

REGULATION OF TRANSCRIPTION OF GENES OF RIBOSOMAL RNA DURING AMPHIBIAN OOGENESIS

A Biochemical and Morphological Study

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ABSTRACT

Natural changes in the transcription of rRNA genes were studied in nucleoli from three oogenic stages of the newt *Triturus alpestris* with electron microscope, autoradiographic, and biochemical techniques. From determinations of the uridine triphosphate pool sizes and [³H]uridine uptake, phosphorylation, and incorporation into 28S and 18S rRNAs in vivo it was estimated that the rate of rRNA synthesis was about 0.01% in previtellogenic oocytes and 13% in mature oocytes when compared to midvitellogenesis. Spread preparations of nucleoli showed significant morphological changes in the transcriptional complexes. The total number of lateral fibrils, i.e., ribonucleoproteins containing the nascent rRNA precursor, were drastically decreased in stages of reduced synthetic activity. This indicates that rRNA synthesis is regulated primarily at the level of transcription. The resulting patterns of fibril coverage of the nucleolar chromatin axes revealed a marked heterogeneity. On the same nucleolar axis occurred matrix units that were completely devoid of lateral fibrils, matrix units that were almost fully covered with lateral fibrils, and various forms of matrix units with a range of lateral fibril densities intermediate between the two extremes. Granular particles that were tentatively identified as RNA polymerase molecules were not restricted to the transcriptional complexes. They were observed, although less regularly and separated by greater distances, in untranscribed spacer regions as well as in untranscribed gene intercepts. The results show that the pattern of transcriptional control of rRNA genes differs widely in different genes, even in the same genetic unit.

Amplification of genes which code for ribosomal RNA (rRNA) takes place during pachytene, very early in amphibian oogenesis (e.g., references 4, 39, and 91). However, this production of an enormous number of extrachromosomal copies of "rDNA" (i.e. DNA units that contain a high number of sequences complementary to the two high molecular weight ribosomal RNAs, the "28S"

and "18S" RNA) does not result in a coincident increase in ribosome synthesis. In *Xenopus laevis*, for example, synthesis of 28S and 18S rRNA, as well as accumulation and storage of ribosomes in the ooplasm, has been detected in significant amounts only later in oogenesis and is particularly predominant during the lampbrush chromosome stage (16, 17, 49). In the period between the

process of rDNA amplification and the onset of vitellogenesis, the major products synthesized are 5S rRNA and transfer RNAs (e.g., references 18, 19, 27, 28, 49, 78, and 80). Beginning at this stage, the production of 28S and 18S rRNA increases and remains at about maximal activity until the oocyte is nearly mature. The average *X. laevis* oocyte produces a total of about 10^{12} ribosomes at a nearly constant rate of 300,000 ribosomes per second during this growth interval, at least under the special growth-promoting conditions of partial ovariectomy and stimulation by gonadotropic hormone (72). According to Davidson and co-workers (17), about 95% of the total RNA synthesized during this stage represents 28S and 18S rRNA. In mature oocytes, on the other hand, the rRNA synthesis declines or, in some cases, falls below the level of detection (8, 9), although the amount of rDNA in the germinal vesicle remains constant (e.g., reference 8).

Several authors have interpreted this pronounced variation in the rate of 28S and 18S rRNA production as being due to an "activation" and "inhibition," respectively, of the transcription of the amplified rRNA genes in the course of oocyte growth (14, 18, 19, 28, 78, 79). In the previtellogenic oocytes of *X. laevis*, 40S pre-rRNA could not be detected (49), which at least suggests the absence of a steady-state concentration of this precursor in a balanced "turn-over" of transcription and degradation of transcriptional products. On the other hand, such data would also be compatible with the concept of control of rRNA formation at the post-transcriptional level if one assumes that the 40S pre-rRNA is made but is then so rapidly degraded that it escapes detection. Both principles of regulation of ribosome biogenesis, the modulation of the transcription and that of the post-transcriptional turnover and transport of synthesized RNA, have been discussed and suggested in other eucaryotic cells. For example, the failure of frog gastrula endoderm cells to produce rRNA seems to be due to a blocked transcription of rDNA (51). Similarly, the rate of synthesis of pre-rRNA has been claimed to decrease in confluent cultures of fibroblasts from various sources (25, 67, 86, 92). On the other hand, regulation at a post-transcriptional level has been suggested in such diverse cells as bovine lens epithelial cells (58), differentiating erythroid cells of the mouse yolk sac (26), cultured human cells (88), lymphocytes (12, 13) and leukemic blast cells (75, 82), hypertrophying mouse kidney cells (37), regenerat-

ing hepatocytes (63), estrogen-stimulated rat uterus cells (47), during thyroxine-induced metamorphosis of *X. laevis* tail muscle cells (69), during mouse spermatogenesis (31), and in cultured cells infected with adenovirus (44) and influenza virus (77). Moreover, a concerted regulation of rRNA formation at two sites, at the transcriptional level as well as in the processing, may be involved (e.g., references 47, 58, 63).

