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Cloned single repeating units of 5S DNA direct accurate transcription of 5S RNA when injected into *Xenopus* oocytes

(purified genes/recombinant DNA/living oocytes/nuclear injection)

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ABSTRACT **Single and multiple repeating units of three types of *Xenopus* 5S DNA recombined with the plasmid pMB9 serve as templates for the accurate synthesis of 5S RNA after their injection into *Xenopus laevis* oocyte nuclei. All 15 cloned single repeating units of *X. laevis* oocyte 5S DNA that were tested supported 5S RNA synthesis. Three cloned fragments of *X. borealis* oocyte 5S DNA and one cloned single repeating unit of *X. borealis* somatic 5S DNA were templates for 5S RNA synthesis. We conclude that the majority of repeating units of 5S DNA in these multigene families contain the information for accurate initiation and termination of 5S RNA synthesis. The ability of this system to detect sequence changes that affect transcription is demonstrated.**

High molecular weight genomic 5S DNA containing many copies of the 5S RNA gene on each molecule serves as a template for 5S RNA synthesis when it is injected into *Xenopus* oocyte nuclei (1). The usefulness of this DNA for determining sequences that influence accurate transcription is limited because of the heterogeneity of the many repeating units in genomic 5S DNA (2) and the difficulty of assaying transcription from any particular gene in the multigene family. In order to identify the nucleotide sequences in 5S DNA and the macromolecules in *Xenopus* oocytes that interact with these sequences to regulate transcription of 5S RNA genes, we will need to test the effect of specific sequence changes on the transcription of cloned variants. As a first step, we report here that cloned single repeating units of 5S DNA recombined with a bacterial plasmid support the accurate synthesis of 5S RNA when injected into *Xenopus* oocytes.

MATERIALS AND METHODS

Description of the DNA Preparations. Fragments of *Xenopus* 5S DNA cleaved with *Hin*dIII were inserted at the *Hin*dIII site of the plasmid pMB9 (3, 4). Three kinds of cloned 5S DNA were tested in these experiments: *X. laevis* oocyte (Xlo) (4), *X. borealis* oocyte (Xbo) (5), and *X. borealis* somatic (Xbs) (6). Genomic Xlo and Xbs 5S DNAs have one *Hin*dIII cleavage site in each repeating unit whereas Xbo 5S DNA is cleaved irregularly by *Hin*dIII at an average of about one site every several repeats (D. Carroll and D. Brown, unpublished data).

Fig. 1 shows a schematic representation of a recombinant DNA molecule containing a single repeating unit of Xlo 5S DNA. The lengths of one repeating unit of Xlo 5S DNA and of the plasmid pMB9 (7) are 700 and 5400 base pairs, respectively. The 5S RNA gene itself comprises 120 base pairs out of the total 6100 base pairs in the recombinant molecule. Inserts can be introduced into the plasmid's *Hin*dIII site in two orientations. In the case of Xlo 5S DNA, insertion in one orientation (using the *Eco*RI site as a marker) does not affect the plasmid's ability to confer tetracycline resistance on the host bacterium. When Xlo 5S DNA is inserted in the opposite orientation, the tetracycline-resistance gene is inactivated (4). Insertion of Xbo or Xbs 5S DNA in either orientation inactivates the tetracycline-resistance gene (3).

The radioactive DNA used in the experiments shown in Fig. 2 and Table 1 is pXlo8. This plasmid contains four adjacent repeating units of Xlo 5S DNA. It was made radioactive during the chloramphenicol induction step by addition of 5 μCi of [^{3}H]thymidine per ml of medium. The plasmid DNA had a specific activity of 110,000 cpm/μg. About 15 ng of DNA was injected into each oocyte.

The original cloning of these recombinant DNA molecules (3) took place under P2 EK1 conditions prior to issuance of the NIH Guidelines but according to the guidelines recommended at the Asilomar Conference. All subsequent growth and handling of recombinant DNA has been carried out according to the NIH Guidelines (P2 EK1). The recombinant DNA was purified by two ethidium bromide/CsCl gradients (8) followed by one neutral CsCl density gradient centrifugation.

Recombinant DNA was cleaved with *Hin*dIII and the 5S DNA was separated from the plasmid DNA by electrophoresis on 1% agarose gels. The DNA was eluted from the agarose gel, and residual agarose was removed by phenol extraction, adsorption of DNA to and elution from a DEAE-cellulose column, and ethanol precipitation as described (4). The isolation of Xlo and Xbo genomic 5S DNA from *Xenopus* erythrocyte DNA has been described (2,9).

