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Transcription of cloned *Xenopus* 5S RNA genes by *X. laevis* RNA polymerase III in reconstituted systems

(recombinant DNA/cell-free extracts/soluble factor/RNA nucleotidyltransferase)

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ABSTRACT **When incubated with a soluble extract from large oocytes of *Xenopus laevis*, recombinant DNA plasmids containing either *X. laevis* oocyte 5S DNA or *X. borealis* oocyte 5S DNA direct the synthesis of discrete 5S RNAs, which by size and sequence analysis are similar or identical to the corresponding 5S RNAs synthesized *in vivo*. Synthesis of the 5S RNAs is mediated by a soluble endogenous RNA polymerase III (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6), which presumably recognizes specific initiation and termination sites in the 5S genes. Optimal conditions for accurate synthesis and the kinetics of the reactions have been determined. A soluble postchromatin supernatant fraction has also been isolated from immature oocytes. Although devoid of a functional endogenous RNA polymerase III, this extract contains a component(s) that effects the accurate transcription of 5S genes (in a plasmid) by a purified RNA polymerase III.**

The most highly reiterated classes of 5S RNA genes in *Xenopus laevis* and *Xenopus borealis* offer many advantages for studies of eukaryotic transcription mechanisms in cell-free systems. Genomic DNAs encoding these genes (5S DNAs) have been purified and characterized (1), and subsets of these DNAs have been cloned in plasmids (2–4) and sequenced (3, 4). These oocyte-type 5S genes are transcribed only during oogenesis (5), and in immature oocytes they account for a large fraction of newly synthesized RNA (6). The stable transcripts of these genes are simple and well characterized (5) and appear to represent primary transcripts (7, 8). Finally, oocytes contain large amounts of RNA polymerase III (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) (9), which is known to transcribe the 5S genes (10, 11) and which has been purified and characterized (12, 13).

Previous studies from this laboratory have demonstrated the selective and accurate transcription of the 5S genes in immature oocyte chromatin by a purified homologous RNA polymerase III (11, 13–15). This specific transcription could not be duplicated with purified DNA templates or with other RNA polymerases, leading to the conclusion that both chromatin-associated components and RNA olymerase III are involved in 5S gene activation. Similar conclusions were reached from analogous studies of the mouse 5S and tRNA (16) and the adenovirus VA RNA (17) genes. In other studies Brown and Gurdon (8, 18) demonstrated the ability of both genomic and cloned 5S DNAs to direct accurate 5S RNA synthesis when injected into living oocytes. Thus these DNAs seemed appropriate for further identification of 5S gene transcription elements via cell-free reconstitution studies.

Here we show that cloned 5S genes are accurately transcribed by components, including an endogenous RNA polymerase III, in extracts of large *X. laevis* oocytes. Analogous results have been obtained for *Xenopus* 5S and adenovirus 2 VA RNA genes with extracts from oocyte nuclei (19) and from cultured animal cells (20,*). In addition, we show here that a component(s) in an immature oocyte extract facilitates the accurate transcription of cloned 5S genes by an exogenous purified RNA polymerase III.

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MATERIALS AND METHODS

Recombinant DNA Templates. The two recombinant DNA plasmids used contain *Hin*dIII fragments of *Xenopus* 5S DNA inserted at the *Hin*dIII site in the plasmid pMB9 (2, 4, 18). One (pMB9X15S, designated here as pXlo) contains a four-repeat-unit fragment of *X. laevis* oocyte (Xlo) 5S DNA. The other (pXbo3, designated here as pXbo) contains a 2700-base-pair fragment of *X. borealis* oocyte (Xbo) 5S DNA with six 5S RNA genes (18). These recombinant DNA plasmids were obtained from D. D. Brown (Carnegie Institution of Washington, Baltimore). All growth and handling of recombinant DNA have been carried out according to National Institutes of Health guidelines (P2, EK1). Supercoiled plasmid DNAs were purified as described elsewhere (2, *).

