CONTROL OF 5S RNA SYNTHESIS DURING EARLY DEVELOPMENT OF ANUCLEOLATE AND PARTIAL NUCLEOLATE MUTANTS OF *XENOPUS LAEVIS*

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ABSTRACT

Ribosomes of all eukaryotes contain a single molecule of 5S, 18S, and 28S RNA. In the frog *Xenopus laevis* the genes which code for 18S and 28S RNA are located in the nucleolar organizer, but these genes are not linked to the 5S RNA genes. Therefore the synthesis of the three ribosomal RNAs provides a model system for studying interchromosomal aspects of gene regulation. In order to determine if the synthesis of the three ribosomal RNAs are interdependent, the relative rate of 5S RNA synthesis was measured in anucleolate mutants (o/o), which do not synthesize any 18S or 28S RNA, and in partial nucleolate mutants (p^{l-1}/o), which synthesize 18S and 28S RNA at 25% of the normal rate. Since the o/o and p^{l-1}/o mutants have a complete and partial deletion of 18S and 28S RNA genes respectively, but the normal number of 5S RNA genes, they provide a unique system in which to study the dependence of 5S RNA synthesis on the synthesis of 18S and 28S RNA. Total RNA was extracted from embryos labeled during different stages of development and analyzed by polyacrylamide gel electrophoresis. Quite unexpectedly it was found that 5S RNA synthesis in o/o and p^{l-1}/o mutants proceeds at the *same rate* as it does in normal embryos. Furthermore, 5S RNA synthesis is initiated normally at gastrulation in o/o mutants in the complete absence of 18S and 28S RNA synthesis.

INTRODUCTION

Ribosomes of eukaryotes contain a single molecule of 5S, 18S, and 28S RNA (8, 17, 33). In view of this equimolar accumulation in ribosomes it is reasonable to expect that equal numbers of the three ribosomal RNAs would be synthesized, i.e., regulation of the relative amounts of 5S, 18S, and 28S RNA would be at the gene level rather than at the level of ribosome assembly. In support of this view, the kinetics of labeling of ribosomal RNA (rRNA) in bacteria indicate that 5S, 16S, and 23S RNA are synthesized coordinately and that their genes may be located in the same operon (9, 10, 14). In higher organisms however, the genes coding for 18S and 28S RNA are located on different chromosomes than the genes coding for 5S RNA (2, 5, 32). For example, in *Xenopus laevis*, cytological hybridization has shown that the 5S RNA genes are located at the ends of all chromosomes (27), whereas the 18S and 28S genes are clustered in the nucleolar organizers of a single homologous pair of chromosomes (4).

At present we know very little of how the transcription of functionally related genes on different chromosomes is coordinated. Since

5S, 18S, and 28S RNA are extremely well characterized in higher organisms, the synthesis of these rRNAs provides a model system for studying interchromosomal aspects of gene regulation. It is not known whether or not the three rRNAs are transcribed in a coordinate manner in higher organisms, but some of the available data indicate that 5S RNA can be synthesized in the absence of 18S and 28S RNA synthesis. Perry and Kelley (28) established that low concentrations of actinomycin D inhibited 18S and 28S RNA synthesis but not the synthesis of 5S RNA. This study demonstrated that 5S RNA synthesis could be uncoupled from the synthesis of 18S and 28S RNA. Subsequent work with HeLa cells (16, 20, 34) and ovaries of *Xenopus* (12, 22) and *Drosophila* (31) has substantiated and extended this finding.

In contrast, a clear dependence of 5S RNA synthesis on the synthesis of 18S and 28S RNA was suggested by the finding that anucleolate mutants of *Xenopus*, which do not have any 18S and 28S RNA genes, do not synthesize 5S RNA during later development even though they have the normal complement of 5S RNA genes (5). It is possible that newly synthesized 5S RNA could not be detected in anucleolate mutants because the methods used were not sensitive enough or because only late stages were tested. Therefore, the important question of whether or not the anucleolate mutants synthesize 5S RNA during early development was investigated using polyacrylamide gel electrophoresis. The rate of 5S RNA synthesis was also measured in partial nucleolate mutants of *Xenopus* which synthesize 18S and 28S RNA at approximately 25% of the normal rate (19). The partial nucleolate embryos have about one-quarter of the normal number of 18S and 28S RNA genes (24), but like anucleolate embryos they most likely have the normal number of 5S RNA genes. The anucleolate and partial nucleolate mutants thus provide a unique situation for studying the interdependence of 5S, 18S, and 28S RNA synthesis without the use of specific RNA inhibitors. Unexpectedly, it was found that both the anucleolate and partial nucleolate mutants synthesize 5S RNA at the *same rate* as normal embryos!