Oocyte Injection Methods, RNA Extraction, and Analysis. DNA was dissolved in 0.015 M NaCl/0.5 mM EDTA/5 mM Tris, pH 7.8, at a concentration of 0.1–1 mg/ml and mixed with [^{3}H]GTP at 10 mCi/ml (15 Ci/mmol from Radiochemical Centre, Amersham). About 50 nl was injected into each oocyte, aimed at its nucleus (10). After 5 hr of incubation at room temperature, the oocytes were frozen at −70°. About 20 oocytes were used for each experimental point.

Conditions for RNA extraction from frozen oocytes and polyacrylamide gel electrophoresis of the deproteinized RNA have been described (1). Nondenaturing polyacrylamide gels (8% or 10%) were used for 5S RNA separations. Gels were sliced and the RNA was extracted for assay of radioactivity (1) or prepared for fluorography (11).

Radioactive RNA extracts were hybridized to DNA immobilized on nitrocellulose filters. The strand selectivity of transcription was determined by hybridization of the radioactive RNA to separated strands of high molecular weight genomic

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Abbreviations: The three kinds of genes coding for 5S ribosomal RNA (5S DNA) used in these studies, Xlo, Xbo, and Xbs, are *Xenopus laevis* oocyte-type, *Xenopus borealis* oocyte-type, and *Xenopus borealis* somatic-type. In recombinant form with a plasmid they are referred to with the prefix "p" (i.e., pXlo, pXbo, and pXbs).

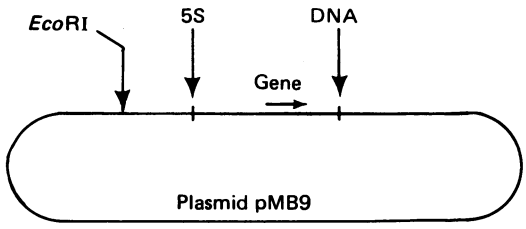

FIG. 1. The structure of a recombinant DNA molecule containing a single repeating unit of Xlo 5S DNA inserted at the *Hin*dIII site of the plasmid pMB9. The location of the *Eco*RI site in the plasmid is about 350 base pairs from the *Hin*dIII site and helps to establish the orientation of the insert. The horizontal arrow indicates direction of transcription of the 5S RNA gene. In this orientation an insert of Xlo 5S DNA inactivates the tetracycline-resistance gene of the plasmid.

Xlo 5S DNA (9). The amount of spacer transcripts was estimated from the amount of labeled RNA hybridizing to 5S DNA in the presence of at least 100-fold excess of unlabeled 5S RNA prepared from *X. laevis* oocytes (12). The amount of plasmid DNA transcription was estimated by using ^{32}P-labeled complementary RNA transcribed from pMB9 with *Escherichia coli* RNA polymerase (13). The [^{32}P]cRNA was added to the hybridization mixture, and the efficiency of hybridization to pMB9 DNA on filters was assumed to be the same as the efficiency of hybridization of the [^{3}H]RNA synthesized by oocytes.

RESULTS

Fate of Injected 5S DNA. A uniformly ^{3}H-labeled recombinant DNA containing four repeating units of Xlo 5S DNA (pXlo8) was injected into oocytes as intact circular molecules or as linear fragments generated by a complete *Hin*dIII digestion. DNA samples were purified at 0, 8, and 21 hr after injection and electrophoresed on agarose gels. The original DNA consisted mainly of relaxed circles (form II) as determined by agarose gel electrophoresis. The changes in the configuration of the DNA with time after injection (Table 1) show that most of the DNA was converted to supercoiled circles (form I), as has been found for simian virus 40 DNA injected into oocytes (14). Some of the radioactive material isolated after 8 and 21 hr of incubation did not migrate into the gel. The nature of this material was not determined. The linear DNA molecules were gradually degraded after injection (Fig. 2). However, even after 22 hr, some full-length linear molecules could be detected.