Large Oocyte S-100 Preparations. Ovarian tissue from adult *X. laevis* was digested with collagenase (21), and the largest of the released oocytes were purified by several washes (sedimentation at 1 × *g*) in 50 mM Tris·HCl (pH 7.9)/50 mM KCl/0.1 mM EDTA/25% (vol/vol) glycerol. Purified oocytes (predominantly stage V and VI) were resuspended in twice the volume of the same buffer containing 1 mM dithiothreitol and disrupted in a Kontes glass–Teflon homogenizer. The crude homogenate was centrifuged for 15 min at 1900 × *g* at 0°C. The resulting supernatant was centrifuged for 2 hr at 100,000 × *g* to yield a high-speed supernatant fraction (designated S-100).

Immature Oocyte Chromatin Supernatant Fraction. Stage I oocytes 75–150 μm in diameter were isolated from immature *X. laevis* ovaries and freed from follicle cells (11, 14). The oocytes from 10 ovaries were resuspended in 0.25 ml of 0.34 M sucrose/100 mM KCl/50 mM Tris·HCl (pH 7.8)/5 mM $MgCl_2$ and disrupted in a Kontes glass–Teflon homogenizer. The homogenate was mixed with 1 ml of the same buffer containing 2.0 M sucrose and centrifuged for 20 min at 45,000 rpm in a Beckman SW-60 rotor. The supernatant was removed from the chromatin pellet and dialyzed for 2 hr versus 50 mM Tris·HCl (pH 7.9)/50 mM KCl/0.1 mM EDTA/25% (vol/vol) glycerol/1 mM dithiothreitol. The chromatin pellet (not used here) is equivalent to that used for previous transcription experiments after washing and swelling (11, 14).

***In Vitro* Transcription.** The standard reaction volume was 50 μl and contained: 45 mM Tris·HCl (pH 7.9); 8–10% (vol/vol) glycerol; 60 mM KCl; 5 mM $MgCl_2$; 0.4 mM dithiothreitol; 0.6

Abbreviations: Xlo and Xbo 5S DNAs refer, respectively, to the oocyte-type 5S DNAs from *Xenopus laevis* and *Xenopus borealis*. pXlo and pXbo refer to the recombinant plasmids containing these DNAs.

* P. A. Weil, J. Segall, B. Harris, S. Ng, and R. G. Roeder, unpublished.

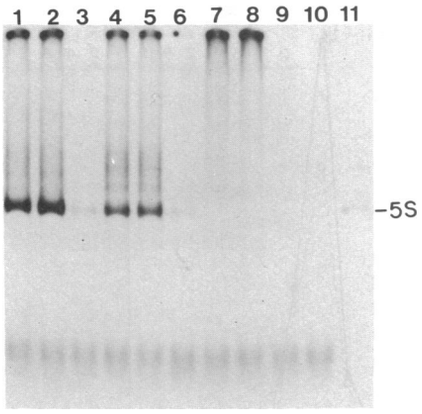

FIG. 1. Polyacrylamide gel electrophoresis of RNAs synthesized during incubation of oocyte S-100 extracts with recombinant plasmids containing 5S DNAs. Reaction conditions were standard except that the α-amanitin concentrations were zero μg/ml (lanes 1, 4, 7, and 10), 0.24 μg/ml (lanes 2, 5, and 8), or 400 μg/ml (lanes 3, 6, and 9). The plasmids added were pXbo (lanes 1–3), pXlo (lanes 4–6), pMB9 (lanes 7–9), or none (lane 10). A 5S RNA marker labeled in intact *X. laevis* cells is shown in lane 11.

mM ATP, CTP, and UTP; 25 μM [α-^{32}P]GTP (5–15 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels); α-amanitin at 0.24 μg/ml; 0.5 μg of DNA; and either 20 μl of the large oocyte S-100 or 20 μl of the dialyzed immature oocyte chromatin supernatant fraction plus 200 units (approximately 1 μg) of purified *X. laevis* oocyte RNA polymerase III (12, 13). After incubation for 60 min at 30°C, reactions were terminated (17) and small aliquots were removed for determination of total GMP incorporation by the DEAE-paper disk method (9). For the fingerprint analyses reactions were scaled up 10- to 24-fold and incubation was for 4 hr.

