to +114 is shown in Figure 7B (lanes 1 and 2). Again the analysis reveals an intragenic protected region, the limits of which are at positions 45 and 92. Although no reproducible intensified cleavages are observed, there are unprotected regions within these boundaries at positions 49–52 and 64–67. The apparent differences in the protected regions between Xbs1 and Xbo1-gene 1 may reflect the fact that the experiments were done with fragments end-labeled on different strands for technical reasons (see Experimental Procedures and Figure 5). Asymmetric protection of two complementary strands has been reported previously (Schmitz and Galas, 1979). The current data, however, only show that the protein interacts with the same general region on the two genes, and do not indicate whether the interactions with the two genes are identical.

An analysis of the Xbo1 gene 3 region extending from positions 32 to 128 is shown in Figure 7C (lanes 1 and 2), although it is obvious that the digestion pattern is more difficult to interpret due to blurring. This effect persisted in three different preparations of end-labeled Xbo1-gene 3 fragment (Figure 5), including the same preparation of Xbo1 DNA which gave clear results for the Xbo1 gene 1 fragment (above). It is probable that length heterogeneity at the 3′ end is responsible for this phenomenon, but no improvement was obtained when labeling (end-filling) conditions were varied. The use of Taq I instead of Hha I to remove the left (5′) end of the fragment also did not alleviate the problem. In spite of this difficulty, digestion patterns in the region of gene 3 shown in Figure 7C could often be interpreted and deserve comment. In particular, the data indicate a protected region between positions 75 and 96 which is nearly identical to the region protected on the same strand of the Xbs1 gene. This region includes an intensified cleavage at position 92. Intensified cleavages are also apparent at positions 61–62; that is, at sites equivalent to those observed on the same strand in this region of the Xbs1 gene (see above). In contrast to the results observed for the Xbs1 gene and the Xbo1