The transcriptional activity of cistrons of rRNA, especially when amplified, can be directly demonstrated and analyzed in the electron microscope by the spreading technique (54). This technique has been particularly successful in work with cells with a very high transcriptional activity, such as during vitellogenesis in amphibia (53-55, 73) and insects (83, 84), during spermiogenesis in *Drosophila* (50), as well as during other forms of giant cell growth in animals and plants (21, 22, 76, 85). We have recently shown that this technique is also valuable in studying stages of reduced or blocked transcriptional activity, such as during the actinomycin D-induced inhibition of transcription (74). In order to study processes of *natural* activation and inactivation of the transcription of a defined gene, we have now chosen the oocyte as a model system that provides stages of increasing and reduced ribosome formation. Since in *X. laevis* the natural seasonal cycle of oocyte growth and ovulation is blocked under laboratory conditions and can be induced only by relatively high doses of gonadotropic hormones, we have restricted this study to newts freshly collected during their breeding season.

MATERIALS AND METHODS

Preparation and Classification of Oocytes

Newts (*Triturus alpestris*) were collected in ponds in the surroundings of Heidelberg or in the Black Forest from March to June. Ovaries were removed from the anesthetized animals (0.1% MS 222; Serva Feinbiochemica, Heidelberg) and placed in Eagle's minimal essential medium diluted 1:1 with distilled water, and oocytes of different size classes were manually isolated. For the purpose of the present study, oocyte development was divided into three basic stages (cf. also reference 23). Small transparent oocytes with a diameter of up to 0.35 mm (nuclear diam ca. 150 μ m) are referred to as *previtellogenic*. For preparative reasons, oocytes smaller than 0.2 mm could not be used; therefore, the definition of the previtellogenic stage as used here is restricted to oocytes within the size range of 0.2 to 0.35 mm. Oocytes 0.5-1.4 mm in diameter (nuclear diameter 0.25-0.6 mm)

are considered *vitellogenic*. In the early phase of this stage, yolk is deposited first in the periphery of the oocyte. In later stages (1.2–1.4 mm large oocytes), the yolk platelets fill the entire cytoplasm, except for a small juxtannuclear zone. Nuclei (germinal vesicles) of *mature* or postvitellogenic oocytes (1.6–1.8 mm diam, nuclear diam ca. 0.65 mm) are characterized by the central aggregation of most of the extrachromosomal nucleoli clustered around the typical condensed chromosomes.

RNA Labeling

Newts were intraperitoneally injected on two subsequent days with 0.5 mCi [³H]uridine each time (49 Ci/mmol; The Radiochemical Centre, Amersham, England). 5 days after the last injection, the animals were decapitated and the ovaries were transferred to diluted Eagle's medium. For autoradiography, vitellogenic oocytes were removed from the animal 4 hr after a single injection of 1 mCi of [³H]uridine into the body cavity.

Electron Microscopy and Autoradiography

Radioactively labeled oocytes of the different size classes were fixed in 4% glutaraldehyde buffered with 0.05 M Na cacodylate to pH 7.2 for several hours at 4°C, washed in the same buffer, and postfixed in 2% OsO₄ (29). Manually isolated nuclei were fixed by the same procedure. They were dehydrated in graded ethanol solutions and propylene oxide and embedded in Epon 812. For light microscope autoradiography, 1- μ m thick sections were coated with Kodak AR 10 stripping film. Ultrathin sections (Reichert ultramicrotome OmU2) were coated with Ilford L4 emulsion and developed in Kodak D19 (for methodological details, see reference 34). Electron micrographs were taken with a Siemens Elmiskop 101 and a Zeiss EM 10.

For spread preparations, nuclei were manually isolated from the different oocyte classes, the nuclear envelope was opened with fine needles, and the nuclear contents, together with pieces of the nuclear envelope, were spread and positively stained according to the method of Miller and Beatty (54; for technical details see references 53 and 73). To enhance contrast, some phosphotungstic acid-stained preparations were rotary shadowed with Au-Pd (75%:25%) at an angle of 7° (Balzers Mikrobendamplungsanlage BA3; Balzers, Liechtenstein).

Gel Electrophoresis of Oocyte RNA

20–30 oocytes of each size class were used per experiment. The follicle epithelium was carefully removed by micromanipulation, and the presence of epithelial nuclei was routinely spot-checked by light microscopy of sections (only in previtellogenic oocytes were minor quantities of follicle residues occasionally noted). The oocytes were homogenized at 4°C in a medium consisting of 50 mM Tris-HCl (pH 7.6), 1% NaCl, and 2% (wt/vol) sodium triisopropyl naphthalene sulfonate

in a motor-driven Potter-Elvehjem homogenizer. Phenol extraction was carried out according to Kirby (40) as modified by Loening (46). The ethanol-precipitated nucleic acids were centrifuged, and the pellet was drained of ethanol and redissolved in 100 μ l of electrophoresis buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 2 mM EDTA; cf. reference 62) containing 0.2% SDS. rRNA extracted from *Triturus alpestris* oocyte ribosomes was added, as an internal marker, to preparations of previtellogenic oocytes, and transfer RNA from *Escherichia coli* was added to each sample. The RNA was analyzed on 1% agarose gel slabs (15). After the run, the two bands of the rRNAs and the band corresponding to the 4S and 5S RNA were marked under UV light; the gel was cut into 1.1-mm thick slices and the radioactivity was counted as described (73). To separate transfer RNA from 5S RNA, 7.5% acrylamide gel slabs were used according to Egyhazi et al. (24).