RNA Purification and Analysis. RNA was extracted (17, *), dissolved in electrophoresis buffer [50 mM Tris borate (pH 8.3)/1 mM EDTA], and electrophoresed into 12% polyacrylamide slab gels (10.5 cm) crosslinked with ethylene diacrylate (17), and the resulting gels were subjected to autoradiography.* To quantitate radioactivity in 5S RNAs, appropriate gel regions were excised, incubated overnight with 0.1 M NaOH at 37°C, neutralized, and assayed in Kinard scintillation counting fluor. 5S RNAs for fingerprint analyses were purified from preparative gels (24 cm, 15% polyacrylamide) and digested with RNAse T_1 as described (22, 23). Oligonucleotides were frac-

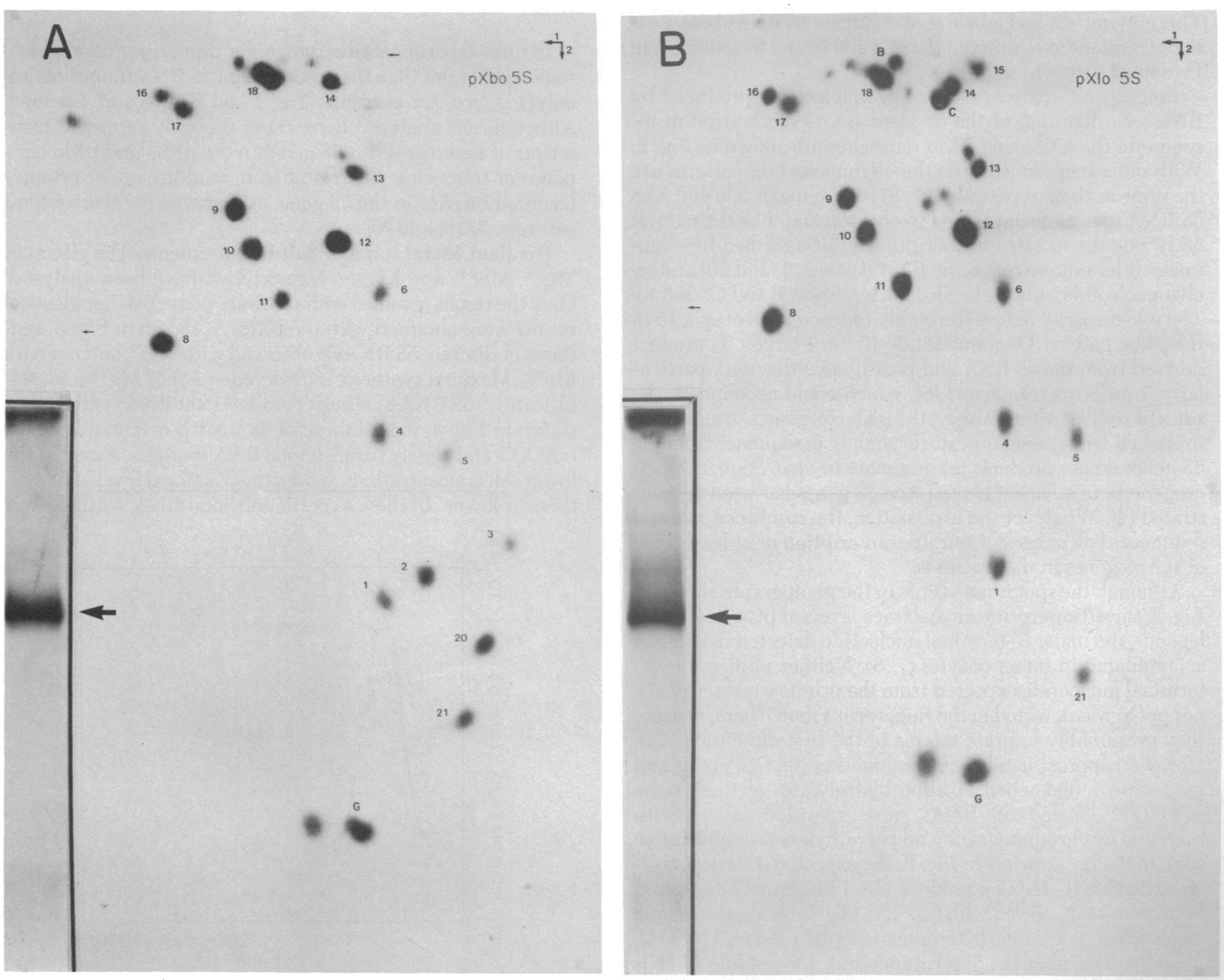

FIG. 2. Fingerprint analyses of RNase T_1 oligonucleotides present in 5S RNA synthesized in oocyte S-100 extracts in response to either pXbo DNA (*A*) or pXlo DNA (*B*). The molar ratios of the numbered oligonucleotides (according to ref. 8) were all within 30% of the expected values (see text and ref. 8). Trace levels of oligonucleotide 22 (pGp) are detected to the left of spot 8 (see arrows). In *A*, the upper arrow indicates a faint level of spot 15 and the molar ratios of the unidentified oligonucleotide adjacent to spots 13 and 14 were 0.25–0.35. The *Insets* show the preparative gels from which the fingerprinted RNAs were obtained.

tionated by ionophoresis on cellulose acetate (first dimension) and DEAE-cellulose (second dimension) by the Sanger and Brownlee method (22) as detailed elsewhere (23).

RESULTS

Transcription of 5S DNA in extracts from large oocytes

Accurate Transcription by RNA Polymerase III. The class I, II, and III RNA polymerases found in *X. laevis* oocytes are recovered nearly quantitatively in the S-100 fraction, which is equivalent to that used previously for the purification of these enzymes from mature ovaries (refs. 12 and 13; D. Engelke and R. G. Roeder, unpublished observations). When this extract is incubated with plasmids containing Xbo 5S DNA (pXbo) or Xlo 5S DNA (pXlo), radioactivity is incorporated