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Differential Kinetics of Transcription Complex Assembly Distinguish Oocyte and Somatic 5S RNA Genes of *Xenopus*

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Differential transcription of the *Xenopus* gene families encoding the oocyte and somatic 5S ribosomal RNAs can be reproduced in vitro with cell-free extracts prepared from *Xenopus* oocytes and unfertilized eggs. The transcriptional activities of these genes as assayed in these in vitro systems are a consequence of large differences in the rates of assembly of active transcription complexes. The somatic 5S genes sequester limiting transcription factors much more rapidly than the corresponding oocyte 5S genes and, as a consequence, are far more active. However, once transcription complexes are formed, these complexes are stable on both of these genes. Previous studies have established that transcription factors IIIA and IIIC are sufficient to form a stable protein-DNA complex on the somatic 5S gene. The rate of formation of the stable TFIIIA + C complex for the oocyte gene is far slower than that for the somatic 5S gene. Insertion of the DNA binding site for TFIIIC2 (the B-block promoter element from tRNA genes) into the 3′ flanking region of a synthetic oocyte 5S gene increases the transcription efficiency and rate of transcription complex assembly of this gene relative to the parent gene lacking the B-block element. Our results support a model in which competition for limiting transcription factors plays a pivotal role in establishing differential transcription of the two classes of 5S genes during early embryogenesis.

5S RNA genes Pol III transcription TFIIIA TFIIIC

TRANSCRIPTIONAL regulation of the 5S ribosomal RNA genes of the frog *Xenopus laevis* has been a subject of intensive research in several laboratories for nearly two decades [reviewed in (33,35)]. Both the highly abundant oocyte 5S RNA gene family, with 20,000 copies per haploid genome, and the somatic 5S RNA gene family, with 400 copies per haploid genome, are actively transcribed during oogenesis, giving rise to approximately equal numbers of transcripts on a per gene basis from the two gene families. Upon oocyte maturation, transcription by RNA polymerase III ceases and only resumes after fertilization at the mid-blastula stage of embryogenesis. The repression of transcription observed upon meiotic maturation of the oocyte is likely due to protein phosphorylation of a component of the general class III gene transcription factor TFIIIB by the cdc2-cyclin B kinase [maturation promoting factor (MPF)] (9,32). At the mid-blastula transition, 50 times more 5S RNA molecules are produced from the somatic genes than from the oocyte genes (on a per gene basis). This somatic gene bias increases as development proceeds such that essentially complete repression of the oocyte gene family is seen in later stage embryos and in the somatic cells of the adult organism. Transcription experiments performed in vitro with chromatin templates isolated from somatic cells showed that active transcription complexes are present on the somatic genes whereas the oocyte genes are transcriptionally inert and presumably are not complexed with transcription factors (5,14,23,25). These studies have also implicated histone H1 as a

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major chromatin determinant for repression of the oocyte genes (25). Wolffe and colleagues have provided convincing in vivo evidence that repression of the oocyte 5S genes is due to the chromatin environment of these genes and, in particular, to histone H1-mediated repression (6). These studies provide compelling evidence for the mechanism whereby the repressed state of the oocyte genes is maintained in somatic cells, but these studies do not fully address the mechanism(s) whereby the differential transcription of the two classes of 5S genes is established during early embryogenesis.

In vitro studies have demonstrated that 5S RNA genes of both classes require a similar set of *trans*-acting protein factors, including the 5S gene-specific factor TFIIIA and the general class III factors TFIIIB and TFIIIC, as well as RNA polymerase III for transcription (31). Further, the differential transcription of these two gene families can be reproduced in vitro with a variety of cell-free extracts prepared from oocytes, unfertilized eggs, or embryos (19–21,36). Wolffe and Brown (35,36) have provided evidence indicating that transcription complexes formed on oocyte 5S RNA genes in a low-speed extract prepared from unfertilized eggs are not stable whereas transcription complexes formed on somatic 5S RNA genes are stable. These authors suggest that the selective loss of transcription complexes from the oocyte genes leads to the assembly of these genes into transcriptionally inactive chromatin domains whereas active transcription complexes persist on the somatic genes. In contrast, Seidel and Peck (27) showed that differential transcription of the 5S RNA genes in a whole oocyte extract is due to different rates of transcription complex assembly. Seidel and Peck also suggest that, once formed, transcription complexes on both somatic and oocyte 5S RNA genes exhibit the same remarkable stability of class III transcription complexes as originally defined by Brown and colleagues (5,8).

An explanation for the difference between the results of Wolffe and Brown and those of Seidel and Peck is that the former authors used a single brief incubation time in their experimental protocol for stable transcription complex formation. It is conceivable that this time period was not sufficient to allow for transcription complex assembly on the oocyte genes. However, different extracts were used by the two groups and hence these two studies might not be directly comparable. In the present communication, we have undertaken a reexamination of differential transcription of the two classes of 5S genes in the unfertilized egg extract. Our results suggest that the preferential transcription of the somatic genes in this extract is due to differences in the rates of transcription complex assembly on the two classes of 5S genes. We further explore the DNA sequence elements and protein factors responsible for this difference in rates of transcription complex assembly. Our results are in full agreement with a model for differential transcription of the 5S genes based on competition for limiting transcription components and different affinities for common transcription factors (33).

MATERIALS AND METHODS

DNA Templates

The following plasmids containing the 5S rRNA genes from *Xenopus laevis* have been described in detail elsewhere: the somatic 5S RNA gene [pXls11 (22)]; the major oocyte 5S RNA gene [pXloΔ3′ + 176 (4)]; oocyte and somatic 5S genes with heterologous 5′ and/or 3′ flanking sequences (27); and synthetic oocyte and somatic 5S genes lacking natural flanking sequences cloned in pUC18 (38). The transcripts of the synthetic 5S genes are the 121-nucleotide-long 5S RNA and a 194-nucleotide-long read-through transcript. This longer transcript is due to a mutation in the natural terminator sequence in these clones (24), and transcription by RNA polymerase III terminates at a downstream T-run in the pUC18 vector sequence (13). Ratios of transcripts per gene in reactions with the synthetic genes are corrected for the larger number of G residues in the longer transcripts (36 in the 121-nucleotide-long somatic 5S RNA and 51 in the 194-nucleotide-long transcript of the synthetic genes). A double-stranded oligonucleotide, corresponding in sequence to the consensus B-block element within tRNA-like class III genes (12,31), was inserted into the *Bam*HI site of the synthetic oocyte 5S gene, and the sequence of the resulting clone was confirmed. The B-block sequence was in the 5′ to 3′ orientation relative to the 3′ end of the 5S gene sequence. The major transcripts from this clone are 220 nucleotides in length, due to the insertion of the B-block sequence between the 3′ end of the 5S gene and the pUC18 T-run terminator.