MATERIALS AND METHODS

Origin and Labeling of Embryos

Anucleolate embryos (o/o) were obtained from a mating of two frogs with one nucleolus per cell ($+/o$, formerly 1-Nu [11]). The partial nucleolate embryos (p^{l-1}/o) were obtained from a cross between a female $+/o$ frog and a male $+/p^{l-1}$ frog. The latter mutant has a nucleolar organizer ($\pm$) which produces a normal nucleolus and an organizer (p^{l-1}) which forms a partial nucleolus. The superscript $l - 1$ is short for lethal and is the first such mutant we discovered (23). Embryos carrying only the p^{l-1} nucleolar organizer (p^{l-1}/o embryos) die at stage 42 (26), an early swimming tadpole stage. The embryos were micro-injected with a solution containing radioactive RNA precursors and then incubated 5–25 h in one-tenth Barth's saline (3). The radioactive solution was prepared by evaporating equal volumes of [5-^{3}H]-uridine (28 Ci/mM) and [8-^{3}H]guanosine (11 Ci/mM) to dryness and redissolving it in Barth's saline free of Mg^{2+} and Ca^{2+} at 10–20 mCi/ml. In some experiments the embryos were injected with L-[*methyl*-^{3}H]methionine (5 Ci/mM). Near the end of the incubation time the nucleolar phenotype of each embryo was determined by excising a small piece of tissue and examining it by phase contrast microscopy. The embryos were pooled according to phenotype and frozen in homogenization medium.

Extraction of Nucleic Acids

Labeled embryos were homogenized in 5–8 ml of 0.1 M sodium acetate, pH 5.0, containing 4 μg/ml polyvinyl sulfate and 0.5% sodium dodecyl sulfate (7). 75–100 μg of unlabeled ovary RNA was added and the homogenate extracted three times at 10–12°C with an equal volume of phenol saturated with homogenization buffer. After a final extraction with an equal volume of chloroform-octanol (24:1, wt/vol), the aqueous phase was made 3% (wt/vol) in NaCl and nucleic acids precipitated with 2 Vol of ethanol for 16–20 h at 4°C. The nucleic acid precipitate was washed with ethanol, dried by an air stream, and stored at −20°C.

Ribosomes of mouse neuroblastoma cells (clone NB2a) were prepared by lysing approximately 5×10^7 cells in 3 ml of 0.24 M sucrose, 5 mM $MgCl_2$, 10 mM tricine, pH 7.5, containing 0.1% Nonidet P-40 (Shell Chemical Co., New York). After centrifugation at 10,000 *g* for 10 min the supernate was made 10^{-3} M in disodium ethylenediaminetetraacetic acid (EDTA) by adding solid EDTA and layered over 3 ml of 2.0 M sucrose. A ribosome pellet was obtained by centrifugation at 50,000 rpm for 25 h in a Spinco L3-50 equipped with a Ti-50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). RNA was extracted from the ribosome pellet as described above.

Polyacrylamide Gel Electrophoresis

Gels composed of 2.7 or 8% acrylamide cross-linked with ethylene diacrylate (K and K Labora-

tories, Inc., Plainview, N. Y.) were cast in 6 mm diameter plexiglass tubes (18, 21). The final concentration of N,N,N',N'-tetramethylethylenediamine was 0.18% in 2.7% gels and 0.09% in 8% gels. Discontinuous gels were prepared by casting a 4 cm 2.7% gel directly on top of a polymerized 6 cm 8% gel. After a prerun of 30–60 min at 5 mA per gel in E buffer (20 mM Tricine [California Biochemical Corp., Los Angeles, Calif.], 30 mM NaH_2PO_4, 1 mM EDTA, 5% glycerol, 0.2% sodium dodecyl sulfate, pH 7.8) the nucleic acid samples were applied to the gels in 50–100 μl of E buffer containing 24% glycerol. After 2.5–6 h the gels were frozen in liquid N_2 and sliced in 1 mm sections. Each slice was placed in a scintillation vial containing 0.5 ml NCS (Nuclear Chicago Solubilizer Nuclear Chicago Corp., Des Plaines, Ill.) or 0.4 ml NCS plus 0.2 ml of toluene. After 1–2 h at room temperature 12 ml of scintillation fluid consisting of 5 g 2,5-diphenyloxazole and 0.3 g 1,4-di 2-(5 phenyloxazolyl) per liter of toluene was added. The slices of 2.7% gels dissolve completely in 1–2 h and can be counted immediately after adding the scintillant. 8% gels had to be heated in the NCS-scintillant mixture for 12–16 h at 45°C before the slices dissolved and could be counted.