into high molecular weight RNA that does not enter the gel, into discrete 5S RNA species equivalent in size to natural 5S RNA, and into low molecular weight RNA about 4 S in size (Fig. 1, lanes 1 and 4). Synthesis of the high molecular weight and 5S RNA species is mediated by RNA polymerase III, because it is sensitive (>80%) to high (lanes 3, 6, 9) but resistant (>90%) to low (lanes 2, 5, 8) concentrations of α-amanitin (cf. refs. 10 and 16). A comparable level of high molecular weight RNA synthesis, but no 5S RNA synthesis, is observed with the pMB9 cloning vector, which contains no 5S DNA (lane 7). In the absence of exogenous DNA (lane 10) radioactivity is incorporated into only the 4S material. This may reflect end labeling of endogenous RNAs because it is also resistant to α-aminitin (lanes 3 and 6) and to actinomycin D (data not shown).

Fingerprint analyses of the oligonucleotides produced by RNase T_1 digestion of the 5S-sized RNAs synthesized in response to the pXbo and pXlo templates are shown in Fig. 2. With one exception (below) the oligonucleotide patterns are the same as those reported (5, 7, 8) for the major Xlo and Xbo 5S RNA species accumulated in oocytes; and, like the natural 5S RNAs, the *in vitro* transcripts are distinguished by oligonucleotides unique to Xbo 5S RNA (spots 2, 3, and 20) and by oligonucleotides unique to Xlo 5S RNA (spots B and C) (ref. 8). The sole disparity here is the highly reduced level of spot 15 in the pXbo pattern. Oligonucleotide 15 is the largest T_1 product derived from the 5S RNA and is easily overdigested, particularly in *in vitro* transcripts (8), which could account for the present results. Alternatively, the pXbo 5S gene(s) transcribed in the cell-free system may differ slightly in sequence from the 5S genes whose products predominate *in vivo*. Sequence heterogeneity in adjacent cloned Xbo 5S genes has been demonstrated (4). Whatever the explanation, the combined size and sequence data suggest accurate transcription of at least some of the 5S genes in the plasmids.