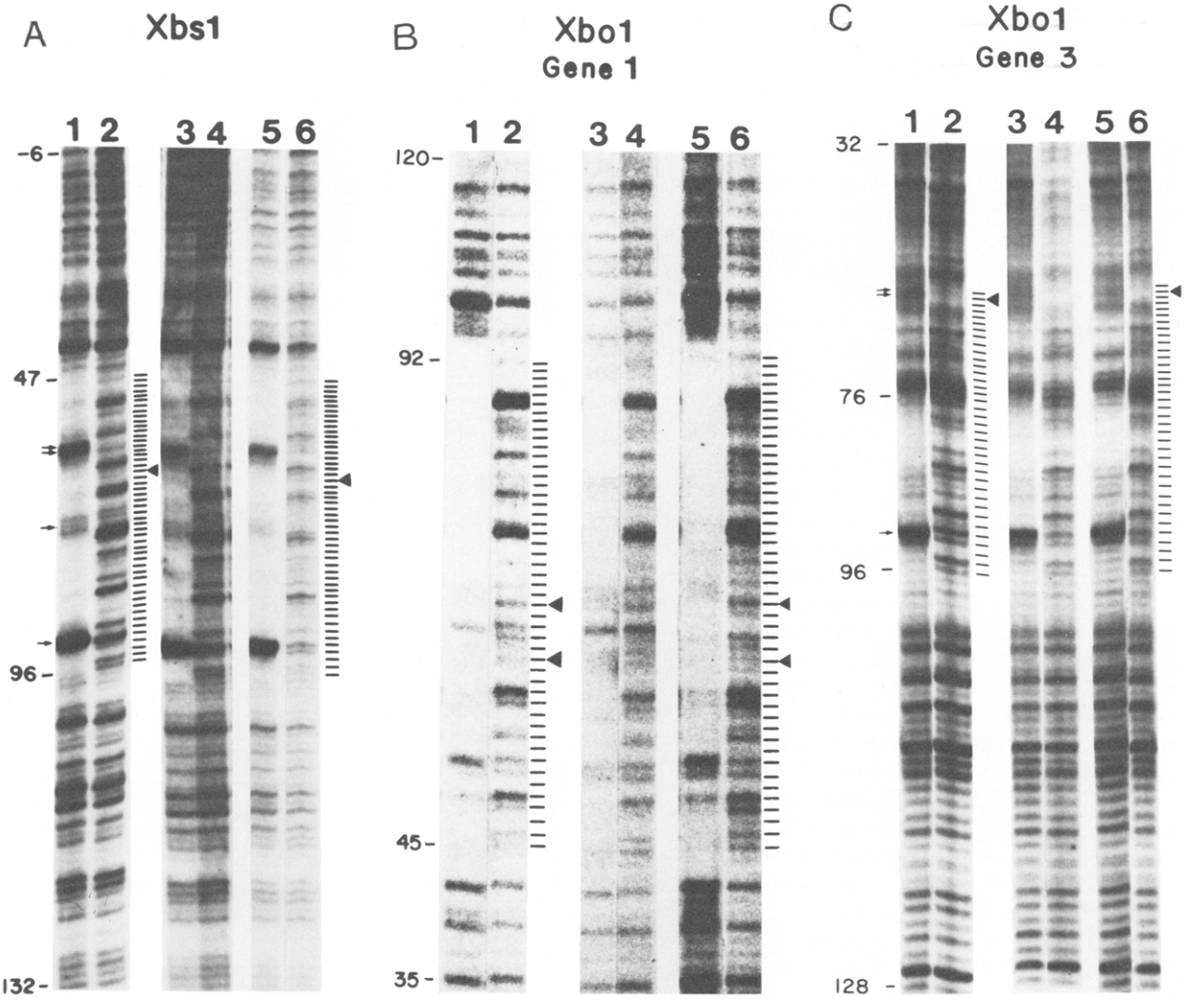

Figure 7. Xbs1, Xbo1 Gene 1 and Xbo1 Gene 3 Footprint Analyses with Purified Transcription Factor and Other Transcription Components

The Xbs gene (A), Xbo1 gene 1 (B) and Xbo1 gene 3 (C) footprints were performed with fragments which were end-labeled as summarized in the legend to Figure 5. Fragments were preincubated in the presence or absence of the various protein fractions, partially digested with DNAase and analyzed on polyacrylamide-urea sequencing gels as detailed in Experimental Procedures. Each assay contained 15 ng of the Xbs1-derived DNA fragment, 40 ng of the Xbo1-derived DNA fragment for gene 1 or 50 ng of the Xbo1-derived DNA fragment for gene 3. Digestions were performed with 1 ng (lane 1 in each panel), 4 ng (lanes 2–4 in each panel) or 130 ng (lanes 5 and 6 in each panel) DNAase. Since the various protein fractions differentially and nonspecifically reduce the level of digestion of DNA, these experimentally determined variations in DNAase concentration were necessary to give the same overall levels of digestion in the various experiments. For each DNA fragment, assay preincubations contained the following proteins in addition to 0.1 mg/ml bovine serum albumin: (lane 1) 0.1 μg purified transcription factor; (lane 2) none; (lane 3) 0.3 μg RNA polymerase III plus 0.1 μg factor; (lane 4) 0.3 μg purified RNA polymerase III; (lane 5) 20 μg egg extract protein plus 0.3 μg RNA polymerase III plus 0.1 μg factor; (lane 6) 20 μg egg extract plus 0.3 μg RNA polymerase III.

For each DNA, the autoradiographs show different lanes from the same gel. Partial Hae III digests of the end-labeled DNAs were run as length markers in adjacent lanes (not shown) and nucleotide positions counted from the known (Korn and Brown, 1978) restriction sites within the genes. Fragment band positions are indicated to the right of lanes 2 and 6. These indicate the boundaries of the protected region for Xbs1 (positions 47–96) and for Xbo1 gene 1 (positions 42–95), while in the case of Xbo1 gene 3 they extend from one end of the protected region (position 96) to the other (position 76) and beyond (to an intensified cleavage band at position 60). The positions of Hae III marker bands within the protected sequences (positions 66 of Xbs1; 61 and 66 of Xbo1 gene 1; 61 of Xbo1 gene 3) are denoted by closed triangles at the appropriate bands. Small arrows in the left margins indicate bands which are intensified in the presence of transcription factor. Numerals in the left margins are marked relative to the first nucleotide in the gene (Korn and Brown, 1978) and indicate the extents of the DNA fragments shown in the figures (outermost numerals) and the boundaries of the intragenic protected regions (inner numbers).

gene 1, however, the region between positions 47 and 73 is protected very poorly, if at all, in Xbo1 gene 3 (the differences in this region of the Xbo1 gene 3 are more clearly discerned on the original autoradiographs of several experiments, although they are difficult to see in the photographic reproductions in

Figure 6C).

Factor Interactions with 5S DNA in the Presence of Other Cellular Components

In addition to the experiments described above, we have also used the footprint methodology to search for possible interactions of other transcription components with 5S DNA sequences in the presence and absence of the purified 5S transcription factor. The other components analyzed included purified RNA polymerase III and the crude egg extract, which is known to contain additional factors necessary for the accurate transcription of the 5S gene by RNA polymerase III. The results of these experiments are shown in Figure 7 (lanes 3–6 in panels A–C) and in Figure 8. Figure 7 shows magnified views of the gene regions, while Figure 8 shows the full-length gels. The digestion patterns observed in the presence of purified RNA polymerase III alone (lane 4 in each panel of Figure 7) or in the presence of the egg extract plus RNA polymerase III (lane 6 on each panel of Figure 7 and lane 3 in each panel of Figure 8) are unchanged from the pattern produced by DNAase alone (lane 1 in each panel of Figures 7 and 8). These components (RNA polymerase and egg extracts) apparently afford a nonspecific protection from digestions, however, since increased levels of DNAase are required to achieve comparable levels of digestion of the DNA fragments (see legends to Figures 7 and 8).