RESULTS

Identification of 5S RNA

The anucleolate mutant does not synthesize any new ribosomes during its limited lifespan and therefore it is unlikely that newly synthesized 5S rRNA would be found in the ribosome fraction. In order to detect newly synthesized 5S RNA, total RNA was extracted from embryos and analyzed on 8% polyacrylamide gels. In all of the RNA samples from early embryos which were analyzed on 8% gels three radioactive peaks were found which migrated slower than 4S RNA (Figs. 1–3). The radioactive peak which runs directly behind the 4S RNA was identified as 5S rRNA. This peak coelectrophoresed with the 5S RNA extracted from neuroblastoma ribosomes (Fig. 1). It also coelectrophoresed with the 5S RNA present in the soluble portion of total tadpole RNA treated with 2 M NaCl according to the procedure described by Roshbash and Penman (29). 5S RNA is the only rRNA which is not methylated. In +/+ embryos after a 5 h labeling period with [^{3}H]nucleosides 4S, 5S, 18S, and 28S RNA peaks are well labeled (Fig. 3 A) but when embryos are labeled with [*methyl*-^{3}H]methionine all of the RNA peaks except 5S RNA are labeled (Fig. 3 B and C). In *o/o* embryos

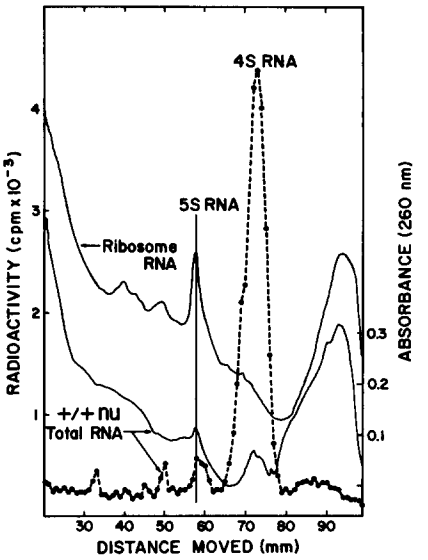

FIGURE 1 Identification of 5S RNA. Total RNA was extracted from embryos labeled for 5 h from stage 12 to 17 (26) and electrophoresed for 5 h on an 8% polyacrylamide gel. RNA extracted from ribosomes of neuroblastoma cells was electrophoresed at the same time on an adjacent gel. The absorbance of both gels was determined by scanning in a Gilford Recording Spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The gel containing total embryo RNA was sliced into 1-mm sections and each section counted in a Packard Model 3385 Liquid Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

4S and 5S RNA become labeled with [^{3}H]nucleoside precursors but only 4S RNA can be detected isotopically when [*methyl*-^{3}H]methionine is used to label embryo RNA (Fig. 3 D). These results confirm that the peak running directly behind the 4S RNA is 5S rRNA. The two radioactive peaks which migrate slower than 5S RNA on 8% polyacrylamide gels were found at all embryonic stages which were examined and were present in +/+, p^{l-1}/o, and *o/o* embryos (Figs. 1–3). The nature of these peaks is unknown.