Although the spot is not visible in the photographs shown in Fig. 2, these fingerprints reveal trace levels of pGp (see figure legend), the major 5′-terminal nucleotide detected in 5S RNA accumulated in intact oocytes (7, 8). Neither pppGp, the 5′-terminal nucleotide expected from the primary transcript (7), nor ppGp was detected in the fingerprints shown here, because they presumably migrate too far in the first dimension (22). However, approximately equal amounts of pppGp, ppGp, and pGp were found when alkaline hydrolysates of these same [α^{32}P]GTP-labeled 5S RNAs were analyzed (along with markers) by chromatography on polyethyleneimine-cellulose (26). In the fingerprints in Fig. 2, the expected 3′-terminal oligonucleotide (C-U-U_{OH}, residues 118–120) cannot be detected because it is not labeled with [α-^{32}P]GTP. However, the unique oligonucleotide near the 3′ terminus (spot 10, residues 114–116), as well as that near the 5′ terminus (spot 13, residues 2–7), is present in the expected molar ratio. The oligonucleotide expected if readthrough had occurred at the 3′ terminus (see ref. 8) is not detected, but this does not necessarily exclude post-transcriptional processing.

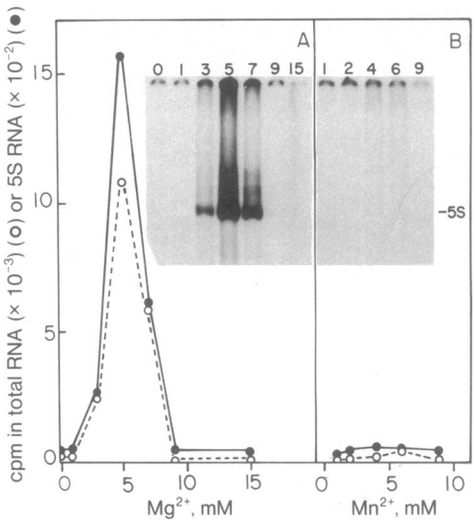

FIG. 3. Effect of divalent cations on total and 5S RNA synthesis in oocyte S-100 extracts. Standard reaction mixtures contained pXbo DNA and the indicated metal ion concentrations. At 5 mM Mg^{2+}, the total amount of GMP incorporated was 0.8 pmol. The *Insets* in this and in later figures show the upper two-thirds of the actual electrophoretic analyses of the *in vitro* transcripts at the indicated abscissa values.

In most experiments discrete minor transcripts (usually two) somewhat larger than the predominant 5S RNA transcripts are detected (see, for example, Fig. 1 and Figs. 3 and 4 below). Although not analyzed here, these probably represent transcripts of heterogeneous 5S genes in the pXbo and pXlo templates or transcripts that result from readthrough of primary termination sites in the 5S gene, as observed in other systems (see refs. 7, 19, and *).

Divalent Metal Ion and Salt Requirements. The effects of Mg^{2+}, Mn^{2+}, and KCl on transcription have been analyzed. Only the results obtained with pXbo are presented, but identical results were obtained with the pXlo. As shown in Fig. 3, synthesis of discrete 5S RNAs is observed with Mg^{2+} and not with Mn^{2+}. Maximal synthesis is observed at 5 mM Mg^{2+} and, significantly, 5S RNA synthesis parallels total RNA synthesis. As shown in Fig. 4, the synthesis of 5S RNA is maximal at 60–80 mM KCl and closely parallels total RNA synthesis, except at the lower salt concentrations, where the fractional level of 5S synthesis is lower. In these experiments total RNA synthesis was