Unfertilized Egg Extracts

Low speed cytosolic extracts from calcium ionophore A23178-activated, unfertilized *Xenopus laevis* eggs were prepared as described (3), with the modification that after the first centrifugation

step at 9000 rpm in the SW50Ti rotor, the extract was further clarified by centrifugation in a microfuge for 15 min prior to freezing aliquots (50 μl) in liquid nitrogen. High-speed egg extracts were prepared either as described (10) or by subjecting the low-speed extract to centrifugation for 90 min at 45,000 rpm in the SW50Ti rotor.

Transcription Factors and Reaction Conditions

TFIIIA was purified from immature oocytes as described (30), with the exception that heparin sepharose was used in place of Bio-Rex 70 (7). Partial purification of transcription factors TFIIIB and TFIIIC and RNA polymerase III from oocyte S-150 extracts (20) was carried out using phosphocellulose chromatography as previously described (26). TFIIIB was further fractionated by sequential chromatography on DEAE Sephadex and Mono Q fast protein liquid chromatography as described (9,29). Transcription reaction conditions were as described previously (13). Briefly, for reconstituted transcription reactions, each reaction contained in a final volume of 20 μl, 9–18 ng of TFIIIA (generally yielding a 1.5- to 3-fold molar excess of TFIIIA over 5S genes), 6 μl of phosphocellulose C fraction (approximately 6 μg total protein, containing TFIIIC and RNA polymerase III), and 2 μl of TFIIIB (approximately 200 ng total protein). Volumes of low- and high-speed egg extracts used in transcription reactions are indicated in the figure legends and these reactions also contained 9 μg/ml TFIIIA. Nucleoside triphosphates were included at final concentrations of 0.6 mM for ATP, UTP and CTP; GTP was included at a concentration of 0.02 mM along with 10 μCi of [α-^{32}P]GTP. Reactions were performed at final concentrations of 12 mM HEPES-OH (pH 7.5), 60 mM KCl, 6 mM $MgCl_2$, 25 μM $ZnCl_2$, and glycerol at 6–8% (v/v). Reactions were for the time periods indicated in the figure legends at ambient temperature and RNA was purified by extraction with RNAzol (Tel-Test, Inc., Friendswood, TX). RNAs were analyzed by electrophoresis on 0.35-mm-thick 6% polyacrylamide sequencing gels containing 8.3 M urea or on partially denaturing polyacrylamide gels (12.5%, w/v, at 29:1 acrylamide to bisacrylamide, measuring 40 cm in length by 0.35 mm in thickness) containing 2.5 M urea (14,21,27). The latter gels were prerun at 1200 V for 2–4 h prior to electrophoresis for 15 h at 500 V (5 mA). This gel system separates oocyte and somatic 5S RNAs of identical size (121 nucleotides) that differ in sequence at five positions. The difference in gel mobility of the two species of 5S RNAs is presumably due to differences in secondary structure under partially denaturing conditions (14). The oocyte gene gives rise to the 121-nucleotide-long transcript and two additional longer RNA transcripts due to inefficient termination at the first T-run at the 3′ end of the gene (4). For quantitation of the relative transcription efficiencies of two 5S gene templates in the same reactions, autoradiograms (taken within the linear range of the X-ray film) were scanned with an LKB laser densitometer or dried gels were analyzed by storage phosphorimage autoradiography and data analysis was performed using ImageQuant software (Molecular Dynamics).

RESULTS

Transcriptional Activities of the Oocyte and Somatic 5S RNA Genes in the Low-Speed Egg Extract

Differential transcription of the oocyte and somatic 5S RNA genes in the low-speed extract prepared from *Xenopus* eggs (3,36) was monitored as a function of the input ratio of the two genes. In experiments not shown, we determined that 300 ng of the oocyte 5S gene plasmid DNA in a 20 μl reaction (containing 5 μl of extract) gave maximal levels of transcription. Thus, the total DNA concentration in each reaction was held at 15 μg/ml and the ratios of the two genes was varied in separate reactions. Figure 1 shows an autoradiogram of a partially denaturing polyacrylamide gel that was used to separate and quantitate the transcripts from the two types of 5S RNA genes (14,21,27). In this gel system, the somatic gene gives rise to two sets of bands (denoted S in Fig. 1) whereas the same somatic 5S RNA gives rise to a single band when analyzed on a denaturing polyacrylamide gel (Fig. 3); thus, the two somatic RNA bands reflect conformational isomers of the same RNA which are resolved on the partially denaturing gel. The oocyte gene gives rise to three sets of bands (denoted O) due to inefficient termination of transcription at the 3′ end of the oocyte gene (4). The relative amounts of transcripts generated by the two genes was determined by phosphorimage analysis. In agreement with previous studies (36), when these mixtures of oocyte and somatic 5S RNA genes were incubated in the egg extract, the somatic genes showed a ~50-fold higher level of transcription on a per gene basis than the oocyte genes (S:O ratio = 50:1). This ratio is largely independent of the input ratio of the two gene types. We observe significantly higher levels of 5S RNA