Analysis of the Nucleotide Pool

(a) In order to compare the uptake and the phosphorylation of the added uridine in the different oocyte classes, newts were injected intraperitoneally with 1 mCi [³H]uridine. After varying times (5 h, 1 day, 3 days), 10 mature, 10 vitellogenic, and 30 previtellogenic oocytes were collected, freed from follicle epithelium, and homogenized in 0.2 ml each of 0.5 N perchloric acid (PCA) at 4°C in a small, motor-driven Potter-Elvehjem homogenizer. After 20 min, the solutions were centrifuged for 10 min at 3,000 *g* in the cold, and the supernates were neutralized with KOH in the presence of 50 mM Tris-HCl (pH 7.2). After a second centrifugation, in order to remove the potassium perchlorate, 20 μ l of each supernate were applied to PEI-cellulose precoated plastic sheets (Polygram CEL 300 PEI; Macherey-Nagel, Düren, Federal Republic of Germany). A mixture of unlabeled uridine mono-, di-, and triphosphate ribonucleotides (UMP, UDP, UTP) was applied to the same spot. For better resolution, the thin-layer plate was soaked in methanol for 10 min after application of the samples. One-dimensional chromatography was performed as described by Randerath and Randerath (61). For counting the radioactivity, the spots corresponding to the particular nucleotides were identified under UV light, cut out from the plastic sheet, and directly placed into scintillation vials to which 1 ml NCS (Nuclear Chicago Solubilizer, Amersham/Searle Corp., Arlington Heights, Ill.) was added. After 12 h of incubation at 40°C, toluene-based scintillation fluid was added and the radioactivity was determined in a liquid scintillation counter (Betaszint 5000; Berthold-Frieseke, Karlsruhe, Federal Republic of Germany). (b) Determination of the endogenous UTP pool sizes of the different oocyte classes was carried out by a modification of the very sensitive method described by Sasvári-Székely et al. (70). Oocytes without the surrounding epithelium were homogenized in 0.5 N PCA at 4°C as described above (10

mature and 10 vitellogenic oocytes each in 1 ml, 100 previtellogenic oocytes each in 0.2 ml of PCA). 10–20 μ l of the neutralized acid-soluble fraction were used for the enzyme assay. The reaction mixture consisted of the standard buffer (Tris-HCl, 32 mM, pH 7.9; MgCl₂, 8 mM; EDTA, 0.08 mM; dithiothreitol, 0.08 mM; KCl, 120 mM) containing the following components, in a final volume of 0.1 ml: 0.15 μ g poly-d (AT) (Boehringer, Mannheim); 250 or 500 pmol ATP; 2 μ Ci [³H]ATP (16.4 Ci/mmol, The Radiochemical Centre); 0.2 μ g RNA polymerase from *E. coli* (sp act ca. 1,400 U/mg as defined by reference 10); and, as the limiting nucleotide, 0–200 pmol UTP (all nucleotides from Boehringer, Mannheim). With 200 pmol of UTP per assay, the radioactivity incorporated into the reaction product increased linearly with time for at least 50 min at 37°C. Therefore, all assays were incubated at 37°C for 40 min. The reaction was stopped by rapid cooling in an ice-water bath, and 100 μ g serum albumin were added to each tube. The precipitate obtained after the addition of 1 ml cold 10% trichloroacetic acid (TCA) containing 10 mM sodium pyrophosphate was collected onto Whatman GF/C glass fiber filters. The filters were thoroughly washed with cold 5% TCA containing 10 mM Na pyrophosphate, dried, and the radioactivity was determined as described above. The amount of UTP that was not rendered soluble during the PCA-precipitation was determined with [³H]UTP added before homogenization and was found to be negligibly low (less than 3.5%). Specific radioactivities of UTP could be calculated from the determinations described under (a) and (b).