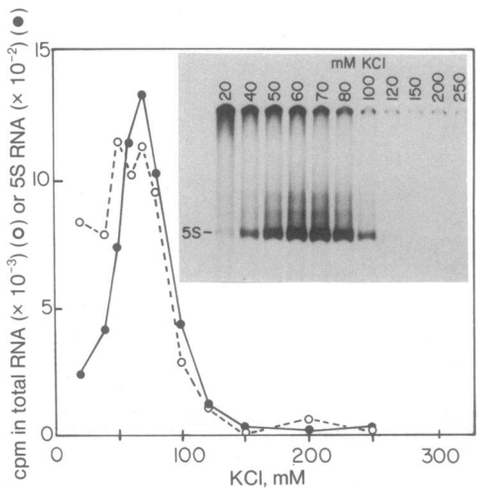

FIG. 4. Effect of KCl on total and 5S RNA synthesis in oocyte S-100 extracts. Standard reaction mixtures contained pXbo DNA and the indicated KCl concentrations. At 60 mM KCl, the total amount of GMP incorporated was 0.7 pmol.

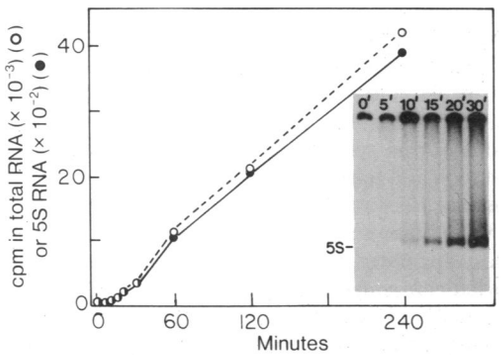

FIG. 5. Kinetics of total and 5S RNA synthesis in oocyte S-100 extracts. Standard reaction mixtures contained pXbo3 DNA incubated for the times indicated. At 60 min, the total amount of GMP incorporated was 1.2 pmol.

monitored by DEAE-paper binding, which scores small oligonucleotides. Thus the fractional value of 5S RNA synthesis as indicated graphically (usually about 10%) appears less (usually severalfold) than is apparent from the autoradiograph. Typically, radioactivity in 5S RNA may account for as much as 40% of the total radioactivity in ethanol-precipitated RNA samples obtained from preparative reactions.

Kinetics. As is shown in Fig. 5, the synthesis of 5S and total RNA proceeds in a near linear fashion for prolonged periods of time (up to 4 hr). Although somewhat of a lag in RNA synthesis is apparent during the early phase of the reaction, 5S synthesis is clearly detectable as early as 10 min (see *inset*).

Effects of DNA Concentration. As shown in Fig. 6 for the pXbo template, 5S RNA synthesis is maximal at low DNA concentrations (about 10 μg/ml) and is inhibited at higher DNA concentrations even though a high level of overall synthesis is preserved. The RNA synthesized at high DNA concentrations is of high molecular weight (see *inset*) but has not been further characterized. Identical results have been obtained with the pXlo template (data not shown).

Transcription of cloned 5S DNA by purified RNA polymerase III in the presence of immature oocyte components

The 5S genes in plasmids containing 5S DNA were previously shown, by DNA-RNA hybridization analyses, to be randomly transcribed by a purified oocyte RNA polymerase III (13, 14). Polyacrylamide gel electrophoretic analyses also failed to reveal the synthesis of any discrete 5S RNA species in this purified system, as is shown in Fig. 7 *left*. Subsequent reconstitution studies with additional components revealed a low level of accurate 5S RNA synthesis in this system in response to DNA-free

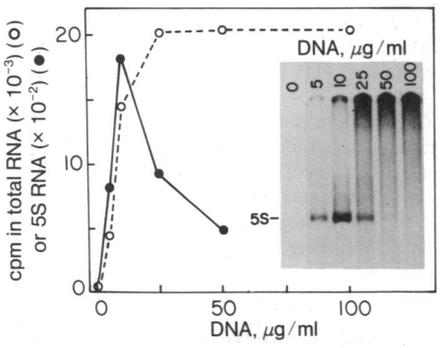

FIG. 6. Effect of DNA concentration on the synthesis of total RNA and 5S RNA by oocyte S-100 extracts. Standard reaction mixtures contained the indicated amounts of pXbo DNA. At 10 μg of DNA per ml the total amount of GMP incorporation was 1.1 pmol.