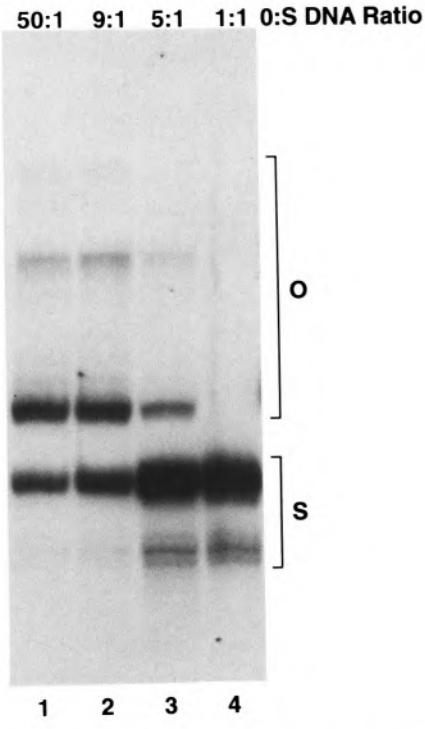

FIG. 1. Transcriptional activity of oocyte and somatic 5S genes in a low-speed extract prepared from unfertilized *Xenopus* eggs. The following DNA mixtures were incubated with TFIIIA (18 ng) for 15 min prior to the addition of extract. After an additional 30-min incubation, nucleoside triphosphates were added and transcription was allowed to proceed for 2 h. Lane 1: 294 ng oocyte, 6 ng somatic; lane 2: 272 ng oocyte, 30 ng somatic; lane 3: 250 ng oocyte, 50 ng somatic; lane 4: 150 ng each DNA. The positions of oocyte (O) and somatic (S) 5S RNA transcripts, separated on a partially denaturing polyacrylamide gel, are indicated and the input ratio of oocyte to somatic 5S genes in each reaction is shown.

gene transcription with the low-speed egg extract when the 5S RNA gene-containing plasmid DNAs are incubated with TFIIIA prior to addition of the extract; however, the relative ratios of oocyte and somatic 5S RNA transcripts remain constant whether the DNA is preincubated with TFIIIA prior to addition to the extract or the DNA templates are added to the extract supplemented with TFIIIA (data not shown). Wolffe and Brown (36), however, found that the S:O ratio observed with their low-speed extracts was highly dependent upon TFIIIA concentration and very high concentrations of TFIIIA (1 mg/ml) were required for oocyte 5S transcription. In our experiments, we used only an approximately threefold molar excess of TFIIIA over 5S genes (9 μg/ml). Thus, our present extracts and those prepared by Wolffe and Brown (36) differ significantly in this requirement for TFIIIA.

Rate of Formation of Stable Transcription Complexes

As discussed above, Wolffe and Brown (36) suggested that the difference in transcriptional activity of the two gene types in the low-speed egg extract is a consequence of differential stability of transcription complexes formed on these genes. The direct test for the formation of a stable transcription complex is a template exclusion assay (5). Briefly, one DNA template is preincubated with transcription factors or extract prior to the addition of a second DNA template. Transcriptionally active templates are then assayed by a subsequent incubation in the presence of radiolabeled nucleoside triphosphates and analysis of the labeled RNAs transcribed from the respective genes. Active transcription from the first template, but not the second, implies the formation of stable transcription complexes on the first gene. In the experiments reported by Wolffe and Brown (36), a 30-min incubation of a plasmid DNA containing a somatic 5S RNA gene was shown to exclude transcription from a subsequently added oocyte 5S RNA gene template. In contrast, an identical incubation of a plasmid DNA containing an oocyte 5S RNA gene did not exclude transcription from a second somatic 5S RNA gene template. We examined the time required to form stable complexes on both the oocyte and somatic 5S RNA genes in the low-speed extract: when the somatic gene was the first DNA added to the extract, transcription of the secondarily added oocyte gene was excluded after only brief incubation periods (Fig. 2A, lane 2, 30 min). In contrast, if the oocyte gene was incubated with the extract for the same 30-min period (Fig. 2A, lane 6), the oocyte gene failed to exclude transcription of the somatic gene, thus confirming the experimental results of Wolffe and Brown (36). Significantly, however, we found that preincubation of the oocyte 5S RNA gene in the egg extract blocked transcription from a subsequently added somatic 5S RNA gene if sufficient time was allowed for the assembly of transcription complexes on the oocyte genes (Fig. 2A, lanes 7 and 8, 2 and 3 h, respectively). We have noted that the time required to form template-exclusion complexes on the oocyte 5S RNA genes varies between extracts prepared from the eggs of different individual frogs; however, all extracts we have examined (10 extracts from the eggs of different animals) were capable of forming stable template-exclusion complexes on the oocyte genes given sufficient incubation times.