RESULTS

RNA Synthesis in Different Oogenic Stages

Labeled RNA was extracted from oocytes of six different size classes, which were taken from both ovaries of the same newt in each individual experiment, and was analyzed by gel electrophoresis (Fig. 1). In all stages the radioactivity was found in 28S and 18S rRNA, although both the total amount of RNA synthesized and the relative proportions of ribosomal to total RNA were greatly different. This indicates that some rRNA synthesis was continuous, taking place in all oogenic stages examined. In midvitellogenic oocytes about 86% of the radioactivity was present in the 28S and 18S rRNA peaks (Fig. 1 c). The remaining radioactivity was evenly distributed over the whole molecular weight range analyzed (approximately 10⁴ to 5 × 10⁷ daltons). This high percentage of rRNA is in close agreement with data obtained in stage 4 oocytes of *X. laevis* (17). In mature oocytes (Fig. 1 a), the radioactivity contained in 28S and 18S rRNA was greatly reduced and, in contrast to

all other stages, only about 45% of the radioactive material applied to the gel could be recovered. Most of the radioactivity ran off the gel and was apparently contained in molecules of very low molecular weight and oligonucleotides (Fig. 1 a). This consistently observed material was characteristic for the mature oocytes and may possibly be indicative of some RNA degradation in such cells (for detailed discussion, see also reference 45). In previtellogenic oocytes, the radioactivity contained in the 28S and 18S rRNA peaks represented only about 13% of the total radioactivity, and the bulk of labeled RNA was found in the 4–5S RNA region (Fig. 1 e, f). In analyses of 7.5% acrylamide gels, this RNA was separated into a 4S component and a larger proportion that comigrated with a 5S rRNA reference.

From the gel electrophoretic patterns shown in Fig. 1, it is obvious that during oocyte development marked differences occurred in the RNA species which were synthesized, similar to what has been described for *X. laevis* (19, 27, 28, 49, 78, 80). In order to estimate the relative rates of synthesis of 28S and 18S rRNAs, we measured the uptake and phosphorylation of [³H]uridine in the three different oocyte stages (Table I) and measured as well the corresponding sizes of the endogenous UTP pools (Fig. 2). The uptake of uridine in vivo was maximal in mature oocytes (for diverging data concerning guanosine uptake into *Xenopus laevis* oocytes kept in amphibian Ringer's solution, see reference 42) whereas intracellular phosphorylation took place at similarly high efficiencies in all stages. If we take into account the specific endogenous pool sizes (Fig. 2 and Table II), the rate of rRNA synthesis was only 0.013% in previtellogenic oocytes and 13% in mature oocytes, relative to midvitellogenesis. In the context of the present study, it is worth emphasizing that rRNA synthesis is never completely suppressed during the oogenic stages examined but that in both previtellogenic oocytes and mature oocytes significant synthesis takes place (for similar observations with *Xenopus laevis* oocytes, see, e.g., references 11, 42, 45, 80, 95; for controversial results concerning mature *Xenopus* oocytes, cf. references 6, 8, 9).

Transcriptional Activity of Nucleoli as Revealed by Autoradiography and Electron Microscopy