The analyses also show the interactions of the factor with the Xbs1 and Xbo1 genes in the presence of purified RNA polymerase III (lane 3 in each panel of Figure 7) or in the presence of the egg extract plus purified RNA polymerase III (lane 5 in each panel of Figure 7 and lane 2 in each panel of Figure 8). Significantly, the digestion patterns observed in these cases are the same as those observed in the presence of the DNA and the factor alone. Thus under the conditions of these experiments, the 5S factor interactions appear not to be influenced by the other components.

Discussion

The availability of soluble extracts which accurately transcribe purified genes now makes it feasible to identify, isolate and characterize components involved in the transcription of specific genes. As a step toward this goal, we have purified a protein factor necessary for transcription of purified 5S genes and have determined its site of action.

Purification and Transcriptional Specificity of the Isolated Factor

Our present and previous (Roeder et al., 1979b) observations suggest that Xenopus oocytes contain multiple components which are necessary, along with those present in purified class III RNA polymerases, for transcription of 5S genes. We have purified one of these by taking advantage of the availability of unfertilized egg extracts which do not contain this factor (in a functional form) but which are otherwise competent for 5S gene transcription. The transcription factor

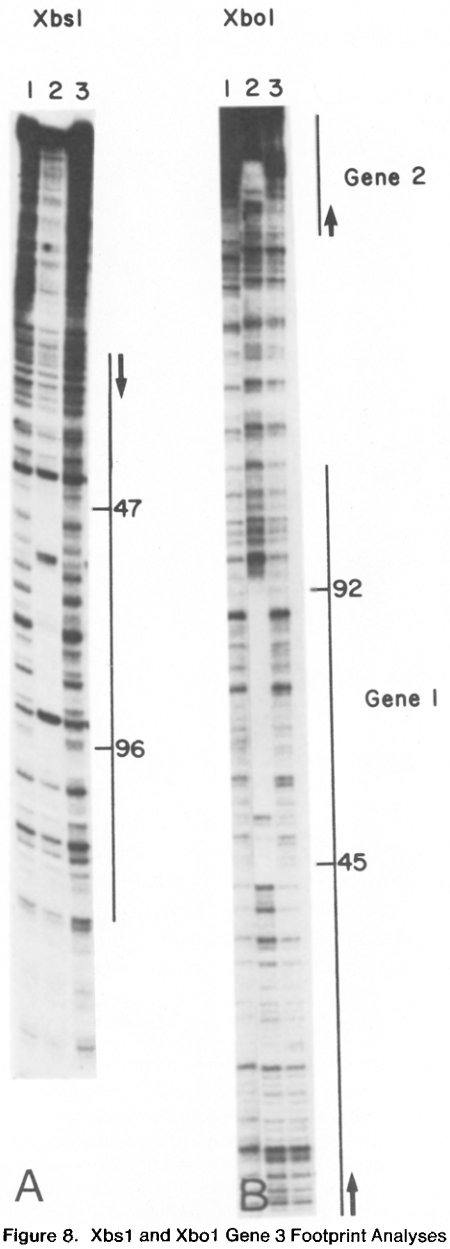

Figure 8. Xbs1 and Xbo1 Gene 3 Footprint Analyses in the Presence of Combined Transcription Components

The Xbs1 gene (A) and the Xbo1 gene 1 (B) footprints were performed with the appropriate terminal labeled fragments as in Figures 6 and 7. Each DNA was preincubated in the presence of the following transcription components: (lane 1) none; (lane 2) egg extract plus 0.3 μg RNA polymerase III plus 0.1 μg purified transcription factor; (lane 3) egg extract plus 0.3 μg purified RNA polymerase III. Continuous lines indicate the 5S gene sequences; heavy arrows indicate the direction of transcription; and numbered horizontal bars at the sides of the gels indicate the minimum boundaries of the intragenic protected regions (refer to Figure 7). The bottom of the Xbo1 gel begins with nucleotide 3 of gene 1.

apparently consists of a polypeptide of 37,000 daltons. This assignment must be considered tentative, since we have been unable to separate this component from much lower amounts of a 34,000 dalton protein. However, considerations of the stoichiometry of protein binding to 5S genes (see below) support the premise that the 37,000 dalton polypeptide (or some multimer thereof) is the active component. The native molecular weight of the transcription factor is presently unknown.

Although it is derived from oocytes, the purified factor is not specific for oocyte-type 5S genes, since it is necessary for selective transcription of both Xbs and Xbo 5S genes in egg extracts. These functional analyses are in agreement with the DNA binding studies described below. In contrast, the factor is apparently not involved in transcription of the $tRNA^{Met_1}$ gene, since egg extracts selectively transcribe this gene in the absence of the purified factor. Moreover, the level of tRNA gene transcription is unaffected by the presence of the purified factor at concentrations which maximally stimulate 5S gene transcription. Although the possible involvement of the factor in the transcription of other genes has not yet been investigated, we tentatively conclude that it is specific for 5S genes.