As a further test for transcription complex sta-

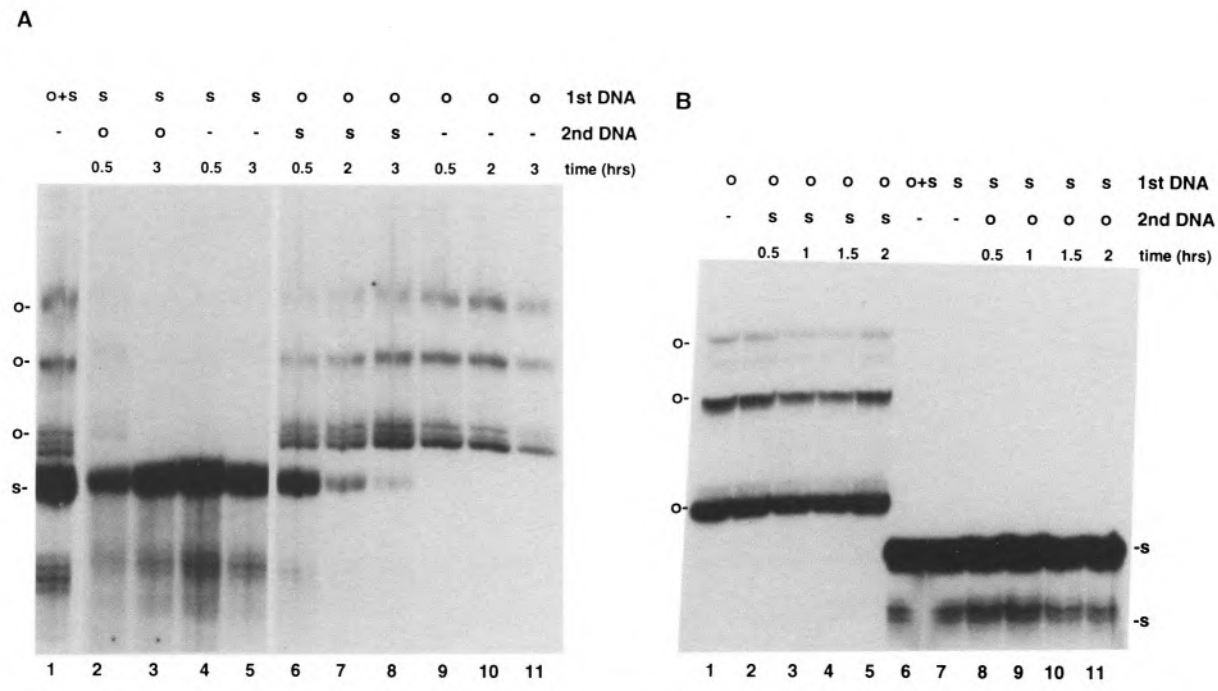

FIG. 2. Transcription complexes formed on oocyte 5S RNA genes are stable in the unfertilized activated egg extract. (A) Formation of template exclusion complexes. The products of transcription from the oocyte (O) and somatic (S) 5S RNA genes were analyzed on a partially denaturing polyacrylamide gel. Lane 1 shows the transcription products from control reaction containing a mixture of oocyte and somatic DNA templates (50:1, oocyte:somatic DNA). Lanes 2–3 show transcription products from reactions where the somatic 5S RNA gene was incubated with the egg extract and TFIIIA for the indicated times prior to the addition of the oocyte 5S RNA gene. Lanes 6–8 show transcription products from similar reactions where the oocyte 5S RNA gene was added to the extract and TFIIIA for the indicated times prior to the addition of the somatic 5S RNA gene. After a subsequent 30-min incubation, labeled and unlabeled nucleoside triphosphates were added and transcription was allowed to continue for an additional 2 h. Lanes 4 and 5 show reactions where the somatic gene was incubated with extract and TFIIIA for the indicated time prior to initiation of transcription. Lanes 8–11 show similar reactions for the oocyte gene. (B) Stability of transcription complexes. Lanes 1, 6, and 7 show the transcription products from control reactions containing the indicated DNA templates. In lanes 2–5, the oocyte 5S RNA gene was incubated with the egg extract and TFIIIA for 2 h prior to the addition of the somatic 5S RNA gene. The reactions containing both templates were then incubated for the indicated times. The transcriptionally active genes were then assayed by the addition of radiolabeled nucleoside triphosphates during a further 2-h incubation. Lanes 8–11 show similar reactions where the somatic 5S RNA genes were added first and the oocyte 5S RNA genes were added second.

bility on the oocyte 5S RNA gene in the egg extract, we first formed complexes on the oocyte genes during a 2-h incubation, and then challenged these complexes with somatic templates for varying times ranging from 30 min to 2 h. Radiolabeled nucleotides were then added and transcripts were analyzed after a subsequent 2-h incubation. This protocol is a slight modification of the standard template exclusion experiment. Figure 2B shows that transcription complexes formed on either the oocyte or somatic genes were stable for greater than 2 h of incubation with the challenge DNA. We conclude that both somatic and oocyte 5S RNA genes form stable transcription complexes in the unfertilized egg extract; however, these two genes differ significantly in the rate of formation of stable transcription complexes.

Titration of Chromatin Assembly Factors Does Not Activate Oocyte 5S Transcription

Extracts from *Xenopus* eggs are highly enriched in the core histones and chromatin assembly factors (3,36). One test for the role of chromatin assembly in 5S transcription is to titrate assembly factors and histones by preincubation of the extract with vector DNA prior to the addition of the 5S gene plasmid DNAs (36). Control experiments were performed to monitor the chromatin assembly activity of the extract: we find that 1 μl of extract will fully assemble nucleosomes on 35–50 ng of plasmid DNA (~2 kbp in length) in a 90-min incubation period (as determined by a DNA supercoiling assay, data not shown). In the experiment shown in Fig. 3, the extract was prein-

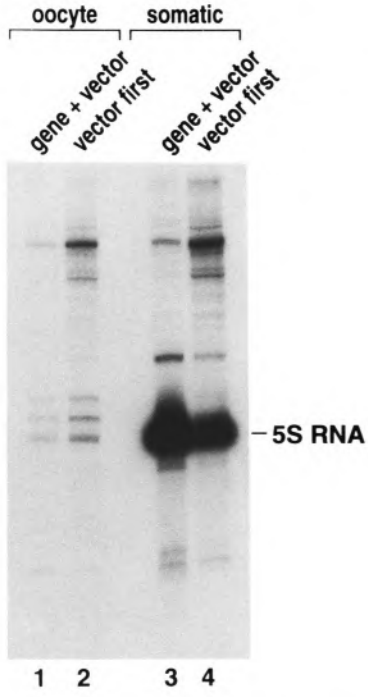

FIG. 3. Titration of chromatin assembly activity does not relieve repression of oocyte 5S gene transcription in the unfertilized egg extract. In the reactions shown in lanes 1 and 3, 100 ng gene-containing plasmid DNA, as indicated, 9 ng TFIIIA, and 300 ng pUC18 DNA were incubated with the egg extract (5 μl in a 16 μl reaction containing 6 mM $MgCl_2$) for 30 min prior to the addition of nucleoside triphosphates and transcription was for 1 h. In the reactions shown in lanes 2 and 4, 400 ng of pUC18 DNA was incubated with 5 μl of egg extract, as above, for 2.5 h prior to the addition of oocyte or somatic 5S DNAs (100 ng) and 9 ng TFIIIA. After an additional 30-min incubation, nucleoside triphosphates were added and transcription was for 1 h.

cubated with an excess of vector DNA for 2.5 h prior to the addition of either the oocyte or somatic 5S gene plasmid DNAs (lanes 2 and 4, respectively). Titration of the chromatin assembly activity of the extract had little effect on the relative transcription activities of the two genes in either separate reactions (Fig. 3) or in mixed template reactions (data not shown). We conclude that the transcription activities of the two 5S genes are not due to differences in chromatin assembly in the egg extract, in agreement with Wolffe and Brown (36).