Initiation of 5S RNA during Gastrulation of Normal and Anucleolate Embryos

The synthesis of 5S, 18S, and 28S RNA is initiated at gastrulation in embryos of the frog *Xenopus laevis* (1, 7, 16, 18). If newly synthesized rRNA precursor or any of the RNA segments released during its processing are required for the initiation of 5S RNA synthesis, the *o/o* mu-

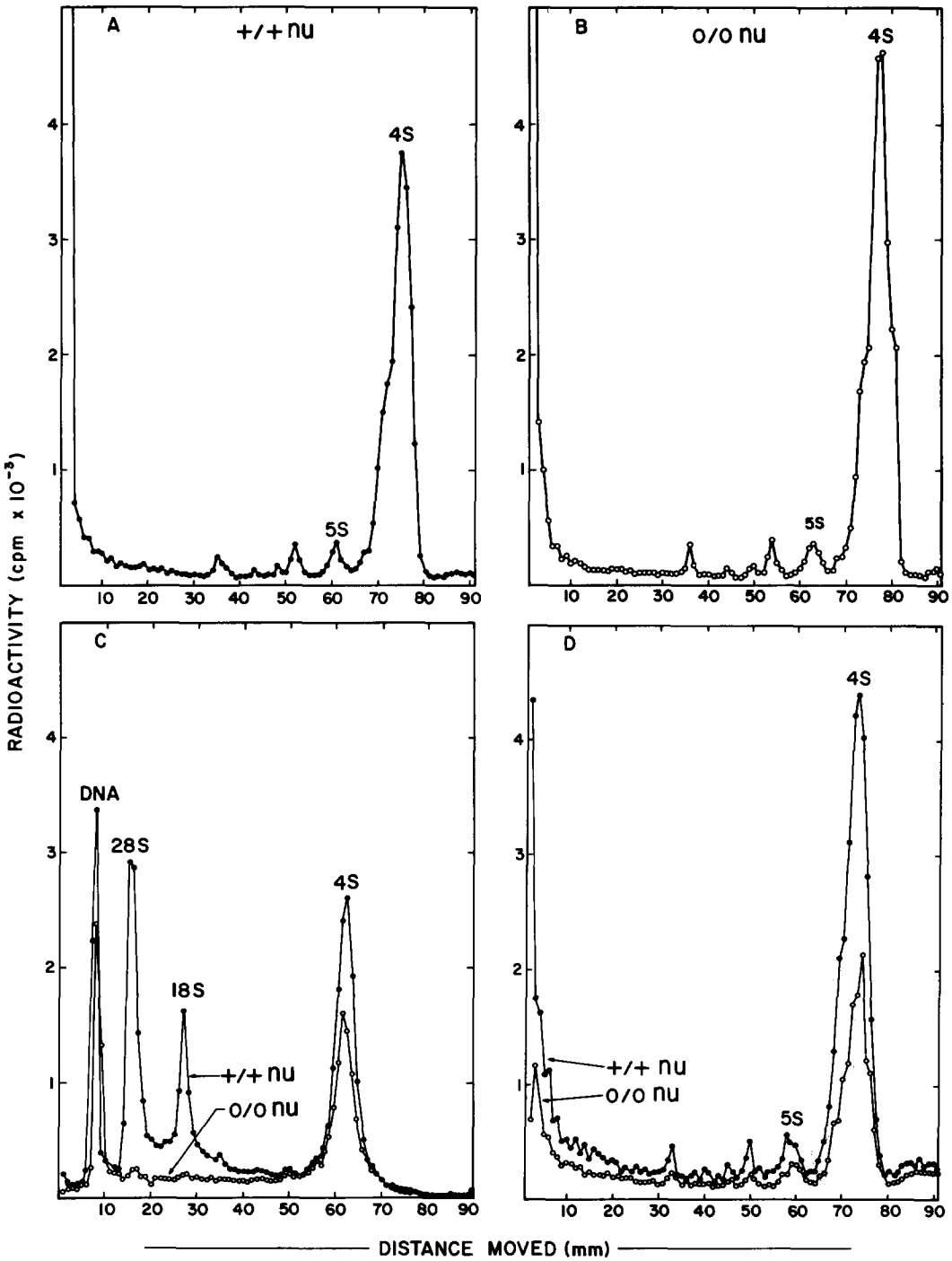

FIGURE 2 5S RNA synthesis during early development. Embryos from a cross of two +/o frogs were injected with a mixture of [5-^{3}H]uridine and [8-^{3}H]guanosine at stage 12. After 5 h or 24 h total RNA was extracted from +/+ and o/o embryos and analyzed by polyacrylamide gel electrophoresis. A and B, total RNA from +/+ (A) and o/o (B) embryos labeled 5 h was electrophoresed on 8% gels for 5 h. C and D, total RNA from +/+ and o/o embryos labeled 24 h was electrophoresed for 2.5 h on a 2.7% gel (C) and for 5 h on a 8% gel (D).