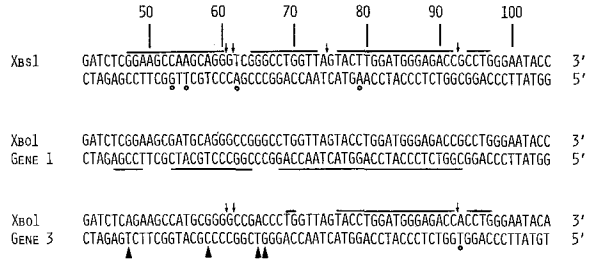

Figure 9. Comparison of Protected 5S Gene Sequences

The base sequences are shown for those regions of Xbs1 and Xbo1 DNAs which are protected from DNAase digestion in the presence of purified transcription factor. The numerals above indicate the nucleotide number counted from the 5′ end of the 5S gene product. Transcription proceeds from left to right. Sequences protected from DNAase cleavage are shown by lines above or below the appropriate (radiolabeled) strand. Arrows indicate induced or intensified DNAase cleavages in the presence of factor. Open circles indicate presumptive noncritical base substitutions, and closed triangles show base differences between the unprotected region of Xbo1 gene 3 and the corresponding protected regions of Xbo1 gene 1 and the Xbs1 gene. The left (5′) limits of Xbs1 and Xbo1 gene 1 protection, and the right (3′) limits of Xbo1 gene 1 and gene 3 protection represent minimum extensions of the protected regions and could be underestimated by 1–3 nucleotides. Some uncertainty was introduced into these assignments due to the inability of DNAase to cleave at every base in the control. The DNA sequences are from Korn and Brown (1978).

Mechanism of Action of the Factor and 5S Gene Transcription

Using the footprinting technique of Galas and Schmitz (1978), we have shown specific interactions between the purified factor(s) and both Xbs and Xbo genes. As summarized in Figure 9, these interactions are reflected in the protection (from DNAase cleavage) of specific DNA sequences *within* the gene regions. These interactions occur at nearly the same positions in both the Xbs and the Xbo (Xbo1 gene 1) genes. These results (protection of intragenic sequences) were quite surprising, but are consistent with recent observations by Brown and co-workers (Bogenhagen et al., 1980; Sakonju et al., 1980). These investigators showed that only the intragenic region extending from positions 50–83 is necessary for accurate transcription initiation at or close to position 1 of the Xbs gene. They also suggested, from more limited data, that a comparable intragenic control region is present in the oocyte-type genes. Since the factor we have purified interacts with these intragenic regulatory sequences, we suggest that it must be involved in transcription initiation. This hypothesis is also consistent with the failure to observe any significant 5S DNA transcription in egg extracts in the absence of the factor. As discussed by Brown and co-workers, the presence of an intragenic control region which in large part determines the site of initiation is in striking contrast to the situation in procaryotes, where promoter regions are localized 5′ to the initiation site.

At present we cannot be certain of precisely which base pairs within the intragenic control region are critical for recognition and/or transcription initiation, but a comparison of base sequences between the Xbs and Xbo genes provides some suggestions. As shown in Figure 9, those positions within the binding site denoted with small circles on the Xbs gene are probably not critical, since base substitutions at those positions in the Xbo1 gene 1 have no discernable deleterious effects on factor interactions or transcription. On the other hand, one or more of the region 45–76 positions which differ in Xbo1 gene 3 (indicated by solid triangles in Figure 9) may be critical for efficient factor binding. This is suggested by the observation that this region (45–76) is protected from DNAase digestion in Xbo1 gene 1 and Xbs1, but not in Xbo1 gene 3, while an adjacent region (76–96) is protected in all cases. The present data provide no further information regarding specific sequences important for factor interactions. Others, however, (Bogenhagen et al., 1980) have speculated that a sequence (AGCAGGGT) present in the intragenic control region of Xbs1 and conserved within other genes transcribed by RNA polymerase III may be of functional significance for transcription initiation within all those genes. Our own observations on the functional specificity of this factor for 5S genes make this seem less probable, although they do not exclude the possibility that the factor reported here might interact with these other genes. This possibility is currently under investigation. It should also be emphasized that at least some sequences within these genes must be related to RNA function, which complicates interpretations of the significance of such sequences in transcription.

