

**PERSISTENCE IN EARLY AMPHIBIAN EMBRYOS  
OF INFORMATIONAL RNA'S FROM  
THE LAMPBRUSH CHROMOSOME STAGE OF OÖGENESIS\***

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The ripe ovarian oöcyte contains a large stockpile of informational RNA's, a direct legacy of the intense gene activity which occurs during the lampbrush chromosome stage of oogenesis. The RNA synthesized at this stage is accumulated so that in *Xenopus* some 47 m<sup>g</sup> of lampbrush-synthesized, template-active RNA is finally present in the mature oöcyte.<sup>1</sup> Estimated by comparing the template activity of oöcyte RNA in a cell-free amino acid incorporating system to that of a known viral RNA standard, this amount of stored template-active RNA is close to 3900× the 4C amount of DNA expected in the terminal ovarian oöcyte chromosomes.<sup>1, 2</sup> Similarly, a massive accumulation of template-active RNA appears to take place in the course of sea urchin oogenesis, according to an estimate of the amount of such RNA in the unfertilized egg recently obtained in the same manner by Slater and Spiegelman.<sup>3</sup> The amount of genetic information potentially encoded in the informational RNA's of the mature oöcyte is as impressive as is their quantity: although only 3 per cent of the total genetic information possessed by the organism is actually transcribed during the lampbrush stage in *Xenopus*, it can be calculated that the amount of DNA active in the chromosomes would be enough to provide no less than  $8.2 \times 10^6$  genes the size of that coding for a hemoglobin  $\beta$  chain.<sup>1, 24</sup> We know that at least 65 per cent of genetic information transcribed during the lampbrush stage is still present in stored RNA's of the terminal oöcyte.<sup>1</sup>

The ultimate fate and function of the enormous stores of informational RNA with which the embryo thus begins life is as yet unknown. By informational RNA's we denote RNA's complementary to the genomic DNA, other than ribosomal and transfer RNA's, without specifying the mode of their utilization. Protein synthesis is required for early morphogenesis,<sup>4, 5</sup> and it is now clear that protein synthesis in cleavage-stage embryos is programmed mainly by "maternal messengers" synthesized during oogenesis. This has been demonstrated in a variety of ways in the sea urchin<sup>6-11</sup> and is likely to be the case in the amphibian as well.<sup>12, 13</sup> The informational RNA stockpile inherited from mid-oogenesis probably includes these "maternal messengers." It is worth noting, however, that the template activity of the stored RNA's in the *in vitro* assay systems used by ourselves and others<sup>1, 3, 10</sup> does not necessarily mean that in life these molecules are to be utilized as templates for early protein synthesis; we know only that they appear capable of such activity. The present experiments represent a first step in the problem thus posed, viz., the actual destiny of the oöcyte's informational RNA stockpile. We have utilized the competitive RNA-DNA hybridization technique to estimate the degree of persistence, in successively later embryos, of maternal RNA's bearing genetic information identical to that transcribed during the lampbrush phase of oogenesis. The experiments show that the load of oogenesis-transcribed genetic information in the maternal RNA stockpile inherited by the embryo remains constant until mid-

blastulation. At this time, however, we find that an abrupt change occurs, and a sizeable fraction of the informational RNA disappears completely from the embryo.

*Methods.—Preparation of nucleic acids:* Highly purified, stable preparations of *Xenopus laevis* H<sup>3</sup>-DNA and P<sup>32</sup>-labeled lampbrush-stage oöcyte RNA were made exactly as described previously.<sup>1</sup> The spectral, thermal denaturation, and equilibrium sedimentation characteristics of DNA prepared in this manner have been reported.<sup>1</sup> All of the RNA preparations used in these experiments, whether of embryo, oöcyte, or tissue origin, possessed A<sub>230</sub>/A<sub>260</sub> and A<sub>280</sub>/A<sub>260</sub> ratios of less than 0.47.