Table 1. Configuration of intact ^{3}H-labeled plasmid DNA at different times after its injection into oocyte nuclei

Duration of oocyte incubation, hr	% of total acid-insoluble cpm: Supercoiled circles	% of total acid-insoluble cpm: Relaxed circles	Total acid-insoluble cpm per oocyte
0	15	77	293
8	40	24	290
21	57	25	217

The radioactive plasmid used here (pXlo8) has four repeating units of Xlo 5S DNA inserted into the *Hin*dIII site of pMB9. Carrier plasmid DNA was added to each extract to visualize the position of relaxed (form II) and supercoiled (form I) DNA. Both of these forms migrated as single bands. The bands were stained with ethidium bromide, and the gels were sliced and assayed for radioactivity. Essentially all of the acid-insoluble radioactivity that was not present in the two forms of DNA did not enter the gel. No radioactive full-length linear molecules were detected nor was there radioactive material that migrated more rapidly than the supercoiled molecules.

Transcription of Intact Plasmid DNA Molecules Containing *X. laevis* Oocyte 5S DNA Insertions. We assayed intact recombinant plasmids with Xlo 5S DNA inserted in either orientation for their ability to serve as templates for 5S RNA synthesis after injection into *Xenopus* oocyte nuclei. Figs. 3 and 4 show gels of labeled RNA synthesized after the injection of cloned Xlo 5S DNA molecules. We have tested 15 different plasmids containing single repeating units of *X. laevis* oocyte 5S DNA (12 are shown in Figs. 3, 4, and 5). All but one acted as template for radioactive RNA that comigrated with known 5S RNA. The one exception (Fig. 3, lane b) gave a band of RNA that migrated slightly faster than mature 5S RNA. In addition to 5S RNA, other radioactive bands were detected in some instances. One of these bands of RNA migrated more slowly than 5S RNA and was estimated to contain about 130 nucleotides. A RNA species of the same length is synthesized *in vivo* by oocytes and contains extra nucleotides at the 3′ end of 5S RNA (15). We have detected the same extra oligonucleotide produced by RNase T1 from the slower moving RNA bands as that found by Denis and Wegnez (15). They showed that this RNA represents a read-through of the normal termination site. Some

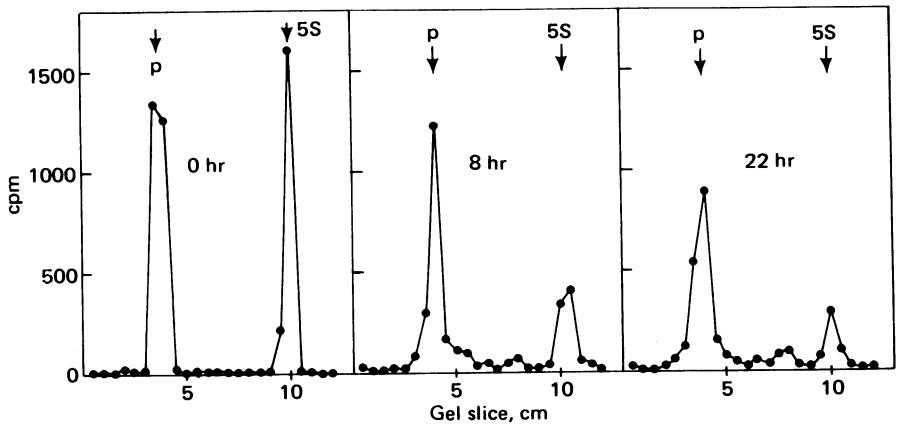

FIG. 2. Digestion of radioactive linear DNA molecules after their injection into *Xenopus* oocytes. Recombinant DNA containing four repeating units of Xlo 5S DNA (pXlo8) was uniformly labeled with [^{3}H]thymidine. A *Hin*dIII digest was injected into the nuclei of *Xenopus* oocytes and incubated for 0, 8, or 22 hr. The purified DNA was electrophoresed on 1% agarose with carrier nonradioactive DNA. The gel was stained with ethidium bromide and sliced for assay of radioactivity. "p" and 5S denote the location of intact linear pMB9 and the monomeric repeating units of Xlo 5S DNA, respectively. These four 5S DNA repeating units migrate as a single band.