Several considerations suggest that more than one 37,000 dalton polypeptide may bind per 5S gene, either individually or as a multimer. First, a helical region of 45–50 bp is apparently too large to be protected by a single 37,000 dalton polypeptide, by comparison with the lengths of DNA protected by E. coli RNA polymerase and the tetrameric *lac* repressor (Schmitz and Galas, 1979). Second, the protected region in Xbo1 gene 3 (positions 76–96) is approximately one half the size of the protected region in Xbo1 gene 1 (45–92) or Xbs1 (47–96). This could reflect a double binding site, although factor titration studies failed to detect any preferential binding to one part of the protected region. Moreover, no obvious sequence is repeated in the 5′ (45–74) and 3′ (76–96) protected regions of the gene, nor is the digestion profile particularly symmetrical. Thus it is unclear whether the partial digestion (protection) pattern observed for Xbo1 gene 3 is due to aberrant binding at a single site or to efficient binding to only one of two sites. Third, maximal DNAase protection is observed at a ratio of ten to twelve 37,000 dalton polypeptides per gene, although the fraction of the polypeptides actually bound is unknown. (The corresponding ratio for the minor 34,000 dalton polypeptide is approximately 0.5, which argues that this protein is by itself insufficient to account for the DNAase protection).

In contrast to our results with the purified factor, we have failed to observe any interactions dependent upon the presence of purified RNA polymerase III or other components present in the egg extracts with 5S DNA. These results could be explained by the presence of limiting amounts of RNA polymerase III or other transcription factors, or by the failure to duplicate transcription conditions in the assays. Alternatively, it may be that the other interactions are simply too weak or transient to be seen by the present methodology. The fact that the RNA polymerase III experiments were conducted at an enzyme:DNA molar ratio of 2–4 argues against the first possibility (although it could be argued that only a fraction of the enzyme molecules are functional). We have also failed to detect any RNA polymerase:DNA interactions (in the presence or absence of factor), even when all four ribonucleoside triphosphates (or combinations thereof) are included in the incubation mixtures (data not shown).

Keeping in mind the complications suggested above, the most straightforward explanation of all the binding studies is that of all the 5S transcription components, the (purified) factor binds most tightly to the gene. The strength of this interaction is further underscored by the fact that it is detected even in the presence of crude egg extracts (supplemented with factor) or concentrated oocyte extracts (our unpublished observations) which contain many other DNA binding proteins. These observations, plus considerations of the large size (approximately 700,000 daltons) of the RNA polymerase III (Sklar and Roeder, 1976; R. G. Roeder, manuscript submitted) suggest a simple model for transcription initiation; namely, that the factor provides the initial DNA recognition event and that its binding directs the subsequent interaction of RNA polymerase III with the complex, such that it is properly oriented for initiation. The apparent requirement for still another transcription factor(s) in the cell-free systems obviously suggests that this model is oversimplified.

Regulation of 5S RNA Synthesis

The factor described here is apparently not directly responsible for the developmental regulation of the relative rates of transcription of the oocytic versus the somatic-type 5S genes. It might well be involved, however, as a general regulator of 5S RNA synthesis either in oocytes or in somatic cells. Its apparent absence (in a functional form) in unfertilized eggs suggests that it may in part regulate 5S synthesis during early embryonic development. A rapid (possibly irreversible) inactivation of an essential component could efficiently curtail 5S RNA synthesis. Additionally, the elaboration of factors specific for one type of class III gene versus another (for example, 5S versus tRNA) would allow independent regulation of these genes; such regulation has been observed in Xenopus embryos (Brown and Littna, 1966).

It should also be stressed that the isolation of the factor described here is only an early step in understanding 5S gene regulation. Apart from understanding the action of this factor in more detail, it will be necessary to define and analyze the other factors implicated in the transcription of purified 5S genes. Currently, it appears that there are at least two such components (our unpublished observations), although these have neither been purified nor assigned specific functions (for example, at initiation versus termination). Additionally, it will be necessary to identify components involved in the developmental regulation of somatic versus oocyte genes. The cell-free systems described to date do not show such regulation (Roeder et al., 1979b; Weil et al., 1979a, 1979b), indicating the existence of regulatory systems or components which may operate at steps (for example, chromatin structural modifications) prior to those which are mediated by the factors described here.

Finally, it appears rather significant that the factor interactions described here can be visualized in very crude cellular extracts. This raises the strong possibility that it may be possible to detect other factors, which interact with specific DNA sequences, without prior purification.

Experimental Procedures

Preparation of Unfertilized Egg and Ovarian Soluble Extracts

Unfertilized eggs were obtained from adult female Xenopus laevis as follows. The dorsal lymph sacs of several toads were injected twice

with 100 units of pregnant mare serum gonadotropin (Sigma) on days 1 and 5 and with 1000 units of human chorionic gonatotropin (Sigma) on day 8. After the last injection, eggs were collected overnight and dejellied (Laskey, Mills and Morris, 1977). Mature ovaries were dissected from adult female X. laevis, rinsed once in cold 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0) and once in cold homogenization buffer [50 mM Tris–HCl (pH 7.9), 25% glycerol, 50 mM KCl, 0.10 mM EDTA, 2 mM dithiothreitol] and minced with scissors. Unfertilized eggs or ovarian tissue were then dispersed in 2 vol of homogenization buffer with several strokes of a Teflon pestle in a Kontes glass homogenizer. High speed supernatant (S-100) fractions were prepared from these homogenates as described by Ng et al. (1979).

