# Ribosomal RNA Synthesis during Cleavage of Ascaris lumbricoides Eggs

Abstract. *The appearance of well-defined nucleoli in 4-celled* Ascaris *embryos is associated with the synthesis of ribosomal RNA.*

Synthesis of ribosomal RNA (rRNA) apparently does not occur in the cleavage stages of eggs as diverse as those of sea urchin, insect, amphibian, and fish (*1*), which suggests that this may be a general feature of animal embryogenesis (*2*). Such eggs do not have typical nucleoli, although they may contain submicroscopic intranuclear bodies that resemble nucleoli in some respects (*3*). There is strong evidence, however, that the presence of nucleoli is associated with rRNA synthesis (*4*), and nucleoli are clearly present in the cleavage cells of equally divergent animals, such as certain coelenterates, annelids, molluscans, crustaceans, and mammals (*5*), whose capacity for rRNA synthesis remains to be explored. Our experiments with the nematode *Ascaris lumbricoides* have shown that in eggs immediately after sperm penetration an active synthesis of rRNA takes place (*6*), and that this unusual synthesis occurs exclusively in the male genome (pronucleus). In later pronuclear stages formation of rRNA is not readily detectable, although small nucleoli, with a poorly developed nucleolonema, can be seen in both pronuclei. At the zygote stage and during the period up to the third cleavage, nucleoli are not a prominent feature of nuclear structure, in that they cannot be demonstrated by light microscopic techniques (*7*). However, cells of the 4-cell egg and subsequent stages contain a clearly defined nucleolus (Fig. 1) with a well-developed reticulate nucleolonema (*8*), and *Ascaris* may be considered to belong to the second group of organisms mentioned above, which do possess typical nucleoli during cleavage (*5*). It was therefore of interest to determine whether rRNA synthesis occurs in cleaving *Ascaris* eggs and whether such synthesis can be correlated with the appearance of clearly defined nucleoli seen in the 4-celled and later cleavage stages.

It was readily shown that a net increase in total RNA occurred in cleavage subsequent to the 4-celled stage (Table 1). One-celled (0-day) eggs were obtained from the proximal region of the uterus and embryonated for periods up to 6 days (*9*), at which time cleavage was completed. Up to the 4-celled stage (72 hours), values for RNA (*10*) were not significantly different; thereafter, all mean values were significantly different from the 4-cell mean, and the increase in RNA at blastulation was 60 percent.

In subsequent experiments, eggs were made permeable, they were labeled with $^3$H-uridine, and purified RNA was isolated from subcellular fractions as previously described (*6*). Five-day (morula) eggs incorporated sufficient label in 1 hour to show that more than half the label in microsomal RNA was associated with the 19*S* and 26*S* peaks characteristic of *Ascaris* rRNA (Fig. 2A). In 4-celled eggs, incorporation of label occurring in 1 hour was insufficient (1.66 pmole of $^3$H-uridine per milligram of RNA) for adequate analysis of the RNA in subcellular fractions; the eggs were therefore exposed to $^3$H-uridine for 15 hours. Control experiments showed that labeled RNA synthesized over the longer period was stable and was, therefore, accumulated. The ultraviolet absorption profile of microsomal RNA from this experiment correlated very poorly with the distribution of label, which was distinctly heterogeneous (Fig. 2B). However, minor radioactive peaks in the 19*S* and 26*S* regions suggested that some rRNA might have been synthesized. This possibility was tested in two ways. First, messenger RNA (mRNA) was separated from ribosomes by removal of $Mg^{++}$ from the isolation medium (*11*), after which ribosomes were sedimented and their RNA extracted. Unfortunately, these additional manipulations resulted in degradation of most of the rRNA, leaving only minor peaks at 19*S* and 26*S*, with which a small amount of label was associated (Fig. 2C). However, the partial degradation of rRNA during prolonged manipulation of ribosomes apparently does not influence the integrity of the ribosomal structure. For example, trypsin-treated ribosomes retain their ability to synthesize protein (*9*), and they can be sedimented by centrifugation. These observations suggested another approach, namely, the dissociation of labeled ribosomes into 40*S* and 60*S* subunits (*12*) by dialysis against $Mg^{++}$-free buffer followed by determination of label distribution on density-gradient centrifugation of the subunits. Since mRNA is known to be associated with the smaller subunit in protein synthesis (*13*), a radioactivity peak coincident with the 60*S* subunit would indicate the synthesis of rRNA, if the possible contribution of labeled transfer RNA could be eliminated from consideration. Experiments showed that this was in fact the case (Fig. 2D). Although RNA isolated from the 60*S* subunit was found to be extensively degraded, analysis of this RNA on sucrose density gradients showed that more than 62 percent of the radioactivity sedimented faster than