Role of Flanking DNA Sequences in Oocyte 5S Gene Transcription

Although the oocyte and somatic 5S genes differ at only five nucleotide positions within their coding sequences, the flanking sequences of these genes differ dramatically (22). To examine a possible contribution of the somatic flanking sequence to differential transcription in the egg extract, we monitored the transcription activity of the wild-type oocyte and somatic 5S genes along with oocyte genes in which either the 5′ or 3′ flanking sequences were replaced with the corresponding flanking sequences from the somatic type gene. As illustrated in Fig. 4, transcriptional activity of the oocyte gene in the egg extract is not markedly influenced by the nature of these flanking sequences, suggesting that the five nucleotide differences between the two genes is responsible for the differential transcription activities of these genes. Similar conclusions were reached by Wolffe and Brown (36).

Protein Factors Required for Assembly of Stable Complexes on the Oocyte 5S Gene

Previous studies have demonstrated that stable TFIIIA + TFIIIC–DNA complexes can be formed on the somatic 5S gene (2,28); however, no similar studies have been reported for the oocyte 5S gene. We wished to determine whether a stable protein–DNA complex on the oocyte 5S gene requires all three components of the transcription complex, TFIIIA, B, and C, or whether a subset of these factors, such as TFIIIA and TFIIIC, would suffice to form a stable complex. Both 5S genes have similar affinities for TFIIIA (18); however, TFIIIA forms a metastable complex with the 5S gene: TFIIIA will readily exchange between templates given sufficient incubation times (2,12,13,28,34).

We used the template exclusion assay to determine the protein factors that are required to form a stable complex on the oocyte gene (Fig. 5). In this experiment, the oocyte gene was incubated with limiting amounts of TFIIIA (lane 2), or TFIIIA and TFIIIC (phosphocellulose C fraction) (lane 3), or TFIIIA, TFIIIC, and TFIIIB (Mono Q fraction) (lane 6) for 30 min prior to the addition of the somatic 5S gene and the remaining factor(s). After a second incubation period (30 min) to allow for exchange of factors between the oocyte and somatic genes, labeled and unlabeled nucleoside triphosphates were added and transcription was allowed to proceed for 1 h. As a control, a mixed template reaction, in which both DNAs and all three factors were incubated for 30 min prior to transcription, is shown in lane 1. Transcripts from separate reactions with the oocyte and somatic genes are shown in lanes 7 and 8, respectively. A 30-min incubation of the oocyte gene with either TFIIIA (lane 2) or with TFIIIA and TFIIIC (lane 3) results in only a modest ~50% reduction of somatic 5S transcription relative to the mixed template reaction (lane 1); however, longer incubation times with TFIIIA and

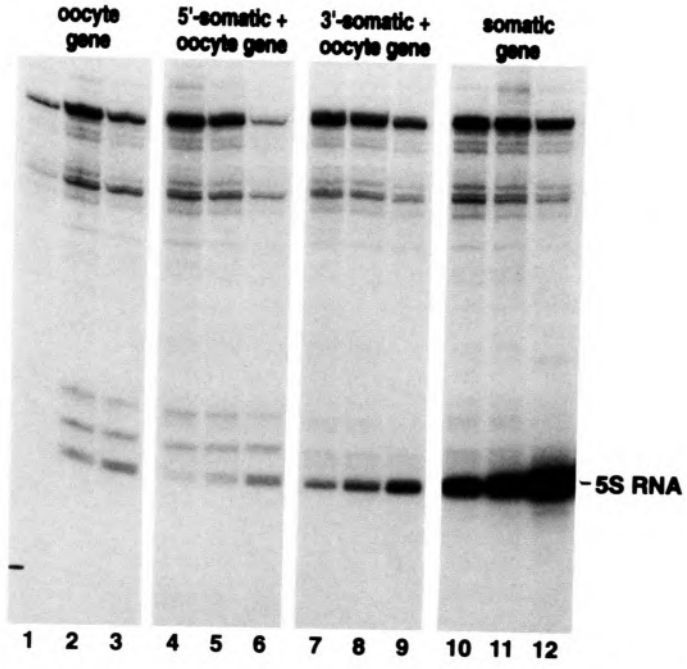

FIG. 4. Flanking sequences do not affect oocyte 5S transcription in the unfertilized egg extract. All transcription reactions contained 5 μl of extract, 18 ng TFIIIA, and the following amounts of DNA: 25 ng gene-containing plasmid plus 275 ng pUC18 (lanes 1, 4, 7, 10); 50 ng gene-containing plasmid plus 250 ng pUC18 (lanes 2, 5, 8, 11); 100 ng gene-containing plasmid plus 200 ng pUC18 (lanes 3, 6, 9, 12). The reactions of lanes 1–3 contained the wild-type oocyte 5S gene pXloΔ3′ + 176; lanes 4–6, oocyte 5S gene with the somatic 5′ flanking sequence; lanes 7–9, oocyte 5S gene with somatic 3′ flanking sequence; lanes 10–12, wild-type somatic 5S gene, pXls11.

TFIIIC (1 and 2 h; lanes 4 and 5, respectively) result in substantial reductions in transcription of the secondarily added somatic gene, indicating that a stable TFIIIA + C complex has been formed on the oocyte gene. In contrast, and in agreement with previous studies (2,28), a 30-min incubation of the somatic gene with TFIIIA and TFIIIC is sufficient to exclude oocyte transcription (data not shown). Thus, the two 5S genes differ in their rates of association of TFIIIC with the TFIIIA–5S DNA complex. This difference may well account for the difference in rates of assembly of transcription complexes observed in the unfractionated extract (Fig. 2A). These results are also consistent with the finding that increasing TFIIIC concentration in an oocyte extract preferentially stimulates oocyte 5S RNA transcription (34) and that TFIIIC exhibits a lower affinity for the TFIIIA complex on the oocyte gene relative to the somatic gene (12,13).