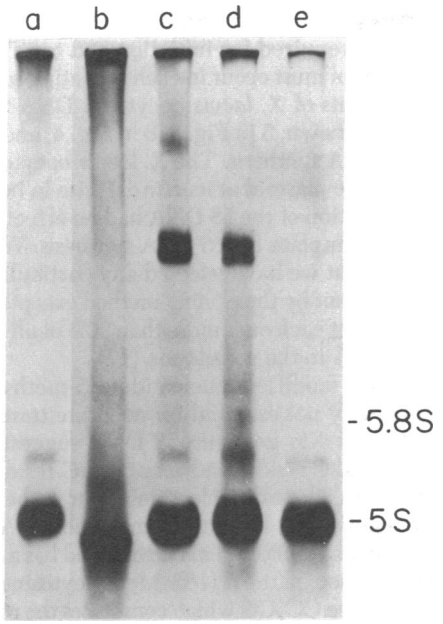

FIG. 3. Polyacrylamide gel electrophoresis of [^{3}H]RNA synthesized by *X. laevis* oocytes after the injection of different recombinant DNAs containing multiple or single repeating units of Xlo 5S DNA. Lanes: a, pXlo8 containing four repeating units of Xlo 5S DNA; b, c, and d, pXlo36, pXlo32, and pXlo33 (containing single repeating units) in the orientation that inactivates the tetracycline gene (see Fig. 1); e, high molecular weight genomic Xlo 5S DNA.

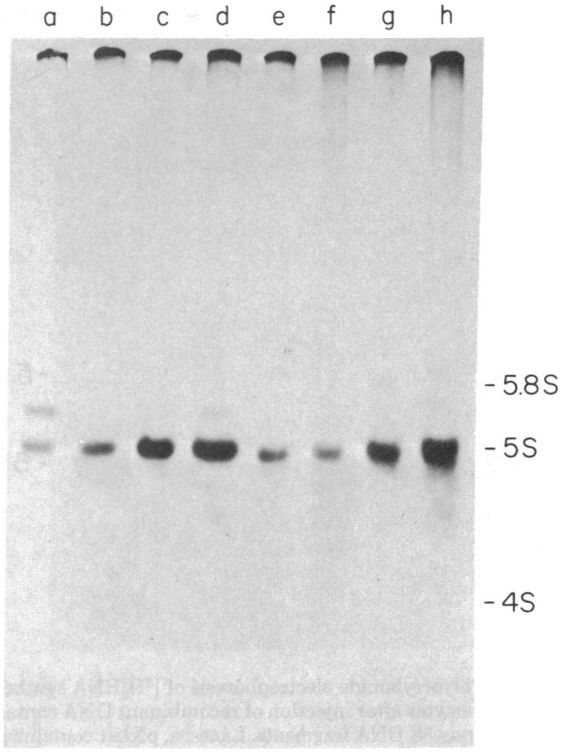

FIG. 4. Polyacrylamide gel electrophoresis of [^{3}H]RNA synthesized by *X. laevis* oocytes after injection of recombinant DNA containing single Xlo 5S DNA inserts. All of these inserts are in the orientation that does not affect the tetracycline resistance conferred by the plasmid. Lanes: a, pXlo6; b, pXlo9; c, pXlo11; d, pXlo12; e, pXlo14; f, pXlo16; g, pXlo18; h, pXlo19.

recombinant DNAs that contained a single 5S repeating unit supported the synthesis of the same small percentage of this longer RNA as did total genomic Xlo 5S DNA (Fig. 3). This result demonstrates that read-through can occur in a minority of transcription events from one cloned repeating unit. Alternatively, a minority of repeating units in the multigene family could have encoded all of the longer RNA molecules. The RNA synthesized from one cloned repeating unit (Fig. 4, lane a) gave two bands of equal intensity—one comigrating with 5S RNA and the other with the longer RNA form. The 5S DNA in this plasmid may have a mutated termination site that permits a higher fraction of polymerases to transcribe past the normal termination sequence. Prominent slower migrating bands of RNA (Fig. 3, lanes c and d) were detected in RNA transcribed from some recombinant DNAs with single Xlo 5S DNA inserts. These RNAs have not been studied further.

Transcription of Single Cloned Repeating Units of *X. laevis* Oocyte 5S DNA. The 5S DNA insert was excised from the recombinant DNA molecule pXlo31 by cleavage with *Hin*dIII and purified by gel electrophoresis. Table 2 compares the transcription from the linear 5S DNA repeating unit with that from the original intact 5S DNA-containing plasmid. Linear repeating units supported less 5S RNA synthesis than did the intact circular recombinant DNA molecules. Fidelity of transcription as measured by strand selection and by the relative abundance of spacer transcripts was about the same for linear molecules and circular templates. Transcripts of the plasmid were detected by hybridization.