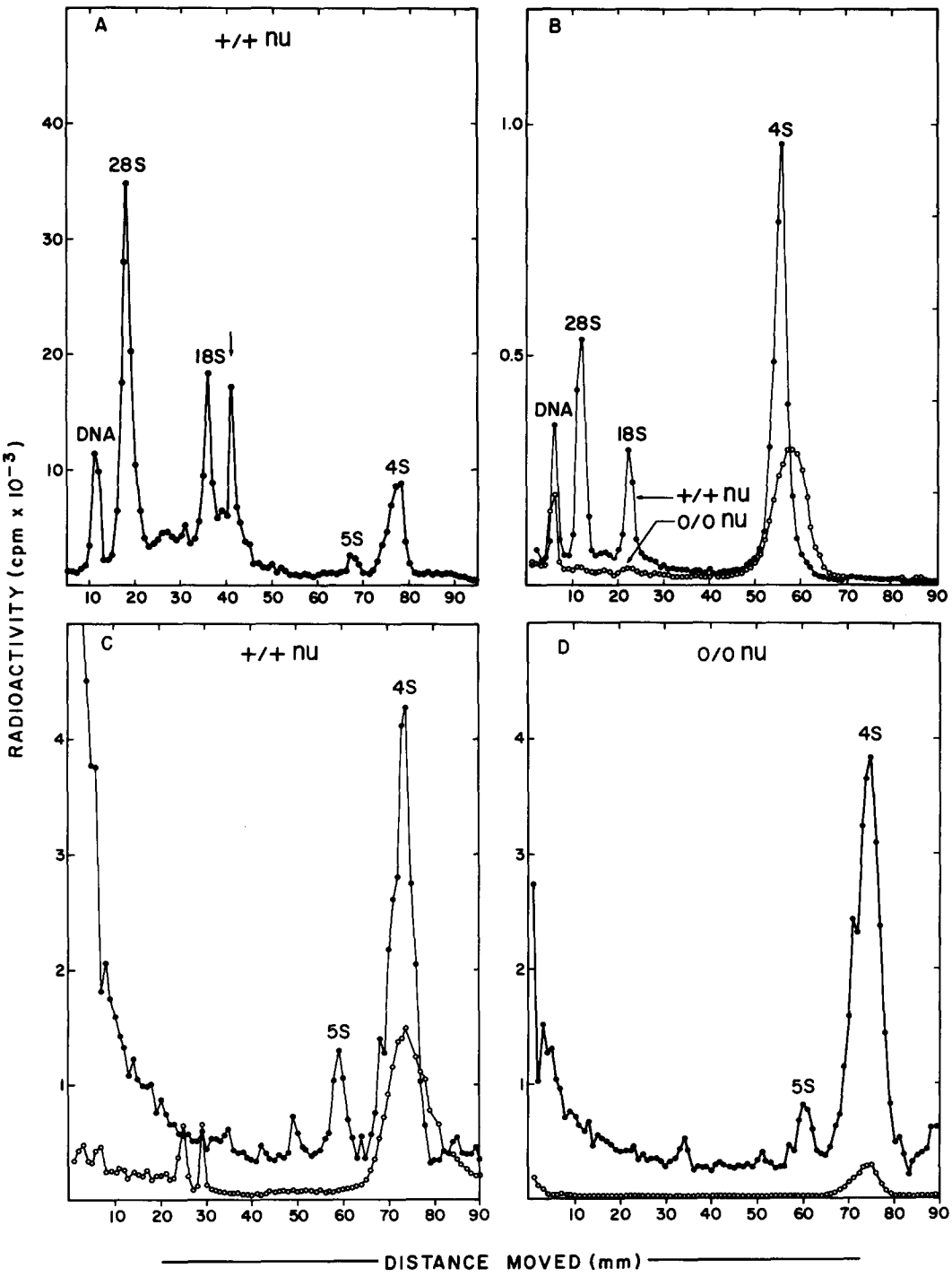

FIGURE 3 5S RNA synthesis during the tailbud stage of development. Embryos from the same cross described in Fig. 2 were labeled for 5 h during stages 27–29. A, total RNA of +/+ embryos labeled with [^{3}H]nucleosides was electrophoresed on a discontinuous polyacrylamide gel for 5 h. The arrow denotes the interface of the 2.7% gel and 8% gel. B, total RNA of +/+ and *o/o* embryos labeled with [*methyl*-^{3}H]methionine was electrophoresed on 2.7% gels for 2.5 h. C and D, total RNA of +/+ (C) and *o/o* (D) embryos labeled with [*methyl*-^{3}H]methionine (○-○-○) or [5-^{3}H]uridine and [8-^{3}H]guanosine (●-●-●) was electrophoresed for 5 h on 8% gels.

tants would not be expected to synthesize 5S RNA at gastrulation. To test this possibility, gastrulating embryos from a cross of two $+/o$ adults were microinjected with [^{3}H]nucleosides and labeled for 5 or 24 h. Three types of embryos which can be distinguished by their nucleolar condition are expected in such a cross: 25% $+/+$ (two equal nucleoli), 50% $+/o$ (one nucleolus per cell) and 25% o/o (no normal nucleoli). The nucleolar phenotype of 125 unlabeled embryos was determined by phase contrast microscopy: 30 were $+/+$; 66, $+/o$; and 29, o/o. Labeled embryos were analyzed in the same way and total RNA was extracted from o/o embryos and pooled samples of $+/+$ and $+/o$ embryos (hereafter, pooled $+/+$ and $+/o$ embryos are designated wild type or $+/+$ embryos). Pooling of $+/o$ and $+/+$ embryos is justified since they synthesize 5S RNA at the same rate (Miller, unpublished) and previous work has shown that they synthesize 18S and 28S at the same rate (6, 19). Even though the anucleolate mutant does not make any 18S or 28S RNA (Figs. 2 C and 3 B) it synthesizes 5S RNA (Fig. 2 B). The relative rate of 5S RNA synthesis was determined by calculating the 4S/5S molar ratio in $+/+$ and o/o embryos. Judging from the similarity of their 4S/5S molar ratios (Table I) o/o mutants synthesize 5S RNA at the same rate as $+/+$ embryos after a 5-h or 24-h labeling period.