PREVITELLOGENIC OOCYTES: Autoradiographs of sections through [³H]uridine labeled

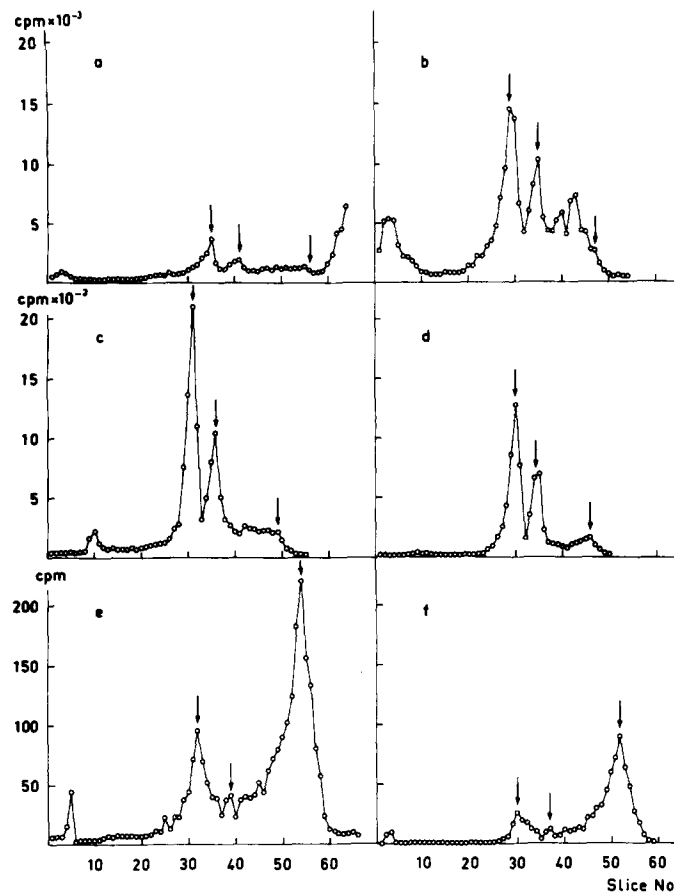


FIGURE 1 Gel electrophoretic analysis (1% agarose gels) of radioactively labeled RNA extracted from different oogenic stages of *T. alpestris*. Newts were injected intraperitoneally with 1 mCi [^3H]uridine. After 6 days of incorporation, both ovaries were removed and the oocytes were divided into six size classes. RNA extraction was carried out as described in Materials and Methods. All values represent the radioactivity from 10 oocytes of each size class. The arrows denote the positions of coelectrophoresed 28S and 18S rRNAs and of 4S RNA (from left to right). In mature oocytes (a, 1.6–1.8 mm oocyte diameter) the radioactivity contained in the ribosomal RNA species is likewise drastically reduced as compared to the various vitellogenic stages (b, 1.2–1.4 mm; c, 0.8–1.0 mm; d, 0.6–0.7 mm oocyte diameter). On the contrary, in previtellogenic oocytes (e, 0.3 mm; f, 0.2 mm oocyte diameter) the predominant product synthesized is the 4S and 5S RNA species, with a disproportionately low labeling of 28S and 18S rRNAs (note the different scales of the ordinates in e and f). The pattern of the RNA extracted from late vitellogenic oocytes (b) exhibits two additional peaks corresponding to RNAs with apparent molecular weights of about 3 and 4×10^6 daltons. The very high molecular weight components revealed in b, c, and e appeared in somewhat variable proportions and might represent non-nucleolar, chromosomal RNA products.

previtellogenic oocytes (for details see Materials and Methods) showed an accumulation of silver grains over the germinal vesicles, with a nearly uniform distribution (Fig. 3). After prolonged exposure, however, the peripherally located nucleoli exhibited an enrichment of radioactivity relative to the nucleoplasm (Fig. 3 c). This finding

of a low but significant level of [^3H]uridine incorporation into nucleoli of young oocytes correlated with the low rRNA synthesis described above. The close association between these nucleoli and the nuclear envelope was particularly conspicuous in ultrathin sections and was apparently maintained by fine fibrillar strands or by somewhat thicker

stalk-like connections (Fig. 4 a). A typical granular cortex was absent, and the nucleolar mass appeared to be almost entirely composed of densely aggregated fibrillar material (see also references 52 and 89; for *Xenopus* oocytes in a comparable stage, cf. references 79 and 87). The

structural component prevalent in the ground cytoplasm was a loose fibrillogranular network with very few interspersed ribosomes (Fig. 4 a; see also references 28 and 79).

In spread and positively stained preparations, the isolated nucleoli from previtellogenic oocytes

TABLE I
Uptake and Phosphorylation of [³H]Uridine in Different Oocyte Classes of *Triturus alpestris* after 5 h of Labeling In Vivo

Oocyte stage	cpm per oocyte in			Σ cpm	Rate of uptake of [³ H]uridine*
	UMP	UDP	UTP		
Previtellogenic	9 (1.6)	20 (3.5)	531 (94.9)	560	2.6
Vitellogenic	184 (1.3)	424 (3.0)	13,522 (95.7)	14,130	66.4
Mature	553 (2.6)	787 (3.7)	19,940 (93.7)	21,280	100.0

The values in parentheses give the distribution of radioactivity in percent of the sum.

* Expressed in percent relative to the rate in mature oocytes.

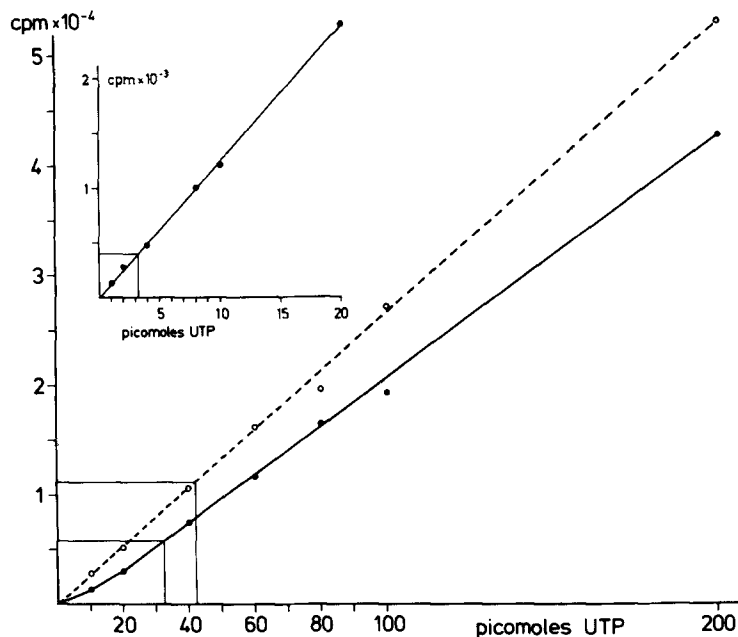


FIGURE 2 Determination of the endogenous UTP pool sizes of three different oocyte classes of *T. alpestris* (for details see Materials and Methods). 10 μ l of the neutralized acid-soluble cell extract were used per assay for the UTP determination in mature (broken line) and vitellogenic oocytes (diameter 0.8–1.0 mm; continuous line), and 20 μ l in the case of previtellogenic oocytes (diameter 0.3 mm; inset). All reactions were carried out in the presence of the cell extract plus known amounts of UTP added. ATP (a defined mixture of ³H-labeled and unlabeled ATP) was present in excess. The values were corrected for the dilution of the specific radioactivity of ATP by the unknown amount of ATP present in the cell extract and represent the radioactivity incorporated into the acid-precipitable poly-AU product minus the radioactivity measured in the presence of the cell extract alone, i.e. without added UTP. By the use of this standard curve the amount of UTP in the specific cell extracts was calculated from the radioactivity incorporated in the presence of cell extract without exogenous UTP added (horizontal bars).