Effect of Increasing TFIIIB Activity on Oocyte and Somatic 5S RNA Transcription

We find that the transcriptional activity of the unfertilized egg extract is markedly enhanced by the addition of recombinant *Xenopus* TATA binding protein, TBP (McBryant and Gottesfeld, in preparation). This added TBP nucleates the assembly of additional active TFIIIB in the extract. Both separate and mixed template transcription reactions show, however, that the relative transcriptional activities of the oocyte and somatic 5S genes are not affected by this increase in TFIIIB activity (data not shown). Thus, differential 5S gene transcription does not appear to rely on differences in TFIIIB affinity or recruitment to the TFIIIA + TFIIIC–5S DNA complexes on the two 5S RNA genes.

Insertion of a TFIIIC2 Binding Site Within or Flanking an Oocyte 5S Gene Increases Transcriptional Activity and Rate of Formation of Stable Transcription Complexes

In a previous mutagenesis study of the oocyte 5S gene, we found that one fortuitous mutation increased the transcriptional activity of the oocyte gene in a whole oocyte extract (13). This mutation introduced a close match to the B-block consensus sequence within the 5S gene. This sequence, found in class III genes such as tRNA and viral RNA

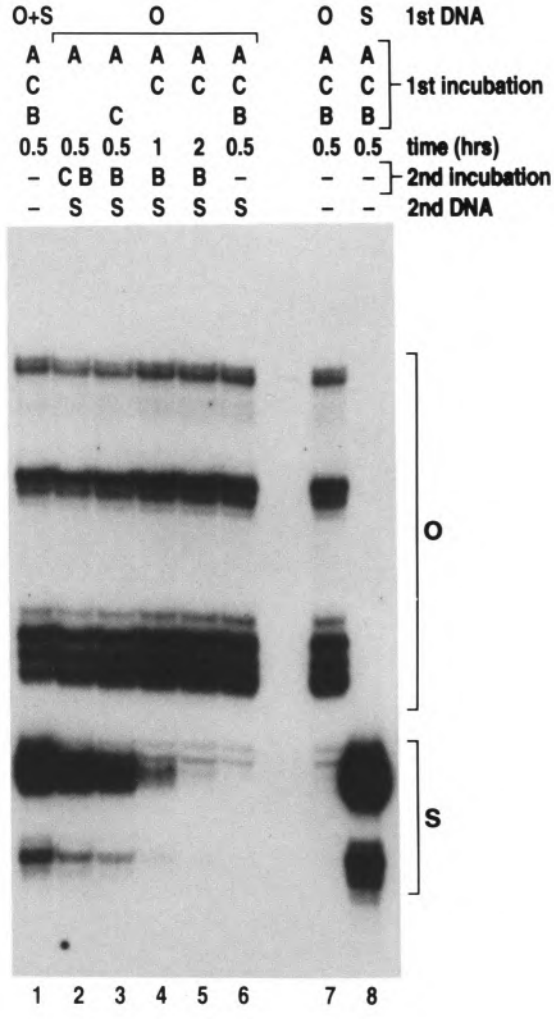

FIG. 5. Rate of association of TFIIIC with the TFIIIA–oocyte 5S gene complex. Reconstituted transcription reactions were performed as described in Materials and Methods and contained the components shown at the top of the figure in the incubation steps. Reactions contained 300 ng oocyte pXloΔ3′ + 176 DNA and/or 60 ng somatic pXls11 DNA, as indicated, and 9 ng TFIIIA (providing a 1.5 molar excess over oocyte genes). Incubation times prior to the addition of the somatic template and remaining transcription components are indicated. After an additional 30-min incubation, transcription was for 1 h. The reaction of lane 8 contained 240 ng pUC18 DNA along with the somatic 5S gene.

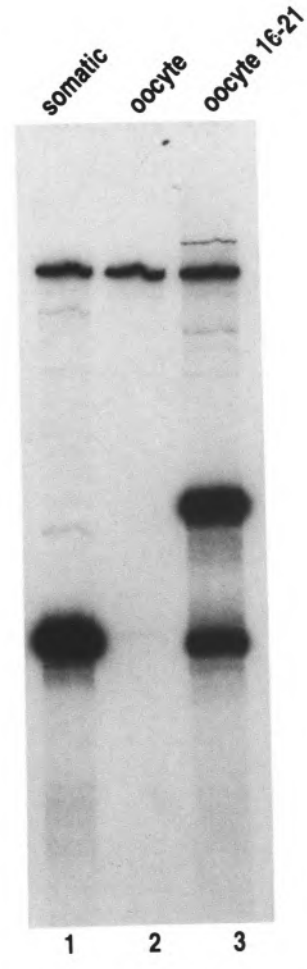

FIG. 6. A fortuitous mutation within a synthetic oocyte 5S gene stimulates transcription. Transcription reactions with the unfertilized egg extract (5 μl) contained 100 ng somatic pXls11 DNA, 3.6 ng TFIIIA, and 400 ng pUC18 DNA (lane 1); 500 ng oocyte pXloΔ3′ + 176 DNA and 18 ng TFIIIA (lane 2); 500 ng pXlo16-21 DNA (38) and 18 ng TFIIIA. Transcription was for 2 h and RNAs were analyzed on a denaturing polyacrylamide gel.

genes (31), represents the primary binding site for the general RNA polymerase III transcription factor TFIIIC2 (15–17,37). We found that this mutant 5S gene had an approximately fivefold higher affinity for TFIIIC than did the parent oocyte gene and we attributed the higher transcriptional activity of this mutant to this increase in TFIIIC affinity (13). When this mutant 5S gene, pXlo16-21, was transcribed in the unfertilized egg extract, the effect of this mutation was greatly enhanced relative to its effect in the oocyte extract (Fig. 6). In this experiment, the S:O ratio for the wild-type genes was > 50:1, whereas the S:O ratio for the pXlo16-21 template was ~2:1 (as determined by phosphorimage analysis, correcting transcriptional activities for transcript lengths).

To confirm that the increased transcriptional activity observed with clone pXlo16-21 is indeed due to enhanced recruitment of TFIIIC at the B-block sequence, we generated a derivative of the synthetic oocyte 5S gene (38) with a consensus B-block sequence inserted downstream from the 5S RNA coding sequence. This clone (termed pXlo + BB), along with the parent synthetic oocyte 5S gene (pXlo), and both the wild-type somatic and oocyte 5S genes, were used as templates for transcription in the high speed egg extract (Fig.