Table 1. Total RNA in cleavage stages of *Ascaris* eggs. Results are given ± the standard error.

| Incubation at 30°C (hr) | Stage | No. of experiments | RNA per $10^6$ eggs ($\mu$g) |
|---|---|---|---|
| 0 | 1-cell | 3 | 90 ± 3.4 |
| 24 | 1-cell | 3 | 82 ± 3.6 |
| 48 | 2-cell | 3 | 79 ± 4.0 |
| 72 | 4-cell | 5 | 96 ± 3.3 |
| 96 | 16-cell | 3 | 108 ± 2.3 |
| 120 | Morula | 3 | 110 ± 1.8 |
| 144 | Blastula | 3 | 152 ± 1.2 |

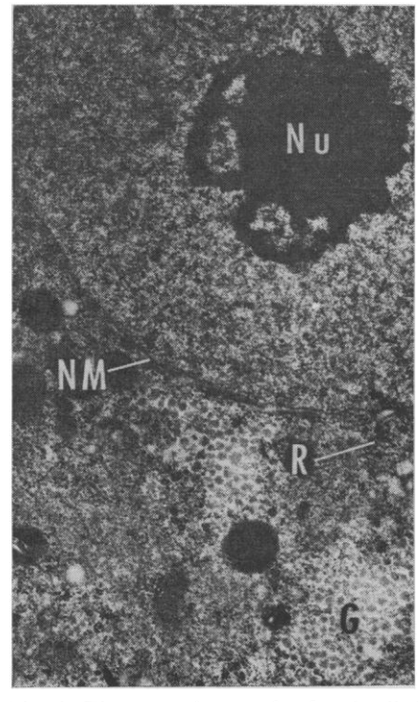


Fig. 1. Electron micrograph of a 4-celled *Ascaris* egg showing a portion of a nucleus and a well-defined nucleolus. *Nu*, Nucleolus; *NM*, nuclear membrane; *R*, ribosomes; and *G*, glycogen (× 20,000).

4*S*, and could therefore be identified as rRNA. These observations could not be duplicated on labeled 1-celled eggs, in which little or no radioactivity could be clearly attributed to 19*S* and 26*S* RNA or to the 60*S* ribosomal subunit. The presence of definite nucleoli in the 4-celled eggs may therefore be correlated with rRNA synthesis. In the late pronuclear stage (0 day), however, the status of the small nucleolus-like bodies is not clear, although they are definitely not associated with rRNA synthesis detectable by present techniques. Similar intranuclear bodies occur in sea urchin eggs (*3*) and are not involved in the synthesis of RNA.

Although the observations described above clearly point to synthesis of rRNA in *Ascaris* eggs that have acquired well-developed nucleoli at the 4-celled and later stages, it should be noted that most of the radioactivity incorporated into cytoplasmic RNA in both 1-celled and 4-celled eggs was nevertheless associated with RNA that was widely distributed throughout the gradients (Fig. 2, A and B). The labeling characteristics, base composition, and high template activity indicate that this consists, in part, of mRNA (*14*). In this feature, therefore, *Ascaris* eggs resemble those of other embryos that have been investigated (*1*).