Transcription Assays

Assays for transcription of recombinant plasmid DNAs containing 5S RNA and tRNA genes were performed as described by Ng et al. (1979). The incubations contained, in a total volume of 50 μl: 40 mM Tris–HCl (pH 7.9) or 10 mM HEPES (pH 7.5), 10% glycerol, 5 mM $MgCl_2$, 0.5 mM DTT, 70 mM KCl or 25 mM $(NH_4)_2SO_4$, 600 μM each of ATP, CTP and UTP, 26 μM α-^{32}P-GTP (4 mCi/mmole, ICN) and 10 μg/ml plasmid DNA. Up to 24 μl of the oocytic fractions and 1 μl (0.05–0.2 μg) purified ovarian RNA polymerase III (where indicated) were added to the other components to start the reactions. For the egg extract complementation assays, 10–20 μl of egg S-100 were included in addition to the oocyte sample to be assayed. After incubation for 60 min at 30°C, the reaction mixtures were terminated and subjected to a phenol-chloroform extraction procedure. After precipitation with 70% ethanol, the RNA samples were resuspended in 50% formamide and subjected to electrophoresis on 12% polyacrylamide slab gels in 0.09 M Tris–borate (pH 8.3), 2.5 mM EDTA buffer for 1 hr at 300 V (Ng et al., 1979). The gels were then dried and used to expose Kodak XR5 or BB5 film on DuPont Cronex intensifying screens.

Recombinant Plasmid DNA

The recombinant plasmids containing 5S DNA were obtained from D. D. Brown (Carnegie Institution of Washington, Baltimore) and J. L. Doering (Northwestern University). The plasmid pXbo3 contains six X. borealis oocyte-type genes (types 1 and 3); the plasmid pXbo1 contains three X. borealis oocyte-type genes (types 1, 2 and 3); and the plasmid pXbs1 contains one X. borealis somatic type gene (Doering, 1977; Korn and Brown, 1978). A recombinant plasmid containing a single $tRNA_i^{Met}$ gene was constructed and provided by A. Lassar of this laboratory. This plasmid (designated $pXItmet_1$) was constructed by the addition of Eco RI linkers to the large Msp I fragment of λt210 (Clarkson, Kurer and Smith, 1978) and the subsequent insertion of this fragment into the Eco RI site of pBR322. All plasmids were propagated in E. coli HB101, and plasmid DNAs were purified as described by Ng et al. (1979). The cloning and growth of recombinant plasmids was carried out according to the NIH Guidelines (P2/EK1 containment).

Purified Enzymes

Xenopus laevis oocyte RNA polymerase III was purified as described elsewhere (R. G. Roeder, manuscript submitted; D. R. Engelke and R. G. Roeder, manuscript submitted). Both enzyme preparations were more than 90% homogeneous and had the same polypeptide composition (compare Parker et al., 1976). The restriction endonuclease Hind III was provided by M. Green of this laboratory. Other restriction endonucleases and the DNA polymerase I large subunit (Klenow and Henningsen, 1970) were supplied by W. Barnes of this institution. RNAase-free pancreatic DNAase I was from Worthington.

Fractionation of Soluble Ovarian Extract

The ion exchangers DEAE-cellulose (Whatman DE-52), DEAE-Sephadex (A-25, Pharmacia), phosphocellulose (Whatman P-11) and Bio-Rex 70 (Bio-Rad) were prepared and used as described by Schwartz and Roeder (1974). All chromatography steps were performed in columns with a height-to-width ratio of 2–3 to 1. Columns were equilibrated immediately prior to use with the buffer in which the sample was applied. Chromatography Buffer A is 20 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA and 1.0 mM dithiothreitol. Buffer B is equivalent to Buffer A with 0.5 mM spermine in place of 5 mM $MgCl_2$. All operations were performed at 0–6°C.