7A, B). We find that both the template concentration (Fig. 7A) and volume of extract (Fig. 7B) strongly influence the transcriptional activities of these genes, in agreement with previous studies with whole oocyte extracts (34). Clone pXlo + BB is far more active at either low template concentrations or low extract volumes than either the parent synthetic oocyte gene or the wild-type oocyte gene and the activity of this gene approaches that of the somatic 5S gene. Wolffe has previously shown that the activity of the oocyte gene is far more dependent on extract concentration than is the somatic gene (34). Our results strongly suggest that insertion of the B-block sequence, either within or flanking the gene, enhances recruitment of TFIIIC to the TFIIIA-DNA complex on the oocyte 5S gene.

To confirm this hypothesis, we monitored the

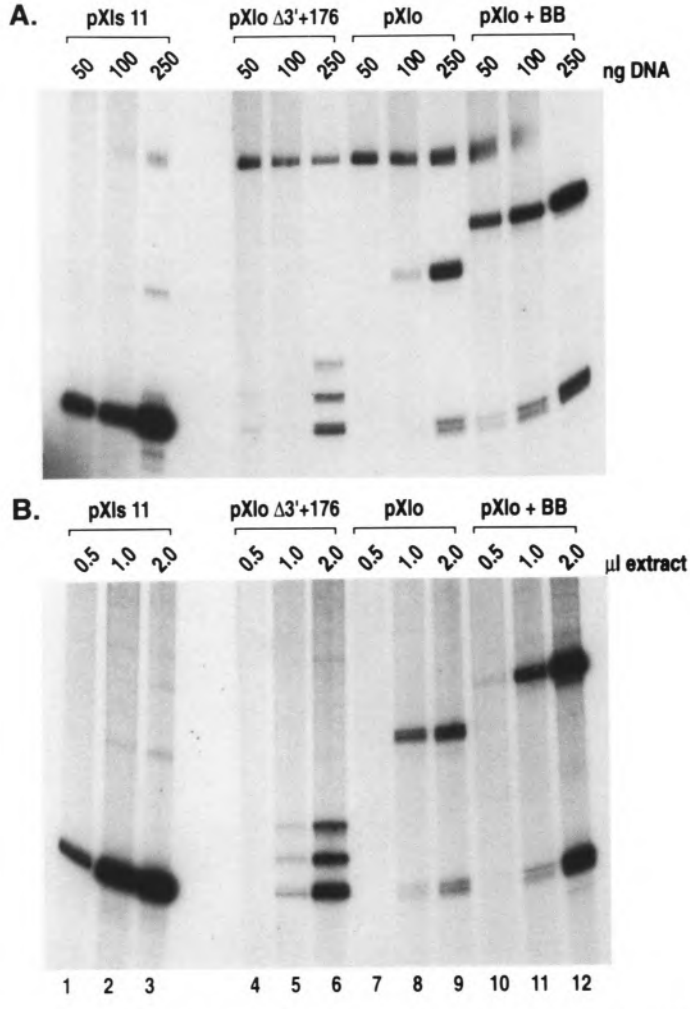

FIG. 7. Insertion of a B-block sequence downstream from the oocyte 5S gene stimulates transcription in the unfertilized egg extract. (A) Effect of template concentration. All reactions contained 2 μl of extract, 9 ng TFIIIA, and the following amounts of DNA: 50 ng gene-containing plasmid plus 200 ng pUC18 (lanes 1, 4, 7, 10); 100 ng gene-containing plasmid plus 150 ng pUC18 (lanes 2, 5, 8, 11); 250 ng gene-containing plasmid (lanes 3, 6, 9, 12). (B) Effect of extract concentration. All reactions contained 100 ng gene-containing plasmid, 9 ng TFIIIA, and 150 ng pUC18 DNA. The reactions of lanes 1, 4, 7, and 10 contained 0.5 μl extract; lanes 2, 5, 8, and 11 contained 1 μl extract; lanes 3, 6, 9, and 12 contained 2 μl extract. For both panels, the reactions of lanes 1–3 contained the wild-type somatic 5S gene, pXls11; lanes 4–6, wild-type oocyte 5S gene, pXloΔ3′ + 176; lanes 7–9, synthetic oocyte 5S gene, pXlo; lanes 10–12, synthetic oocyte 5S gene with the 3′ B-block insertion, pXlo + BB. Transcription was for 1 h and RNAs were analyzed on a denaturing polyacrylamide gel.

time required to form a stable transcription complex on the wild-type oocyte 5S gene and on clone pXlo+BB (Fig. 8). This experiment was performed at low extract concentration (1 μl/20 μl reaction), where very low levels of oocyte transcription are observed after a short (30-min) preincubation (Fig. 7B, lane 5). These conditions yield a S:O ratio of > 50:1 for the wild-type oocyte gene (lane 3); however, an S:O ratio of ~5:1 is observed for pXlo+BB under these same conditions (lane 8). Under these experimental conditions, template exclusion was not observed for the wild-type oocyte gene after either 30 min, 1.5 or 2.5 h of incubation prior to addition of the somatic 5S gene (lanes 4–6). In contrast, clone pXlo+BB was able to sequester limiting transcription factors during either a 1.5- or 2.5-h incubation and effectively excluded transcription of the secondarily added somatic 5S gene in this experiment (lanes 10 and 11, respectively). These results suggest that the oocyte 5S gene is impaired in its ability to recruit TFIIIC2 relative to either the somatic gene or to clone pXlo+BB (Fig. 5).