Embryo RNA's were prepared from 1000 to 2500 Nieuwkoop and Faber<sup>14</sup> stage 2 (2-cell), stage 7 (early blastula), and stage 9 (late blastula) *Xenopus* embryos obtained from gonadotropin-stimulated mating pairs as described previously.<sup>15</sup> The RNA was extracted with the same two-pH extraction procedure,<sup>16</sup> as was utilized in extracting RNA from lampbrush-stage oöcytes, except for the following variations: (a) the initial aqueous-extracting medium contained 0.87% sodium dodecylsulfate and was buffered with 0.10 M acetate to pH 5.0 (rather than pH 7.6); (b) the pH of the second aqueous-extracting medium was 8.5 (rather than 9.2); and (c) following removal of low-molecular-weight contaminants by Sephadex G100 gel filtration, pronase treatment, and two further phenol extractions and ethanol precipitations,<sup>1</sup> the RNA was dissolved in 0.3 M phosphate buffer, pH 6.8–0.05 MgCl<sub>2</sub>. It was then treated with 1.2 µg electrophoretically purified DNase at final concentration of 330 µg/ml, for 2 hr at 37°C. The enzyme was eliminated by two further phenol extractions and the RNA again twice precipitated in ethanol. RNA was extracted from adult *Xenopus* tissues, viz., from heart, and from testis 1 day after gonadotropin stimulation, in exactly the same manner as from embryos.

A critical step in our RNA extraction procedures is the re-extraction at pH 8.5 or 9.2 of the interface remaining after the initial extractions at pH 5.0 or 7.6. We have found that omission of the re-extraction step invariably results in failure to solubilize and extract a tightly bound RNA fraction containing 5–15% of total uridine-5-H<sup>3</sup> counts incorporated in a labeling experiment (cf. ref. 16). With the two-pH procedure, however, virtually all newly synthesized RNA is extracted into the aqueous phase. Some losses of initially extracted RNA occur subsequently, in the course of the lengthy purification procedures we employ, but these losses evidently do not result in change in the total informational RNA content of the RNA preparations. Thus the hybridization frequency at saturation turns out to be relatively invariant whether the P<sup>32</sup>-RNA tested was extracted with a final yield of 98% or of 60% of what was initially present in the starting material.

*Hybridization technique:* A scaled-down variant of the liquid-liquid hybridization method described by Gillespie and Spiegelman<sup>17</sup> was used in these studies. Our procedure was exactly that used before,<sup>1</sup> except that the salt concentration of the annealing mixture was doubled. After annealing, an equal volume of water was added to return the salt concentration to the original level (0.90 M NaCl–0.09 M Na citrate) in order to provide a noninhibitory milieu for RNase A treatment. The hybrids were trapped on nitrocellulose filters and counted.<sup>1</sup>

*Estimation of saturation-point P<sup>32</sup>-oöcyte RNA/DNA ratios:* To estimate the amount of RNA in the embryo which is directly homologous with that synthesized during the lampbrush stage of oögenesis, we challenged the P<sup>32</sup>-lampbrush RNA-DNA hybridization system with increasing amounts of unlabeled total embryo RNA. Competition for DNA binding sites is monitored by measuring the decrease in observable hybridization between constant amounts of P<sup>32</sup>-labeled oöcyte RNA and H<sup>3</sup>-DNA, as competing unlabeled RNA is added, until sufficient competing RNA is present to establish a new plateau level of observable hybridization. This type of experiment, however, cannot yield an accurate estimate of homology unless the P<sup>32</sup>-RNA/DNA ratio is set so that there are no excess DNA sites beyond what are hybridized by the amount of P<sup>32</sup>-RNA added, since excess DNA sites could bind complementary unlabeled RNA molecules without causing an observable decrease in the P<sup>32</sup>-RNA hybridization frequency. Furthermore, in hybridization experiments carried out in the presence of a large excess of DNA, those RNA species present in large quantity will be hybridized in an amount disproportionately greater than the percentage of the DNA genome complementary to them. Thus, only when the amount of P<sup>32</sup>-RNA is at least great enough to saturate the DNA in the annealing mixture can a competition experiment measure the *relative informational content* of the RNA populations being tested. A similar discussion of these points can be found in a recent paper by Denis,<sup>18</sup> who notes that most