A soluble oocyte extract from approximately 100 g of mature ovarian tissue was prepared as described above. To 120 ml of extract was added 36 g of solid ammonium sulfate. The resulting precipitate was collected by centrifugation for 90 min at 95,000 × g and resuspended in Buffer A to a final ammonium sulfate concentration of 50 mM. This fraction was applied to an 80 ml DEAE-cellulose column. The column was washed with Buffer A containing 45 mM ammonium sulfate and step-eluted with Buffer A containing 125 mM ammonium sulfate. Step fractions containing the transcription factor activity (and about one fifth of the input protein) were combined, readjusted to 125 mM ammonium sulfate and passed through an 80 ml DEAE-Sephadex column to remove RNA polymerase III and residual nucleic acids. The flow-through fraction was dialyzed extensively against Buffer B containing 100 mM ammonium sulfate and then applied to a 25 ml phosphocellulose column. The column was washed with Buffer B containing 95 mM ammonium sulfate and eluted with a 5 column volume linear 95–700 mM ammonium sulfate gradient. Column fractions were dialyzed against Buffer B containing 50 mM ammonium sulfate and frozen after the removal of aliquots for activity assays. The peak activity fractions, which eluted between 300–600 mM ammonium sulfate, were thawed, combined and applied to a 4 ml Bio-Rex 70 column. This column was washed with Buffer A containing 50 mM ammonium sulfate and eluted with a 5 column volume linear 50–500 mM ammonium sulfate gradient. Chromatographic fractions were assayed for transcription factor activity and stored at −70°C, where the activity was stable for several months. The protein composition of appropriate fractions was determined by electrophoresis on 5–15% polyacrylamide gels in the presence of sodium dodecylsulfate (Jaehning, Woods and Roeder, 1977).

DNAase Digestion of DNA Fragments

Binding of the transcription factor to 5S DNA sequences was examined by a modification of the "footprinting" techniques of Galas and Schmitz (1979). DNA fragments containing somatic or oocytic 5S rDNA were released from plasmids pXbs1 and pXbo1 by digestions with restriction endonuclease Hind III and purified by preparative electrophoresis on 1.2% agarose gels in Tris–acetate buffer [40 mM Tris–HCl, 5 mM sodium acetate, 1.0 mM EDTA (pH 7.8)]. The released Xbo1 and Xbs1 fragments were extracted from the gels with $NaClO_4$ and purified by hydroxyapatite chromatography as described by Southern (1975). For examining DNAase protection of regions around the somatic gene and oocytic genes 1 and 3, subfragments were generated as described in Figure 5. DNA fragments (2–5 μg) were end-labeled in a 50 μl reaction containing 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 μCi α-^{32}P-dATP or dCTP, 20 μM each of the remaining deoxyribonucleotide triphosphates, 2–5 μg DNA and Klenow enzyme. After incubation for 1 hr at 25°C, labeled DNA fragments were separated from nucleotides by gel filtration on Sephadex G-100 in 2.5 mM Tris–HCl (pH 7.4), 1 mM NaCl. Fragments were lyophilized, resuspended in the same buffer and stored at −20°C.

For the DNAase protection assays, 15–40 ng of end-labeled DNA fragment were pre-incubated for 10 min at 25°C with the indicated protein fractions. Other reaction conditions were identical to those used for the transcription assays, except for the absence of ribonucleoside triphosphates. After preincubation, 2 μl of DNAase (1–130 ng) were added and incubations continued for 30 sec. Reactions were terminated by adding 8 μl 15 mM EDTA, 0.2% sodium dodecylsulfate and incubating at 100°C for 2 min to denature proteins bound to the DNA. Sample volumes were increased to 50 μl with sterile distilled water, extracted with phenol and chloroform (as for RNA samples) and dried in tubes under vacuum. For electrophoretic analyses, the samples were resuspended in 98% formamide, incubated at 100°C for 2 min and run 2 hr at 1300 V on 6 or 8% polyacrylamide-urea sequencing gels (Sanger and Coulson, 1978) with Tris–borate

electrode buffer (see above). Autoradiographic exposures of the dried gels were made with Kodak XR 5 film and DuPont Cronex intensifying screens.

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References

Birkenmeyer, E. H., Brown, D. D. and Jordan, E. (1978). A nuclear extract of Xenopus laevis oocytes that accurately transcribes 5S RNA genes. Cell *15*, 1077–1086.

Bogenhagen, D. F., Sakonju, S. and Brown, D. D. (1980). A control region in the center of the 5S RNA gene directs specific initiation of transcription: II. The 3′ border of the region. Cell *19*, 27–35.

Brown, D. D. and Littna, E. (1966). Synthesis and accumulation of low molecular weight RNA during embryogenesis of *Xenopus laevis*. J. Mol. Biol. *78*, 397–415.

Brown, D. D., Korn, L. J., Birkenmeyer, E., Peterson, R. and Sakonju, S. (1979). The *in vitro* transcription of 5S DNA. In Eukaryotic Gene Regulation, R. Axel, T. Maniatis and C. F. Fox, eds. (New York: Academic Press), pp. 511–519.

Burgess, R. R. (1969). Separation and characterization of the subunits of ribonucleic acid polymerase. J. Biol. Chem. *244*, 6168–6176.

Clarkson, S. G., Kurer, V. and Smith, H. O. (1978). Sequence organization of a cloned $tDNA_1^{met}$ fragment from Xenopus laevis. Cell *14*, 713–724.

Doering, J. (1977). The structure of *X. borealis* oocyte and somatic 5S DNAs. Carnegie Inst. Washington Year Book *76*, 120–125.

Galas, D. and Schmitz, A. (1978). DNase footprinting: a simple method for the detection of protein-DNA binding specificity. Nucl. Acids Res. *5*, 3157–3170.