TABLE I

The 4S RNA:5S RNA and 5S RNA:28S RNA Molar Ratios during Early Development of Normal, Anucleolate, and Partial Nucleolate Embryos

Nucleolar genotype	Duration of labeling	Stages passed	Molar ratio	
			4S/5S	5S/28S
	h			
$+/+$	6	12–17	41.0	—
o/o	6	12–17	43.2	—
$+/+$	9	12–20	36.4	6.0
$+/+$	24	12–28	34.8	—
o/o	24	12–28	31.4	—
$+/+$	5	27–29	13.6	1.9
o/o	5	27–29	20.8	—
$+/+$	5	40	11.1	1.3
p^{l-1}/o	5	40	13.1	5.6
$+/+$	8	40–41	13.8	0.8
p^{l-1}/o	8	40–41	17.2	4.2
$+/+$	72	33–46	7.6	1.0

Total RNA from embryos labeled during different stages (26) of development was fractionated by polyacrylamide gel electrophoresis as described in Materials and Methods. The total counts under the 28S, 5S, and 4S RNA peaks were summed and a correction was made for background radioactivity (15). Molar ratios (cpm in RNA peak/molecular weight of RNA) were calculated assuming a molecular weight of 1.51×10^6 for 28S RNA, 4.0×10^4 for 5S RNA and 2.5×10^4 for 4S RNA.

5S RNA Synthesis in Anucleolate and Partial Nucleolate Mutants during Later Stages of Development

The 5S/28S molar ratio at early stages of development suggests that about six 5S RNA molecules are synthesized for each 28S RNA molecule (Table I). At later stages of development the 5S/28S molar ratio approaches unity in $+/+$ embryos suggesting a coordinate synthesis of 5S and 28S RNA. In o/o embryos at the tailbud stage of development considerable amounts of 5S RNA are made (Fig. 3 D) but compared with $+/+$ embryos the evidence from this single experiment suggests a slight reduction in the relative rate of 5S RNA synthesis (Table I).