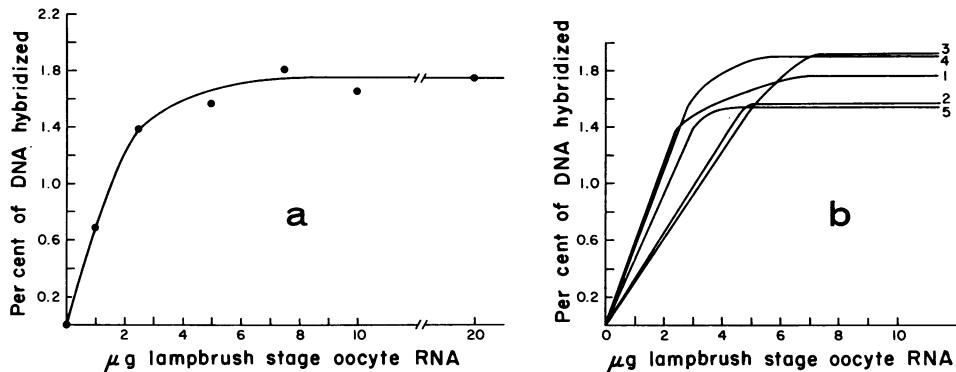


FIG. 1.—Saturation of DNA with lampbrush-stage oocyte  $P^{32}$ -RNA: (a) a single representative saturation curve; (b) superimposed saturation curves for five separate  $P^{32}$ -RNA preparations, each obtained from the lampbrush-stage oocytes of one animal. Each sample contained 5  $\mu$ g  $H^3$ -DNA, and the amounts of  $P^{32}$ -RNA of known specific activity<sup>1</sup> shown on the abscissa, in a total volume of 200  $\mu$ l. Since only one strand of the DNA is active in RNA synthesis, 1.5–1.8% of the DNA hybridized indicates the presence of newly synthesized RNA's bearing 3–4% of the total genomic information in the lampbrush-stage oocyte RNA preparations, as previously reported.<sup>1</sup>

of the competition experiments reported so far suffer from "some ambiguity in the results" due to having been carried out under nonsaturating conditions. If  $P^{32}$ -RNA/DNA ratios very much larger than the saturation point are used, on the other hand, it is conceivable that an apparent insensitivity to unlabeled competing RNA might result, particularly at low concentrations of competing RNA. Therefore the  $P^{32}$ -RNA/DNA ratios in the experiments to follow were adjusted as close to the saturation point as possible, the latter having been determined previously for each  $P^{32}$ -RNA preparation used. Figure 1a reproduces a typical saturation curve for a  $P^{32}$ -oocyte RNA preparation. Figure 1b, in which the saturation curves for the five oocyte RNA preparations used in these experiments are superimposed, shows that the  $P^{32}$ -RNA/DNA saturation-point ratios are slightly different for each preparation, although similar percentages of DNA are hybridized by the various preparations. We have reported previously that RNA preparations from the lampbrush-stage oocytes of different individual females vary somewhat in template-active RNA content, and we interpret the diverse saturation-point ratios of Figure 1b as the consequence of small differences in the ratio of an informational to bulk, or ribosomal RNA, among the five RNA preparations.