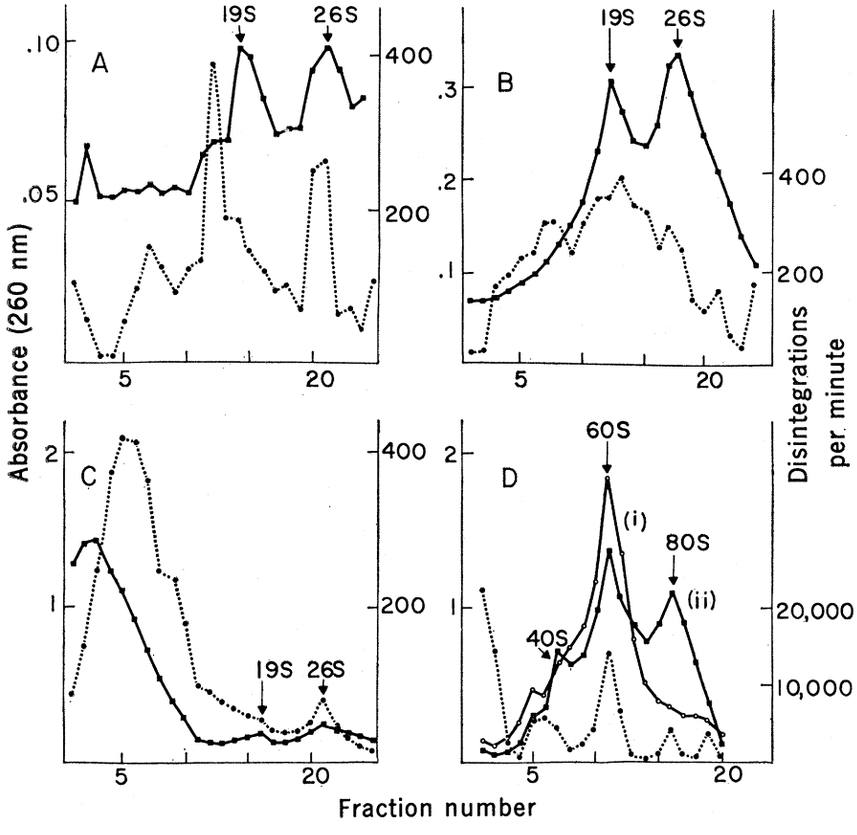


Fig. 2. Ribosomal RNA synthesis during cleavage of *Ascaris* eggs. The direction of sedimentation in all gradients is from left to right. Solid lines depict absorbancy; broken lines, radioactivity. (A) Sedimentation and labeling pattern of microsomal RNA isolated from 5-day (morula) eggs exposed to $^3$H-uridine for 1 hour. RNA centrifuged for 4.5 hours at 178,880*g* in a 5 to 20 percent sucrose gradient; 19*S* and 26*S* peaks are characteristic for *Ascaris* rRNA (*6*). (B) Sedimentation and labeling pattern of microsomal RNA isolated from 3-day (4-celled) eggs exposed to $^3$H-uridine for 15 hours. Centrifuged for 3.5 hours, but otherwise as in (A). Label is widely distributed, although there is a suggestion of peaks corresponding to rRNA. (C) Same as (B), except that mRNA was removed from ribosomes before isolation of residual RNA. Despite extensive degradation of RNA, there is indication of correspondence between absorbancy and labeling profiles at 19*S* and 26*S*. (D) Fractionation of ribosomal fraction into 60*S* and 40*S* ribosomal subunits, after dialysis for 24 hours (*i*) or 12 hours (*ii*) against $Mg^{++}$-free buffer, by centrifugation for 4 hours at 63,581*g* on a 5 to 20 percent sucrose gradient. The radioactivity measurements correspond to (*i*). Correspondence of the 60*S* peak with a major radioactivity peak identifies this portion of the newly synthesized RNA as rRNA.

Our results permit the following conclusions. Fertilized *Ascaris* eggs taken from the terminal portion of the uterus have already synthesized large amounts of rRNA and more modest amounts of mRNA (*6*). When embryogenesis begins with the admission of oxygen (*15*), synthesis of mRNA continues but that of rRNA cannot be detected. With the development of definitive nucleoli in 4-celled eggs, rRNA synthesis begins again and continues at an accelerating pace throughout cleavage. This rRNA is stable to the extent that its net increase at blastulation amounts to 60 percent.

Whether eggs of other species that possess nucleoli in cleavage stages (*5*) also synthesize rRNA is not yet known, although it appears to be probable. If they do, it is evident that molecular events resulting from fertilization are rigidly prescribed only for the species. Among gymnoblastic coelenterates, for example, morphological aspects of development in *Eudendrium* and *Pennaria* are much alike, yet cleavage cells of the former possess nucleoli and those of the latter do not (*16*). In a broader sense, there also seems to be no predictable correlation between a "mosaic" type of development on the one hand and the presence of nucleoli and rRNA synthesis on the other. The correlation in *Ascaris* is positive; but in *Ascidia nigra* and *Ilyanassa* it is negative (*17*).