Various restriction enzyme fragments cleaved from genomic Xlo 5S DNA or from cloned plasmids containing one or more repeating units of 5S DNA have been tested for their ability to serve as template for 5S RNA synthesis in this system. A linear form of the recombinant plasmid is produced by digestion with the restriction endonuclease *Eco*RI (see Fig. 1). There are no cleavage sites for *Eco*RI in any of the 5S DNA inserts used in these experiments. These linear molecules supported 5S RNA synthesis (results not shown). Gel-purified single repeating units of genomic DNA produced by digestion with *Hin*dIII also supported 5S RNA synthesis. However, linear restriction enzyme fragments that contained only parts of the gene (as much as half of the 5′ end) did not support the synthesis of any radioactive RNA that hybridized with 5S DNA.

There was considerable variation in the amount of 5S RNA synthesis from one experiment to the next, an effect attributable in part to the extent to which DNA is successfully deposited in the oocyte nucleus (16). Despite this variation we observed, in several experiments, that injection of circular molecules resulted in more 5S RNA synthesis than did injection of linear molecules. However, linear fragments containing a complete repeating

Table 2. Fidelity of 5S RNA transcription from an intact circular plasmid (pXlo31) or from its isolated 5S DNA linear insert (Xlo31)

	% of total cpm		Strand selection,* coding/noncoding	% competed by unlabeled 5S RNA
	5S RNA†	pMB9 RNA‡		
pXlo31	37	19	19	42
Xlo31	19	—	14	50

† Radioactivity in 5S RNA as percentage of total radioactivity in the gel.

‡ RNA transcripts of pMB9 were estimated by hybridization to pMB9 DNA.

* The coding (light strand) and the noncoding (heavy strand) were purified by alkaline CsCl sedimentation of genomic 5S DNA of *X. laevis* (9) and used for hybridization of the total RNA from injected oocytes.

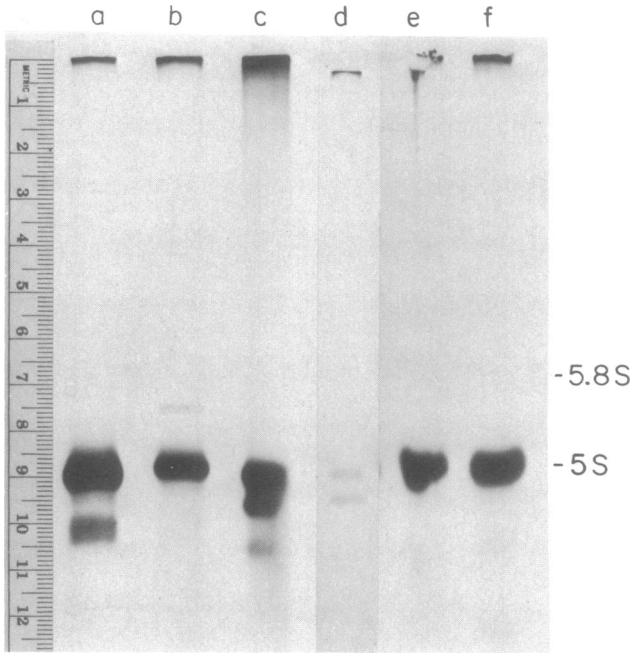

FIG. 5. Polyacrylamide electrophoresis of [^{3}H]RNA synthesized by *X. laevis* oocytes after injection of recombinant DNA containing various *Xenopus* 5S DNA fragments. Lanes: a, pXbs1 containing one repeating unit of *X. borealis* somatic 5S DNA; b, pXlo31 containing one repeating unit of Xlo 5S DNA in the tetracycline-sensitive orientation; c, pXbo1 containing a *Hin*dIII fragment of Xbo 5S DNA (755 base pairs long) with three genes for Xbo1 5S RNA; d, Xbol, the purified 5S DNA insert from pXbo1; e, pXbo3 containing a 2700-base pair fragment of Xbo 5S DNA with six 5S RNA genes; f, pSE1, a plasmid produced first by inserting a large Xbo 5S DNA fragment into the *Hin*dIII site of PSC101 and then cleaving the molecule with *Eco*RI and ligating it to *Eco*RI linear molecules of ColE1. There are about 8400 base pairs of Xbo 5S DNA in each molecule, including more than 10 5S RNA genes (S. Emmons and D. D. Brown, unpublished data).

unit were transcribed as accurately as the same 5S DNA molecule inserted in plasmids (Table 2).