*Comparison between the Informational Content of Newly Synthesized Lampbrush-Stage Oocyte RNA and the Total RNA of Stage 2 and Stage 7 Embryos.*—As noted above, at least 65 per cent of the species of RNA synthesized at the lampbrush stage remain stored in the mature ovarian oocyte.<sup>1</sup> By comparing RNA from the stage 2 (2-cell) embryo to newly synthesized lampbrush-stage oocyte RNA in a competition experiment, we can determine whether the informational RNA of the mature oocyte survives ovulation and fertilization. Similarly, we can compare RNA from the mid-blastula (stage 7) to lampbrush chromosome-synthesized oocyte RNA. Figure 2 shows a typical experiment in which unlabeled stage 2 and stage 7 RNA's are made to compete with saturating amounts of  $P^{32}$ -labeled lampbrush-stage RNA. The experiment shows that there is no detectable decrease in the informational content of the RNA inherited by the embryo between the beginning of cleavage and the mid-blastula stage, when there are 500–2000 cells in the embryo.<sup>19</sup> (New species of RNA may of course be synthesized during this period but they would not affect this experiment.) Furthermore, there is evidently as much homology be-

tween newly synthesized lampbrush-stage oöcyte RNA and early cleavage-stage embryo RNA as between mature ovarian oöcyte RNA and lampbrush-stage RNA. Table 1 includes data from six additional experiments in which stage 7 RNA competes for limited DNA with lampbrush-stage RNA. It can be seen that the average plateau value for hybridization of the lampbrush-stage oöcyte RNA in the presence of excess stage 7 RNA indicates about 64 per cent homology between the two preparations just as is the case with mature ovarian oöcyte RNA and lampbrush-stage oöcyte RNA.<sup>1</sup> Thus the stockpile of lampbrush-transcribed informational RNA's remains constant in informational content from late oogenesis to mid-blastulation.

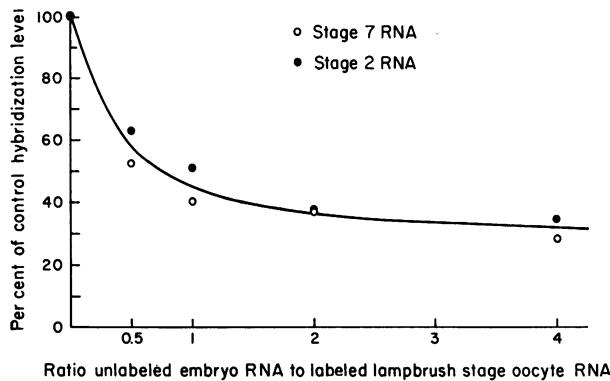


FIG. 2.—A representative competition experiment in which increasing amounts of stage 2 and stage 7 embryo RNA are added to lampbrush-stage oöcyte  $P^{32}$ -RNA and  $H^3$ -DNA under hybridizing conditions. As described in *Methods*, the  $P^{32}$ -RNA/DNA ratio in all the samples is set close to the saturation point, here 6  $\mu$ g of  $P^{32}$ -RNA/5  $\mu$ g  $H^3$ -DNA (preparation 4, Fig. 1b).

*Comparison between Informational Content of Newly Synthesized Lampbrush-Stage Oöcyte RNA and the Total RNA of Stage 7 and Stage 9 Embryos.*—Figure 3 reproduces a competition experiment similar in design to those just described, but comparing early and late blastular RNA preparations for homology with newly

TABLE 1

COMPARISON OF PLATEAU HYBRIDIZATION LEVELS ATTAINED BY LAMPBRUSH-STAGE OÖCYTE  $P^{32}$ -RNA IN PRESENCE OF UNLABELED STAGE 7 AND OF UNLABELED STAGE 9 RNA