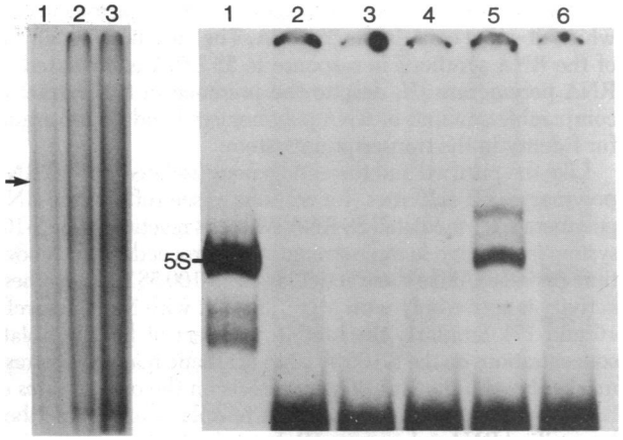

FIG. 7. Polyacrylamide gel electrophoresis of RNAs synthesized in a reconstituted system containing a purified DNA template, purified RNA polymerase III, and an immature oocyte-derived chromatin supernatant fraction. (*Left*) Reaction conditions were identical to those for *Right* (below), except that the chromatin supernatant fraction was omitted and each reaction mixture contained purified RNA polymerase III and 2 (lane 1), 10 (lane 2), or 50 (lane 3) μg of pXbo DNA per ml. The position of a 5S RNA marker run in the same gel is indicated by the arrow. (*Right*) All reaction mixtures contained the immature oocyte chromatin supernatant fraction incubated under standard conditions with the following additions: lane 2, none; lane 3, 200 units of RNA polymerase III; lane 4, pXbo DNA at 10 μg/ml; lane 5, 200 units of RNA polymerase III and pXbo DNA at 10 μg/ml; lane 6, 200 units of RNA polymerase III and pMB9 DNA at 10 μg/ml; lane 1 contained a 5S RNA marker.

protein fractions dissociated from immature oocyte chromatin (C. S. Parker, unpublished observations). However, it was also found that the chromatin supernatant fraction (see *Materials and Methods*) contained a component(s) with this capability. As shown in Fig. 7 *right*, incubation of this fraction in the absence of any other macromolecular components (lane 2), or in the presence of oocyte RNA polymerase III alone (lane 3) or pXbo DNA alone (lane 4), showed no synthesis of any discrete RNA species. In contrast, incubation of this fraction with both pXbo DNA and RNA polymerase III (lane 5) results in the synthesis of a discrete 5S RNA as well as a discrete transcript somewhat greater in size (see above). Under similar conditions the pXlo template directs the synthesis of a discrete *X. laevis* 5S RNA (data not shown) but the plasmid vector (pMB9) (lane 6) yields no discrete transcripts. Hence 5S RNA synthesis in this system requires plasmids that contain 5S DNA, an exogenous RNA polymerase III, and unknown components in the chromatin supernatant fraction. The limited availability of quantities of immature oocytes has hindered further analysis of this component(s). It was for this reason that the studies with mature oocytes (above), which actually followed the experiments with immature oocytes, were initiated.

DISCUSSION

The 5S genes in purified DNA templates appear to be selectively and accurately transcribed in crude extracts from large oocytes on the basis of the following observations: The major discrete transcripts synthesized in response to the pXlo and pXbo DNAs are similar or identical in size and sequence to the corresponding naturally occurring oocyte 5S RNAs, and they account for a large fraction (up to 40%) of the newly synthesized RNA recovered from polyacrylamide gels. The extent to which 5S DNA spacer sequences (1, 3) and the noncoding strand of the gene are transcribed has not been analyzed directly, but the large fraction of the total RNA found in the 5S RNA transcripts suggests that this is minimal. Moreover, most of the newly synthesized RNA not found in the discrete 5S-sized transcripts

is apparently synthesized in response to the plasmid vector, which does not contain the 5S DNA. The fact that virtually all of the RNA synthesis in response to 5S DNA is mediated by RNA polymerase III, despite the presence in this extract of comparable amounts of RNA polymerases I and II, also argues for fidelity in the transcription system.