The p^{l-1}/o embryos were obtained in a cross between a $+/p^{l-1}$ frog and a $+/o$ frog. One-fourth of the embryos of such a cross carry only the p^{l-1} nucleolar organizer. The p^{l-1}/o mutants have only 25% of the normal number of 18S and 28S RNA genes and synthesize 18S and 28S RNA at 25% of the normal rate (19, 24). After a 5- or 8-h labeling period during stages 40–41 (early swimming tadpoles), RNA was extracted from p^{l-1}/o and $+/+$ embryos and analyzed on discontinuous polyacrylamide gels as described in Materials and Methods. This technique makes it possible to resolve the three species of rRNA as well as 4S RNA and DNA in one step (Fig. 4). The relative rate of 28S RNA synthesis in the p^{l-1}/o embryos was 32% of the rate attained by normal embryos agreeing well with previously reported results (19). The 5S/4S molar ratios in $+/+$ and p^{l-1}/o embryos were very similar suggesting that p^{l-1}/o embryos synthesize 5S RNA at the same rate as normal embryos. If this is so, the 5S/28S molar ratio of p^{l-1}/o mutants should be about 3–4 times greater than the same ratio of $+/+$ embryos, because of the 25–30% reduction in the synthesis of 18S

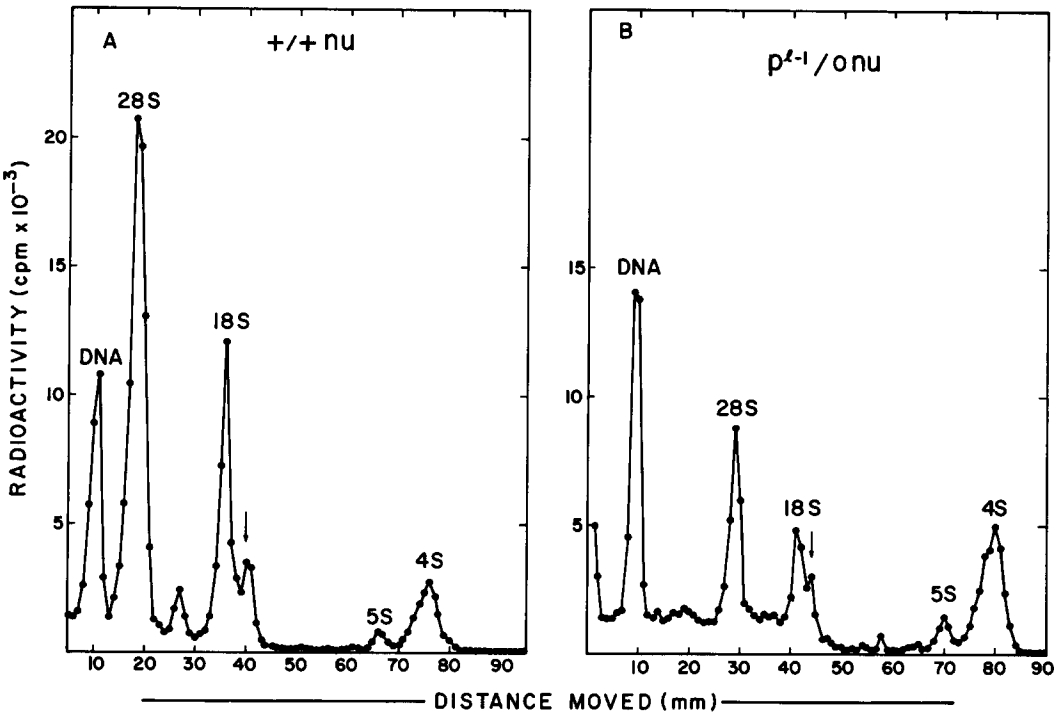

FIGURE 4 5S RNA synthesis in partial nucleolate mutants. Total RNA from +/+ (A) or p^{l-1}/o (B) embryos labeled for 8 h during stages 40–41 was electrophoresed on discontinuous polyacrylamide gels. The arrow indicates the interface of the 2.7% and 8% gels.

and 28S RNA in p^{l-1}/o embryos. In fact, the 5S/28S molar ratios of p^{l-1}/o embryos were slightly higher than expected, being 4–5 times greater than those of +/+ embryos (Table I). This can be explained by the decrease in 18S and 28S RNA synthesis in p^{l-1}/o embryos which reduces the drain of radioactive RNA precursors from the pool (relative to +/+ embryos) and results in the synthesis of RNAs with higher specific activity than comparable RNA species of +/+ embryos (19, 25). The increase in the relative proportion of radioactivity entering 4S RNA of p^{l-1}/o embryos (compare Fig. 4 A and B) can be explained in the same way. The total amount of 4S RNA per embryos is not altered by the p^{l-1} mutation (25). Thus it is likely that p^{l-1}/o embryos synthesize 5S RNA at the same rate as wild type embryos.