Oöcyte RNA: preparation no.	Unlabeled RNA added:	Approximate plateau hybridization level expressed as % of control hybridization	Difference (stage 7 and stage 9) as % of control
1	Stage 7	50	19
	Stage 9	69	
1	Stage 7	43	12
	Stage 9	55	
2	Stage 7	35	21
	Stage 9	56	
2	Stage 7	34	17
	Stage 9	51	
3	Stage 9	51	
3	Stage 7	24	
4	Stage 7	32	
			18

synthesized lampbrush-stage RNA. Five additional such experiments are summarized in Table 1. Only  $1\frac{1}{2}$ -2 hours separate stages 7 and 9 at  $18^{\circ}\text{C}$ , but the data show clearly that during this time a definite amount of lampbrush-transcribed genetic information disappears from the store of embryonic RNA's. Table 1 shows that RNA's complementary to an average of 18 per cent of the DNA sites hybridized by newly synthesized lampbrush-stage RNA are lost in the mid-to-late blastula period. Since RNA complementary to only about 65 per cent of these sites is present at the termination of oogenesis and transmitted to the early embryo in the first place, this means that over 28 per cent (18%/ $65\%$ ) of the genetic information in the RNA's inherited by the embryo from the lampbrush stage of oogenesis is utilized and degraded, or otherwise caused to disappear, between stages 7 and 9 of embryogenesis.

#### *Search for Ubiquitous "Housekeeping Enzyme" Information in Newly*

*Synthesized Lampbrush-Stage Oocyte RNA.*—It is frequently hypothesized that the maternal messenger present in the egg at fertilization is constituted in large part of message for "housekeeping" enzymatic machinery presumably required in all cells for the maintenance of ubiquitous processes such as protein synthesis, glycolysis, etc. Mangan *et al.*<sup>20</sup> have shown that at least one such class of "housekeeping" proteins may be synthesized from maternal messengers in sea urchins during early cleavage, viz., the spindle proteins needed for cell division. A major function of the lampbrush stage of oogenesis could thus be the storage of "housekeeping" messenger RNA's for the maintenance of the high metabolic activity and rapid cell division characteristic of the cleavage stage embryo. In this case we might expect that a competition experiment in which  $\text{P}^{32}$ -labeled, newly synthesized lampbrush-stage oocyte RNA is challenged with unlabeled total RNA from any metabolically active adult tissue would reveal homology between some RNA's in the oocyte and tissue preparations. The degree of competition would then provide a rough approximation of the proportion of the genome active at lampbrush stage which is concerned with ubiquitous "housekeeping" machinery. However, as shown in Figure 4, neither total RNA from heart nor total RNA from hormone-stimulated testes were able to compete noticeably with lampbrush-stage RNA. In the case of the testes, it is certain that DNA synthesis and cell division were taking place when the RNA

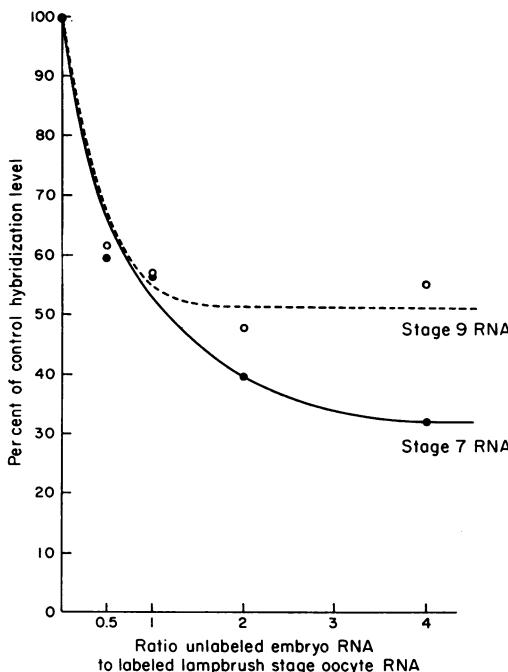


FIG. 3.—A representative competition experiment in which increasing amounts of stage 7 and stage 9 embryo RNA are added to constant quantities of  $\text{H}^3$ -DNA and lampbrush-stage oocyte  $\text{P}^{32}$ -RNA, under hybridizing conditions. The saturation point ratio in this experiment was  $5 \mu\text{g P}^{32}\text{-RNA}/5 \mu\text{g H}^3\text{-DNA}$  (preparation 2, Fig. 1b).

was extracted. The data do not permit us to exclude a low level of competition, of the order of the 0.11 per cent expected from ribosomal RNA,<sup>21</sup> but it is clear that most of the genes operating during the lampbrush stage are not concerned with the production of "housekeeping" proteins also possessed by the adult. We conclude that either the housekeeping information carried in lampbrush-synthesized RNA constitutes a very small part of the total genetic information transcribed during oogenesis, or that most of the housekeeping enzymes of the early embryo are different from those of adult tissues (e.g., they could be isozymes of the adult enzymes).