Transcription of *X. borealis* 5S DNA Plasmids. We tested cloned oocyte and somatic 5S DNAs from *X. borealis* for their ability to support 5S RNA synthesis when the recombinant DNAs (in one case, the purified 5S DNA insert) were injected into *X. laevis* oocytes. Fig. 5 shows fluorograms of gels of the RNA products. Three different plasmids containing *X. borealis* oocyte 5S DNA (Xbo) and one containing *X. borealis* somatic 5S DNA (Xbs) were tested, and all were active templates for 5S RNA synthesis. The plasmid pXbsl has one 5S RNA gene (J. L. Doering and D. D. Brown, unpublished data). There are three 5S RNA genes in the Xbol fragment (L. Korn and D. D. Brown, unpublished data). The plasmid (pXbol) and the purified insert (Xbol) gave two RNA species—one the size of 5S RNA and one migrating slightly faster. Sequence analysis (not shown here) showed that each band contained RNA synthesized mainly from one of the three genes. The faster migrating RNA has not been detected in the 5S RNA synthesized from total genomic *X. borealis* oocyte 5S DNA (see ref. 1). The other two *X. borealis* oocyte 5S DNA-containing plasmids, pXbo3 and pSE1, have 6 and >10 genes, respectively (ref. 5; S. Emmons and D. D. Brown, unpublished data).

DISCUSSION

We have shown that a single repeating unit of 5S DNA with one structural gene contains the information required for synthesis of 5S RNA when injected into oocyte nuclei. Thus, the DNA sequences that are required for initiation and termination of 5S RNA transcription must occur in each repeating unit. All 15 single repeating units of *X. laevis* oocyte 5S DNA that were tested (only 12 are shown: 3 in Fig. 3, 8 in Fig. 4, and 1 in Fig. 5) supported 5S RNA synthesis. The *X. laevis* oocyte 5S DNA was inserted into the plasmid at its *Hin*dIII site in both orientations. The orientation of the 5S DNA had no effect on its capacity to act as a template for 5S RNA synthesis. There is no reason to believe that we have selected any particular kind of 5S DNA repeating unit by the cloning method except those that have a *Hin*dIII site at each end [more than 90% of all repeating units of Xlo 5S DNA in the population (17)].

The pattern of modified nucleotides (5-methylcytosine residues) is probably not essential for accurate transcription. Sequencing data of Xlo genomic 5S DNA suggest that the majority of if not all C residues adjacent to a G residue (CpG) are methylated (18). These dinucleotides occur in the gene and in the spacer regions that flank the gene. The DNA used for these experiments was replicated in bacteria and has a markedly different methylation pattern (18). Methylcytosine residues occur in the sequence CC$^{A}_{T}$GG which constitutes the recognition site for the *Eco*RII restriction enzyme (19). Such sequences occur in the gene and in the pseudogene of Xlo 5S DNA (18). Both *Eco*RII methylated (derived from bacteria) and CpG-methylated 5S DNA (derived from the frog itself) have been shown to support 5S RNA synthesis in oocyte nuclei. We have not ruled out the possibility that the DNA is modified after its injection into the oocyte nucleus.

Linear single repeating units of 5S DNA support less 5S RNA synthesis upon injection into oocytes than do circular plasmid molecules or higher molecular weight genomic 5S DNA having many repeating units on each molecule. The absolute length of flanking sequences on either side of the gene may need to exceed some minimum size for optimal transcription, the specific sequence not being important. Alternatively, the circularity of the DNA may be the most important factor. Circular DNA is more stable than linear DNA in the oocyte nucleus, but that fact alone may not account for all of the difference because a substantial fraction of molecules that comigrate with undegraded linear molecules remain after even 8 hr of incubation (Fig. 2).

Because cloned 5S DNA molecules can support 5S RNA synthesis, we can use the oocyte injection system to detect mutants of various kinds. Among the plasmids tested in these experiments, one Xlo repeating unit (Fig. 4, lane a) differed from the others in the efficiency with which transcription terminates at the end of the 5S RNA gene. Other cloned molecules yielded transcripts that migrated faster than 5S RNA on gels (Fig. 3, lane b; Fig. 5, lanes c and d). The sequence difference between these RNAs and 5S RNA is not yet known. In the case of pXbol (Fig. 4, lane c), preliminary evidence indicates that RNA in the two bands may not differ in size because they comigrate in formamide gels (D. D. Brown and L. Korn, unpublished data). Such a difference could be caused by mutations within the gene that alter the secondary structure of 5S RNA.

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