DISCUSSION

We have found that the process of transcription complex assembly discriminates between the dif-

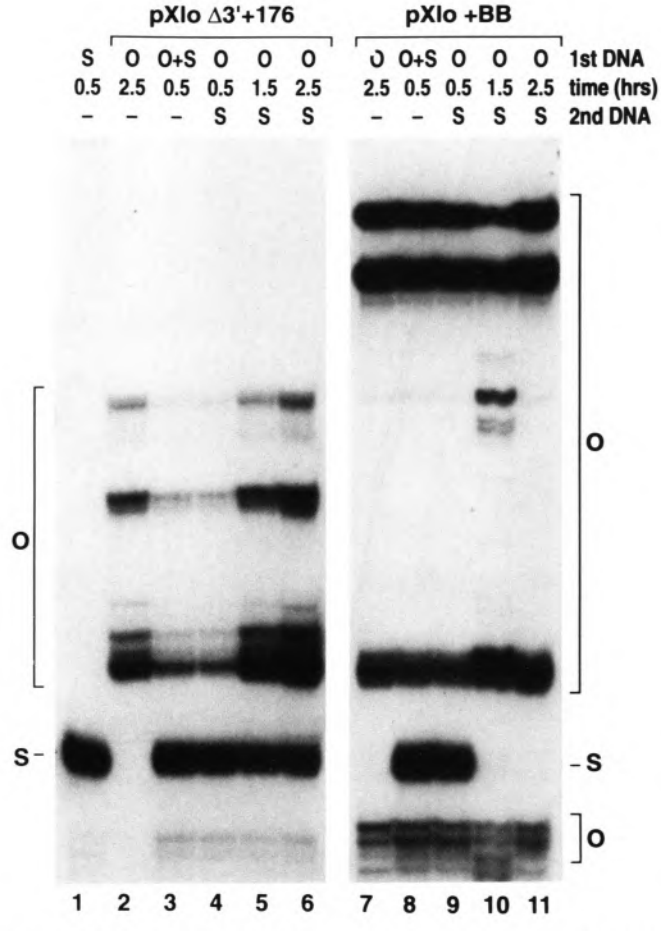

FIG. 8. Insertion of a B-block sequence downstream from the oocyte 5S gene increases the rate of transcription complex assembly. The template exclusion assay was used to monitor the rate of formation of transcription complexes on the wild-type oocyte 5S gene, pXloΔ3′+176 (lanes 2–6), and the synthetic oocyte 5S gene with the 3′ B-block, pXlo+BB (lanes 7–11). Reactions contained 1 μl unfertilized egg extract, 250 ng oocyte DNA, and/or 10 ng somatic DNA and 9 ng TFIIIA. The reaction of lane 1 also contained 290 ng pUC18 DNA. DNAs, incubation times, and the order of template additions are indicated at the top of the figure. After addition of the somatic template and an additional 30-min incubation, transcription was for 1 h.

ferent genes, with somatic 5S RNA genes sequestering the limiting transcription factors much faster than oocyte 5S RNA genes. Our present data and the earlier results of Seidel and Peck (27) support the conclusion that differential 5S RNA gene expression in vitro in both oocyte and egg extracts is a consequence of the different rates of transcription complex assembly on the different genes. Previous studies have suggested that differences in rates of TFIIIC recruitment might play a significant role in the relative activities of the oocyte and somatic 5S genes (12,13,33-35). Indeed, addition of TFIIIC to an oocyte extract has been shown to selectively stimulate oocyte 5S transcription (34). Our present results support these conclusions: we find that TFIIIC is recruited to the TFIIIA-oocyte 5S DNA complex at a lower rate than for the somatic 5S gene (Fig. 5) and that insertion of the DNA binding site for TFIIIC2 (from a tRNA gene) within or flanking the oocyte 5S gene markedly stimulates the transcriptional activities of these synthetic genes and increases the rates of assembly of transcription complexes on these genes relative to the parent oocyte 5S genes (Figs. 7 and 8).

Previous studies suggested that stable transcription complexes are not formed on oocyte 5S genes in a low-speed extract prepared from *Xenopus* eggs (36). Our current results suggest that stable transcription complexes on oocyte 5S RNA genes were not observed in that study due to the slower rate of formation of these complexes on the oocyte genes relative to the somatic genes. As a test for stability of oocyte transcription complexes in the egg extract, we challenged preformed complexes with the somatic 5S gene and assayed for transcription from the two templates after various incubation times (Fig. 2B). If the oocyte transcription complexes were not stable, then with increasing incubation times the oocyte genes would lose transcription factors to the somatic genes. Even if these dissociating factors did not activate the somatic genes, their dissociation would lead to a decrease in the number of active oocyte transcription complexes. We found, however, that the activity of the oocyte transcription complexes remained the same irrespective of the length of time during which these oocyte complexes were incubated in the presence of the somatic 5S RNA genes. In addition, no active somatic transcription complexes were observed. We conclude that transcription complexes on oocyte 5S RNA genes are stable in the unfertilized egg extract (Fig. 2B). However, it is conceivable that our extracts differ from those prepared by Wolffe and Brown in this property of stability of oocyte transcription complexes. It is noteworthy that Wolffe and Brown found that stable oocyte transcription complexes (formed in an oocyte nuclear extract) were destabilized in their egg extract. This result suggests the presence of an activity that preferentially destabilizes the oocyte transcription complex (36). Components of a ribosome fraction were suggested to possess this activity; however, the molecular identity of this factor(s) has not been established.

Our present results confirm many of the aspects of the model for the developmental regulation of the 5S gene families put forward by Wolffe (33). Differences in the kinetics of transcription complex assembly could have profound effects in vivo on the differential expression of 5S RNA genes, as previously suggested (27,33). During early oogenesis, where both the oocyte and somatic 5S genes are actively transcribed, there is an abundance of the general class III transcription factors, TFIIIB and TFIIIC, and especially the 5S gene-specific factor TFIIIA. During the early cleavage stages of embryogenesis there is an increase in cell number without concomitant protein synthesis, thus leading to a situation where transcription factors required for both families of 5S genes become limiting relative to the chromosomal complements of 5S genes (33). Thus, under these conditions and due to the higher rate of transcription complex assembly on the somatic 5S genes, these genes will be more effective competitors for limiting transcription factors than the oocyte 5S genes. The effects of limiting TFIIIA concentration on differential transcription of the two gene families have been established experimentally in vivo (1,6). In addition to competition for limiting transcription components, it is likely that multiple levels of regulation participate in the developmental program of 5S gene expression, including the chromatin environment of these genes, the selective inactivation of oocyte 5S genes by histone H1 (6,11), and the states of postsynthetic modification of the basal RNA polymerase III transcription machinery (32). Competition for limiting transcription components and the selective repression of the oocyte 5S genes by histone H1 likely accounts for the developmental pattern of 5S gene expression established during early embryogenesis (33).

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