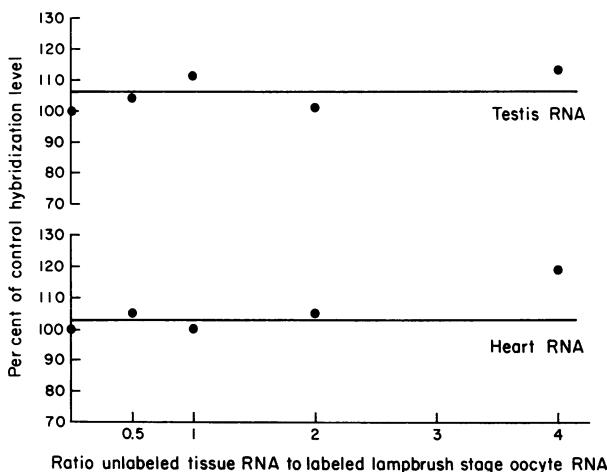


FIG. 4.—Competition experiments in which increasing quantities of total RNA from adult heart and hormone-stimulated testis are added to constant quantities of lampbrush-stage oocyte P<sup>32</sup>-RNA and H<sup>3</sup>-DNA under hybridizing conditions. As before, P<sup>32</sup>-RNA to H<sup>3</sup>-DNA ratios are set near the saturation point for each RNA preparation.

*Discussion.*—These experiments, taken together with those published in our last report,<sup>1</sup> show that in addition to microgram quantities of ribosomal RNA<sup>22, 23</sup> the embryo inherits a massive stockpile of nonribosomal, high-molecular-weight RNA's bearing as much as 2 per cent of the total genomic information possessed by the organism.<sup>24</sup> We know that most of the informational RNA's bequeathed to the early embryo originated long previously, at the lampbrush chromosome stage of mid-oogenesis.<sup>1</sup> The present experiments show that no decrease in the informational content of this inherited RNA stockpile can be detected even after fertilization and the onset of embryogenesis, and it is not until after the 500 to 2000-cell early blastular stage that a change occurs. Then, within a brief period, a significant fraction, some 28 per cent of this long-stored genetic information, disappears. The period of embryogenesis during which at least this class of RNA-born genetic information is utilized must thus be delimited by the stage at which the embryo becomes a late blastula (stage 9).

The mid-to-late blastula is a period at which gene transcription in preparation for the onset of gastrulation appears to take place. This has been shown to be true in echinoderms, in experiments in which actinomycin is added at successively later times,<sup>25</sup> and in teleosts as well.<sup>26</sup> Recently we carried out radioautographic and