TABLE II
Synthesis of rRNA and Transcriptional Activity of Pre-rRNA Genes in Different Oogenic Stages of Triturus alpestris: a Combination of Biochemical and Electron Microscope Analyses

Oocyte stage (oocyte diameter in mm)	UTP pool size (picomoles per oocyte)*	Amount of [³ H]uridine incor- porated into rRNA (cpm per oocyte)†	Relative rate of synthesis of stable rRNA§	Mean number of lateral fibrils per mm nucleolar DNP axis	Relative transcrip- tional activity¶
Previtellogenic (0.3)	0.37	67	0.013 (0.01–0.4)	644 (33:9.8)	3
Vitellogenic (0.8–1.0)	325	15,600	100	22,941 (117; 2.04)	100
Mature (1.6–1.8)	420	2,290	12.9 (10.7–14.0)	3,353 (17; 1.85)	15

* Mean values from three experiments (for related data in *Xenopus laevis* oocytes, see reference 94).

† Calculated from the radioactivities contained in the 28S and 18S rRNAs for one representative experiment out of five (cf. Fig. 1). For experimental variations, see next paragraph.

§ Expressed in percent relative to the vitellogenic oocyte stage. The values have been calculated from the rates of [³H]uridine uptake and phosphorylation (Table 1) and the specific sizes of the endogenous UTP pools. Values given in brackets indicate the range of experimental variation.

|| Numbers in the brackets indicate the corresponding mean number of lateral fibrils per pre-rRNA gene unit and total mm of axial fibril length traced.

¶ Values of the lateral fibril density are expressed in percent of that present in vitellogenic oocytes in which an average of about 95% of the genes showed transcriptional activity. Note, however, that the transcriptional activity of the vitellogenic oocytes does represent only about 90% of the maximally possible activity, assuming that an average repeating unit (5.1 μm; cf. reference 73) of the rDNP axis contains approximately 130 lateral pre-rRNP fibrils.

appeared partially unraveled; their composition was revealed by long "axial" fibrils (Figs. 4 b, c, and 5 a; for nomenclature and interpretation, see below and references 53–55, 73, 76, 84, 85) which probably represent strands of nucleolar chromatin, i.e. deoxyribonucleoprotein (DNP) containing the rDNA. As a consequence of the tendency of largely inactive nucleolar chromatin to aggregate into dense clumps, spreading of nucleoli from previtellogenic oocytes in general was less readily achieved¹ than from oocytes in vitellogenic stages. Fibrillar aggregates as shown in Figs. 4 b, c and 5 a can be positively identified as nucleolar material since in such preparations the spreading was performed with isolated nuclear envelopes, thereby separating the peripheral nucleoli from the more centrally located chromosomes and other nucleo-

plasmic components. In some spread preparations of such nucleolar aggregates, only very few individual "lateral fibrils" (for nomenclature see references 53–55, 73, 76, 84, 85), probably ribonucleoprotein (RNP) containing nascent pre-rRNA, were recognized, the identification of which was facilitated in regions where groups of lateral fibrils were arranged in a pattern resembling that of typical "matrix units" (Figs. 4 c and 5 a). Only very rarely did we recognize individual complete or almost complete matrix units within the aggregates of nucleolar chromatin from previtellogenic oocytes (cf. Figs. 4 c and 5 a). The vast majority of lateral fibrils, however, were separated from each other by distances much larger than 20 nm, and occurred either as isolated fibrils or in series that resembled the "diluted" matrix units described in actinomycin D-treated amphibian oocytes (Fig. 5 b–d; cf. reference 74). The pattern of lateral fibril dilution within such fibril-covered intercepts on the nucleolar chromatin strands was mostly irregular. However, local groupings of lateral fibrils and, correspondingly, fibril-free intervals within largely intact matrix units were also not infrequently observed. Such diluted ("incomplete") matrix

¹Such clumping was especially noted in attempts to spread nucleolar material from early previtellogenic oocytes from young (2 mo after metamorphosis) *X. laevis* in which we were unable to demonstrate any matrix unit-like structures (unpublished results from work done in collaboration with Dr. Christian Thomas, Université Libre de Bruxelles, Brussels, Belgium).