Jaehning, J. A., Woods, P. S. and Roeder, R. G. (1977). Purification, properties and subunit structure of DNA-dependent RNA polymerase III from adenovirus 2-infected KB cells. J. Biol. Chem. *252*, 8762–8771.

Klenow, H. and Henningsen, I. (1970). Selective elimination of the exonuclease activity of the deoxyribonucleic acid polymerase from *Escherichia coli* B by limited proteolysis. Proc. Nat. Acad. Sci. USA *65*, 168–175.

Korn, L. J. and Brown, D. D. (1978). Nucleotide sequence of Xenopus borealis oocyte 5S DNA: comparison of sequences that flank several related eucaryotic genes. Cell *15*, 1145–1156.

Kressman, A., Clarkson, S. G., Pirrotta, V. and Birnstiel, M. L. (1978). Transcription of cloned tRNA gene fragments and subfragments injected into the oocyte nucleus of *Xenopus laevis*. Proc. Nat. Acad. Sci. USA *75*, 1176–1180.

Laskey, R. A., Mills, A. D. and Morris, N. R. (1977). Assembly of SV40 chromatin in a cell-free system from Xenopus eggs. Cell *10*, 237–243.

Ng, S. Y., Parker, C. S. and Roeder, R. G. (1979). Transcription of cloned *Xenopus* 5S RNA genes by *X. laevis* RNA polymerase III in reconstituted systems. Proc. Nat. Acad. Sci. USA *76*, 136–140.

Parker, C. S. and Roeder, R. G. (1977). Selective and accurate transcription of 5S RNA genes in isolated chromatin by purified RNA polymerase III. Proc. Nat. Acad. Sci. USA *74*, 44–48.

Parker, C. S., Ng, S. Y. and Roeder, R. G. (1976). Selective transcription of the 5S genes in isolated chromatin by RNA polymerase III. In Molecular Mechanisms in the Control of Gene Expression, D. P. Nierlich, W. J. Rutter and C. F. Fox, eds. (New York: Academic Press), pp. 223–242.

Roeder, R. G. (1974). Multiple forms of DNA-dependent RNA polymerase in *Xenopus laevis*: levels of activity during oocyte and embryonic development. J. Biol. Chem. *249*, 249–256.

Roeder, R. G. (1976). Eukaryotic nuclear RNA polymerases. In RNA Polymerases, R. Losick and M. Chamberlin, eds. (New York: Cold Spring Harbor Laboratory), pp. 285–329.

Roeder, R. G., Engelke, D. R., Luse, D. S., Ng, S. Y., Segall, J., Shastry, B. S. and Weil, P. A. (1979a). Studies of the transcription of specific class II and class III eukaryotic genes in reconstituted cell-free systems. In RNA Polymerases, Transfer RNA, and Ribosomes: Their Genetics and Evolution, S. Osawa, ed. (Tokyo: University of Tokyo Press), in press.

Roeder, R. G., Engelke, D. R., Harris, B., Ng, S. Y., Segall, J., Shastry, B. S. and Weil, P. A. (1979b). Factors involved in the transcription of purified genes by RNA polymerase III. In Eukaryotic Gene Regulation, R. Axel, T. Maniatis and C. F. Fox, eds. (New York: Academic Press), pp. 521–540.

Ross, W., Landy, A., Kikuchi, Y. and Nash, H. (1979). Interaction of Int protein with specific sites on λ *att* DNA. Cell *18*, 297–307.

Sakonju, S., Bogenhagen, D. F. and Brown, D. D. (1980). A control region in the center of the 5S RNA gene directs specific initiation of transcription: I. The 5′ border of the region. Cell *19*, 13–25.

Sanger, F. and Coulson, A. R. (1978). The use of thin acrylamide gels for DNA sequencing. FEBS Letters *87*, 107–110.

Schmitz, A. and Galas, D. (1979). The interaction of RNA polymerase and *lac* repressor with the *lac* control region. Nucl. Acids Res. *6*, 111–137.

Schwartz, L. B. and Roeder, R. G. (1974). Purification and subunit structure of DNA-dependent RNA polymerase I from the mouse myeloma. J. Biol. Chem. *249*, 5898–5906.

Southern, E. M. (1975). Long range periodicities in mouse satellite DNA. J. Mol. Biol. *94*, 51–69.

Weil, P. A., Luse, D. S., Segall, J. and Roeder, R. G. (1979a). Selective and accurate initiation of transcription at the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell *18*, 469–484.

Weil, P. A., Segall, J., Harris, B., Ng, S. Y. and Roeder, R. G. (1979b). Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates. J. Biol. Chem. *254*, 6163–6173.

Wu, G. J. (1978). Adenovirus DNA directed transcription of 5.5S RNA *in vitro*. Proc. Nat. Acad. Sci. USA *75*, 2175–2179.