DISCUSSION

The results presented indicate that when 18S and 28S RNA are not synthesized, as in anucleolate mutants, or their synthesis is reduced fourfold, as in partial nucleolate mutants, the synthesis of 5S RNA proceeds at the same rate as it does in normal embryos. Therefore, it is clear that during early development of *Xenopus*, newly synthesized rRNA precursor, or 18S and 28S RNA, are not required for the initiation or the changes in rate of synthesis of 5S RNA. The finding that 5S RNA is synthesized by cells in vitro when rRNA precursor synthesis is inhibited with actinomycin D (1, 16, 28) also demonstrated that 5S RNA synthesis can be uncoupled from 18S and 28S RNA synthesis. It is possible, however, that the uncoupling induced by actinomycin D cannot be maintained for an extended period of time, i.e., a certain length of time may be necessary in order to establish the repression of the 5S RNA genes and during this period 5S RNA would be synthesized normally. This problem cannot be resolved with actinomycin D studies because prolonged treatment with this drug results in an overall deterioration in cell metabolism. As described here, however, the excessive synthesis of 5S RNA in o/o and p^{l-1}/o embryos occurs throughout early development, a period of 8–10 days.

A number of other observations on HeLa cells suggest that equal numbers of 5S, 18S, and 28S RNA are not synthesized. A substantial nuclear pool of 5S RNA exists (17) and 5S RNA is synthesized during mitosis whereas 18S and 28S RNA synthesis is repressed (34). More recently it has been shown that about four times as much 5S RNA is made in exponentially growing HeLa cells than is required for the assembly of new ribosomes (20). This latter observation and the clear noncoordinate synthesis of the three rRNAs during oogenesis of *Xenopus* (12, 22) represent the best evidence that in normal situations equal numbers of 5S, 18S, and 28S RNA are not synthesized.

Of course HeLa cells and oocytes are not truly typical eukaryotic cells and it is possible that equal molar quantities of the three rRNAs are made in many cell types. The fact that the 5S/28S molar ratio in older +/+ embryos is close to unity (Table I) supports this view. In earlier +/+ embryos however, excess 5S RNA is made (Table I and unpublished data). Abe and Yamana (1) have also found that excess 5S RNA is synthesized during early development of *Xenopus*, but in hepatocytes of recently metamorphosed animals equal quantities of 5S and 28S are synthesized. Alternative explanations of the high 5S/28S molar ratios in early embryos are that the newly synthesized 28S RNA is not as stable as the 5S RNA or that a different RNA species which co-electrophoreses with 5S rRNA is synthesized during early development. In fact oocyte-specific 5S RNA co-electrophoreses with somatic 5S RNA even though they have a different base composition (13, 30). Since the experiments described here do not differentiate between oocyte 5S RNA and somatic 5S RNA it is possible that the excess 5S RNA made by gastrula is oocyte 5S RNA and that somatic 5S RNA and 18S and 28S RNA are synthesized in equal molar quantities. Likewise, in *o/o* mutants the genes coding for somatic 5S RNA may be repressed and all of the 5S RNA detected may be oocyte 5S RNA. Thus the data from in vivo experiments do not rule out coordinate synthesis of the three rRNAs. The equimolar synthesis of the three rRNAs, if it exists, could be controlled by a specific regulatory molecule, possibly by a specific ribosomal protein or nucleolar protein. If so, then the regulatory molecule may be present in normal amounts in *o/o* and p^{l-1}/o embryos which would account for the normal transcription of 5S RNA in these mutants. The synthesis of normal amounts of 5S RNA in *o/o* and p^{l-1}/o embryos and the finding that in HeLa cells newly synthesized 5S RNA is found initially in a soluble form in the cytoplasm (20) are also consistent with the idea, originally suggested by Knight and Darnell (17), that 5S RNA has a function other than its role in ribosomes.

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