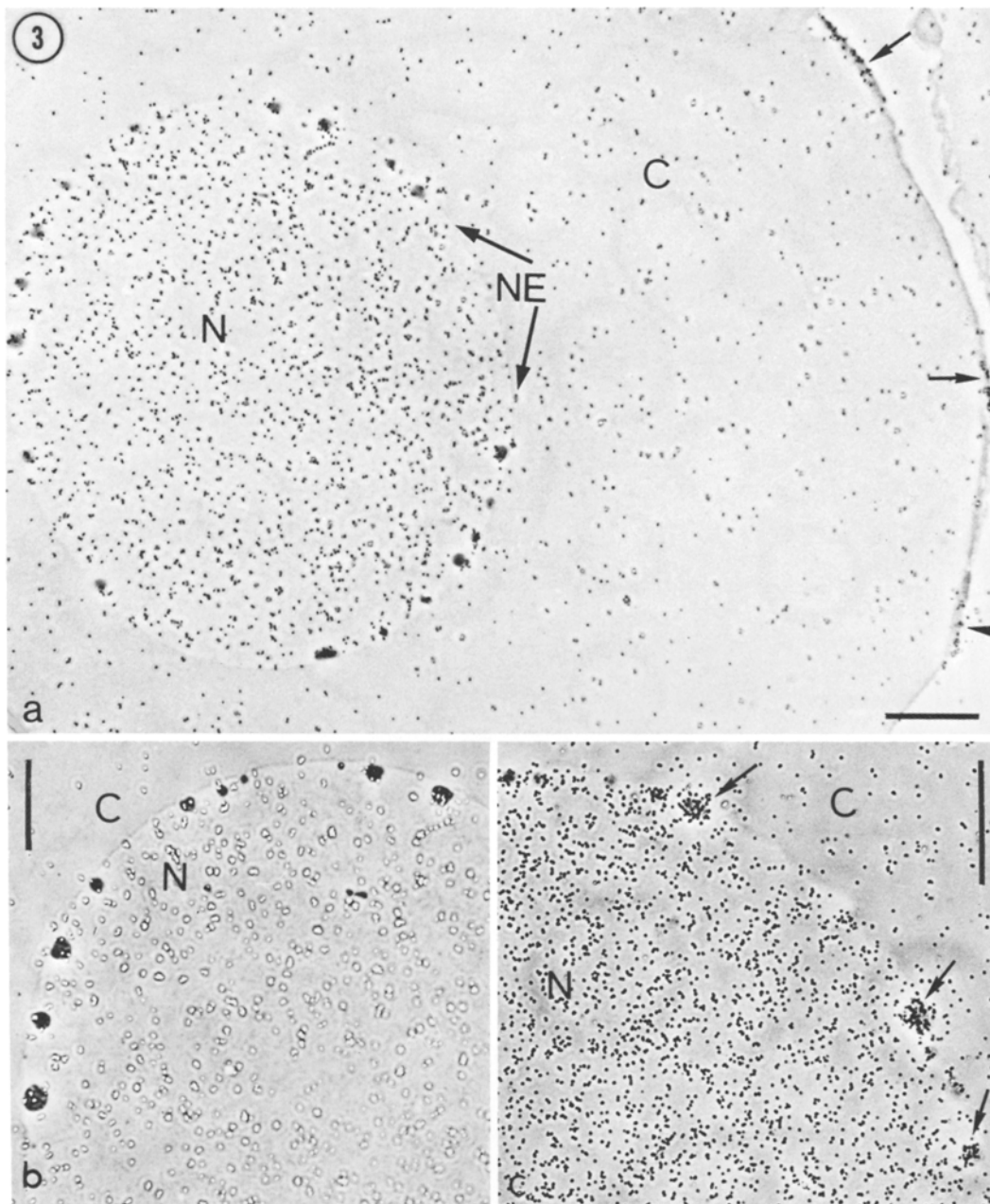


FIGURE 3 *a-c* Light microscope autoradiographs of 2- μ m sections through previtellogenic oocytes of *T. alpestris* newts that were injected with [3 H]uridine and allowed to incorporate for 6 days (see Materials and Methods). When the sections were exposed for 3 days (*a* and *b*), the majority of grains appeared enriched over the nuclei (*N*) of the oocyte and the follicle epithelium (small arrows). Preferential labeling of the nucleoli which are almost exclusively located in the nuclear periphery was not noted (*b*). However, after prolonged exposure (e.g., *c*; 7 days), some of the oocyte nucleoli showed a significantly higher grain density (arrows, *c*), indicative of some synthesis of precursors to ribosomal RNA. *N*, nucleus (germinal vesicle); *NE*, nuclear envelope; *C*, cytoplasm. (*a*) and (*b*) \times 650; (*c*) \times 860; scales indicate 20 μ m.