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## Adrenal Cholesterol: Localization by Electron-Microscope Autoradiography


Abstract. *As determined by electron-microscope autoradiography of adrenal glands containing tritiated cholesterol and by modified differential centrifugation techniques, 70 to 80 percent of adrenal cholesterol is contained within lipid droplets of rat adrenal cortical cells.*


Five percent of the wet weight of the adrenal cortex of the rat is comprised of cholesterol, most of which is esterified. This large pool of cholesterol is depleted after adrenocorticotropic hormone (ACTH) stimulation of adrenal steroidogenesis (*1*); cholesterol is a direct precursor of adrenal steroid hormones (*2*). Although adrenal cholesterol has not been definitely localized, mitochondrial fractions prepared by differential centrifugation contain considerable quantities of cholesterol (*3*). Histochemical studies have shown a correlation between the presence of adrenal cholesterol and the cell layers producing corticosterone (*4*) and a depletion of sudanophilic material in the adrenal after administration of ACTH (*5*). Lipid droplets are also depleted after administration of ACTH (*6*). These histologic results suggest that adrenal cholesterol is located, at least in part, in the lipid droplets. The light-microscopic methods for localization of tissue cholesterol lack the specificity or resolution needed to determine subcellular localization (*7*), and biochemical studies on isolated adrenal lipid droplets have not been reported. Because there is evidence that the rate-limiting reaction in production of adrenal steroid hormones is the transformation of cholesterol to pregnenolone and that the enzyme system responsible for the reaction is present within the mitochondrion (*8*), determination of the intracellular location of the substrate (cholesterol) is important.

Sprague-Dawley rats (200 g) were injected intravenously with 500 $\mu$c of $H^3$-cholesterol previously conjugated to rat serum protein (*9*). Eighteen hours later, the specific activities of adrenal and plasma cholesterol were within 90 percent of equilibrium, and maximum incorporation of radioactivity into adrenal cholesterol was obtained (*10*). Chromatography of adrenal lipids revealed that more than 99.5 percent of the radioactivity contained in the adrenal was present as cholesterol (*11*). Adrenals were fixed with 1 percent glutaraldehyde in modified Tyrode's solution (*12*) perfused through the abdominal aorta. The adrenals were cut into small blocks and rinsed in phosphate-buffered 7.5 percent sucrose (*p*H 7.2). Osmium fixation was essential for the retention of cholesterol. When osmium fixation was omitted entirely, almost all the cholesterol was extracted by the dehydrating fluids (Table 1); less than 1 percent remained in the embedded tissue (*14*). With a standard 1-hour fixation in 1 percent osmium tetroxide, 34 percent of the cholesterol was retained in the embedded tissue. These findings are similar to studies by Korn (*15*) which indicate that loss of lipid-soluble substances occurs during preparation of tissue for electron-microscopic studies.

Because osmium may react with lipids by forming complexes with unsaturated lipids (*16*) and because highly unsaturated fatty acids occur in adrenal cholesterol esters (*17*), we treated adrenal tissue with 2 percent osmium tetroxide in a phosphate or barbital buffer at room temperature for 12 to 24 hours. Prolonged fixation with osmium doubled the retention of cholesterol after standard processing for electron microscopy (Table 1). Retention was increased to more than 90 percent by omitting absolute ethanol and propylene oxide and using instead Epon 812 as the final dehydrating agent after 95 percent ethanol [a modification of the procedure of Idelman (*18*)]. This was the best method tried and was used to prepare adrenal tissue for autoradiography. For the latter procedure, lightly carbonized 90-nm sections of Epon-

Table 1. Extraction of $H^3$-cholesterol from adrenal glands during processing for electron microscopy. Percentage of disintegrations per minute (dpm) retained (second column) is expressed as percent of total dpm (dpm extracted plus dpm retained). The "embedding mixture" was composed of Epon 812, acid anhydrides, and amine (*13*). Specimens described in the bottom row were partially dehydrated up to 95 percent ethanol, were then soaked in Epon 812 alone (three transfers), and finally were infiltrated with the complete embedding mixture.

| Fixation after glutaraldehyde | Disintegrations per minute (% dpm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Retained | Extracted | | | | | | |
| | | Aqueous solutions | Ethanol | | | Propylene oxide | Epon 812 | Embedding mixture |
| | | | 70% | 95% | Abs. | | | |
| None | 0.6 ± 1.0* | 0.1 | 7.6 | 36.1 | 54.1 | 1.4 | | 0.1 |
| 1 percent $OsO_4$ for 1 hr | 32.0 ± 2.9 | 0.1 | 0.6 | 10.4 | 28.4 | 28.0 | | 0.5 |
| 2 percent $OsO_4$ for 24 hr | 67.2 ± 4.9 | 0.2 | 0.5 | 1.9 | 7.8 | 25.7 | | 1.2 |
| 2 percent $OsO_4$ for 24 hr | 90.3 ± 2.1 | 0.2 | 0.5 | 1.8 | | | 5.7 | 1.5 |

* ± 1.0 S.E.M.