Like the purified and the endogenous (isolated nuclei) RNA polymerase III activities (for references see ref. 10) the RNA polymerase III-mediated 5S RNA synthesis reaction in the S-100 system is sensitive to ionic strength and proceeds for extended time periods. Unlike these activities, the S-100 5S RNA synthesis activity is active only with Mg^{2+} and not with Mn^{2+} (cf. refs. 16 and 17). Similarly the inhibitory effect of high template concentrations on the S-100 5S RNA synthetic reaction contrasts markedly with the lack of such effects on the overall rates of RNA synthesis when general DNA templates are transcribed by purified RNA polymerase III. In general, these observations emphasize the need to optimize and carefully control conditions when the transcription of specific genes is under study. Additionally, the kinetic studies suggest that any modifications of the 5S DNA template (e.g., folding within a chromatin structure, see below) or the RNA polymerase III that are essential for transcription must occur relatively rapidly. Moreover, while the somewhat surprising template dosage effects can be explained by trivial effects (such as increased numbers of nonspecific initiation sites), they could also reflect nontrivial effects that are related to the relative affinities of the various transcription components (RNA polymerase or other factors) for each other or for specific (promoter) and general binding sites on the DNA. The inhibition of 5S RNA synthesis by secondary DNAs (pMB9) that do not contain 5S genes (S. Ng, unpublished observations) also argues for interaction of essential transcription components with nonspecific DNA sites.

The large-oocyte extract employed here contains most of the large RNA polymerase pools (I, II, and III) localized in the germinal vesicles in intact oocytes (9) and probably contains other components such as the free histones (24) and the chromatin assembly activity (25). However, we presently do not know what fraction of the exogenous DNA, if any, becomes organized within a chromatin structure, nor do we know whether such organization is a prerequisite for the specific transcription observed. It does appear that most of the added plasmid DNA is converted to a relaxed form within minutes in the crude extract (S. Ng, unpublished observations). Attempts to ascertain the nature of the transcribed template are complicated by the apparently low transcription efficiency in the system. For example, during a 1-hr incubation under standard conditions only about one 5S RNA is generated per 50 5S genes present, suggesting that only a minor fraction of the exogenous DNA molecules actually serve as templates. This could reflect the sequestration of most of the added DNA within structures (e.g., nucleoprotein complexes) that cannot serve as templates or the presence of limiting quantities of necessary transcription components. While there is only about 1 RNA polymerase III molecule (assuming quantitative recovery) present per 10 5S genes under standard conditions, the RNA polymerase itself does not appear to be limiting because the addition of a functional (14, 15) exogenous RNA polymerase III does not enhance 5S gene transcription (S. Ng, unpublished observations). In contrast, an exogenous RNA polymerase III is required for accurate transcription of exogenous 5S DNA in the presence of a soluble postchromatin fraction from immature oocytes. Presumably the active components in this fraction are the same as those in the large-oocyte extract. These results and previous chromatin transcription studies (13–15) suggest that the active component(s) does not copurify with the RNA polymerase III, although they do not exclude a primary interaction with the enzyme prior to transcription.

The systems described here and the analogous oocyte germinal vesicle (19) and somatic cell systems (20, *) are appropriate for answering many questions relating to the transcription of various genes—e.g., the functionality of naturally heterogeneous or mutated forms of the 5S genes (cf. refs. 8 and 18). Additionally, the systems should prove valuable for further fractionation and for identification of the various transcription components. Because the components whose effects are manifest in these systems may reflect only part of the transcriptional regulatory apparatus, it will be important to analyze the effects of purified components on the transcription of specific genes both in purified DNA templates and in minichromosomes reconstructed from various histones and these same DNA templates.

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