biochemical studies on RNA synthesis at successively later blastular and gastrular stages in *Xenopus*.<sup>15, 27</sup> These investigations revealed that a dramatic, near embryo-wide activation of nuclear RNA synthesis occurs between stages 8 and 9, i.e., in the mid-to-late blastular period. Nuclei of both presumptive endoderm and presumptive ectoderm increase their rates of what is apparently messenger RNA synthesis over 20-fold within an hour at this stage, and by stage 9-10 (beginning of gastrulation) the embryo has attained per-cell RNA synthesis rates close to those which are to persist throughout the remainder of gastrulation.<sup>28</sup> Our finding that stage 7 RNA competes with new oöcyte RNA significantly better than stage 9 RNA indicates that *utilization of a large class of gene products stored ever since the lampbrush phase of oogenesis must be completed within the critical mid-to-late blastular nuclear activation period.* An obvious speculation suggests that the utilized genetic information, the product of enough DNA to include 148,000 hemoglobin genes,<sup>24</sup> is in some way involved in this embryonic activation phenomenon. It is of course also conceivable that early RNA synthesis in the young embryo represents the activity of the same genomic loci as were functional during the lampbrush stage, and that part of the competition registered in our experiments is due to newly synthesized embryo RNA's.<sup>29</sup> However, it seems very unlikely that a significant portion of the striking competition we obtain with total stage 2 embryo RNA could be due to newly synthesized embryo RNA, since, according to Denis,<sup>18</sup> the newly synthesized RNA of stage 0 *Xenopus* embryos hybridizes insignificantly, and since the amount of RNA synthesis between stage 0 and stage 2 is extremely small. Our present experiments thus show that most of the RNA-borne genetic information the embryo has inherited from oogenesis is still present in the embryo even later than stage 9. In early embryogenesis the RNA molecules bearing this information are clearly those synthesized at the lampbrush stage of oogenesis, and most probably the same molecules are responsible for the residual competition we obtain with stage 9 RNA. If part of this competition is due to newly synthesized embryo RNA's, it must be considered that specification of the pattern of embryonic gene activity is also inherited from oogenesis. In either case our findings lend an interesting new reality to the classical conception<sup>30</sup> that embryogenesis begins in oogenesis.

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- <sup>24</sup> Since about 3% of the genome is active at the lampbrush stage of oogenesis<sup>1</sup> and 65% of the genetic information transcribed at that time is present in the early embryo,  $65\% \times 3\% = 1.95\%$  or about 2% of the total genetic information is stored in the maternal RNA stockpile. Eighteen per cent of the total lampbrush-transcribed genetic information, or  $18\%/65\% = 28\%$  of the information inherited by the embryo, is used by stage 9. The amount of DNA active in the lampbrush chromosomes is about  $0.03 \times 12 \times 10^{-12}$  gm =  $3.6 \times 10^{-13}$  gm, considering that the 4C amount of DNA is present in these chromosomes and including as active DNA both the strand serving as template for the RNA molecule and the complementary strand. (In our previous publication<sup>1</sup> we used the value 1.38% of DNA hybridizing with  $P^{32}$ -oocyte RNA exclusive of DNA complementary to ribosomal RNA, rather than the 1.5% value used here. This accounts for the slight difference in the amount of active DNA calculated here as opposed to that in ref. 1. As Fig. 1b shows, saturation values for oocyte  $P^{32}$ -RNA preparations actually vary from about 1.5% of DNA to about 1.8%.) A structural gene for the 146 amino acid hemoglobin  $\beta$  chain would have a mass of about  $(438 \times 302/6.02 \times 10^{23}) \times 2 = 4.39 \times 10^{-19}$  gm, given 3:1 coding ratio and considering that the DNA is double-stranded. Therefore the total amount of DNA active in the lampbrush chromosomes is equivalent in mass to about  $3.6 \times 10^{-13}/4.39 \times 10^{-19} = 8.2 \times 10^5$  hemoglobin-sized genes and 18% of this number, or  $1.48 \times 10^6$  hemoglobin-sized genes could be represented in the RNA used by stage 9.
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- <sup>28</sup> Rapid increase in per cell synthesis of nonribosomal, "DNA-like" RNA just before stage 10, followed by stabilization of the per cell RNA synthesis rates is also described in *Xenopus* by Brown and Littna. (Brown, D. D., and E. Littna, *J. Mol. Biol.*, **20**, 81 (1966).) Furthermore, according to the extensive hybridization studies recently reported by Denis,<sup>18</sup> newly synthesized RNA's complementary to 2.4% of the DNA are accumulated in the *Xenopus* embryo as early as stage 12 (late gastrula).
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