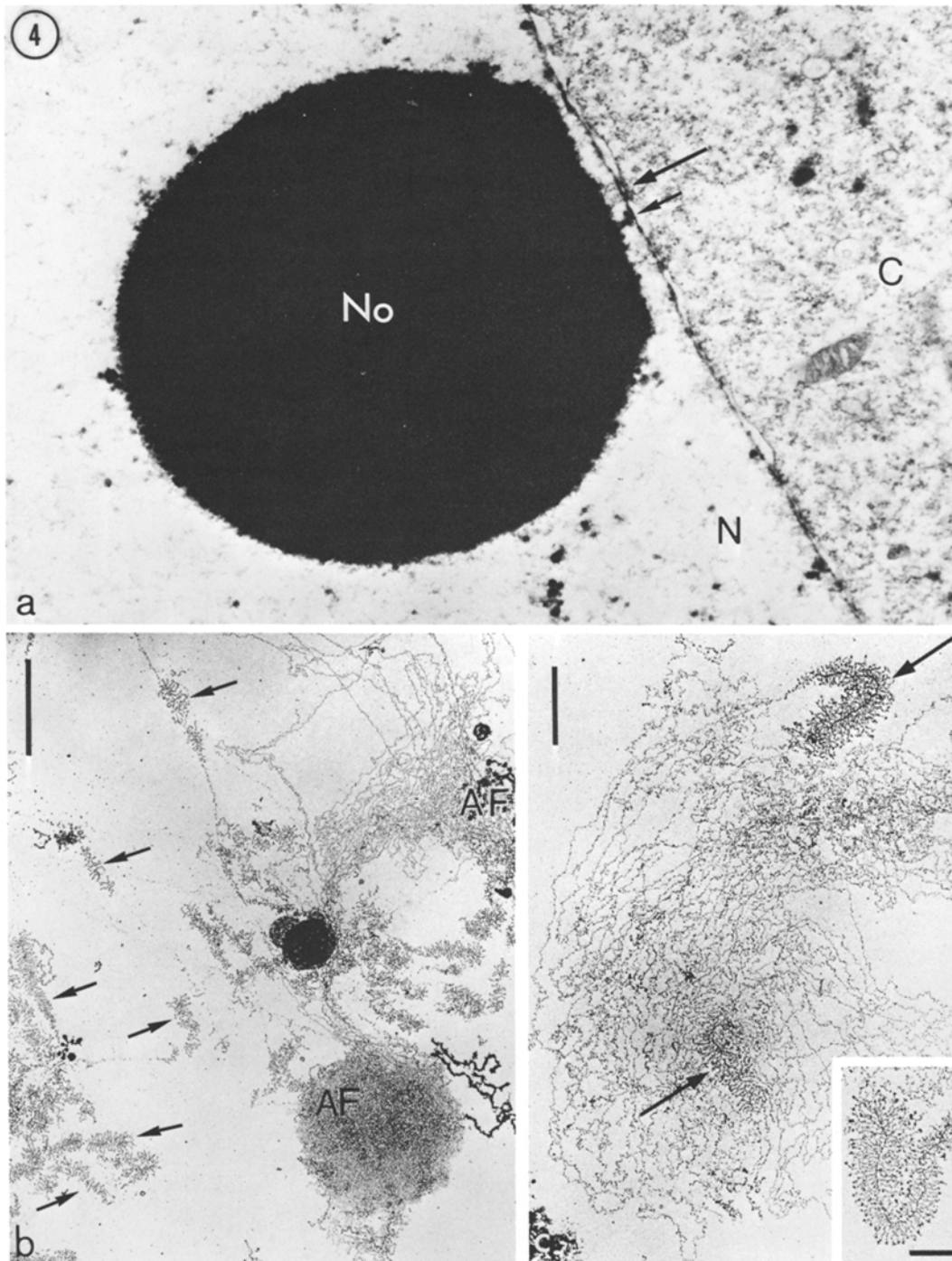


FIGURE 4 *a-c* Electron micrographs of an ultrathin section through the nuclear periphery of a previtellogenic oocyte (*a*) and of spread and positively stained nucleolar material from the same oogenic stage (*b, c*). (*a*) shows the typical firm attachment of the nucleoli to the nuclear envelope via thin fibrils or stalk-like connections which terminate either at the pore complexes (e.g., as indicated by the long arrow) or in interpore regions (short arrow). At this stage, the nucleolar fine structure shows a rather uniform appearance and represents an aggregate of densely packed fibrils. Note the low concentration of ribosomes in the cytoplasm (*C*). In the spread preparations (*b, c*), aggregates of "naked" axial fibrils predominate. Occasionally, however, individual typical matrix units occur on such fibrils (arrows, *c*), thus indicating their nucleolar nature. Some of these matrix units that occur on the nucleolar chromatin axis of previtellogenic oocytes are (almost) completely set with lateral fibrils (*c* and *inset*; note also the occurrence of "terminal knobs" on the lateral RNP fibrils), whereas others show more or less "diluted" fibril distributions (arrows, *b*). Note the local differences of transcriptional activity within one nucleolus as illustrated by the contrast of aggregated fibrils (*AF*) and matrix unit-containing fibrils in *b*. *N*, nucleus; *No*, nucleolus. (*a*) $\times 22,000$; (*b*) $\times 7,000$; (*c*) $\times 11,500$; *inset*, (*c*) $\times 14,000$. Scales indicate $1 \mu\text{m}$ (*a, c*), $2 \mu\text{m}$ (*b*), or $0.5 \mu\text{m}$ (*inset, c*).

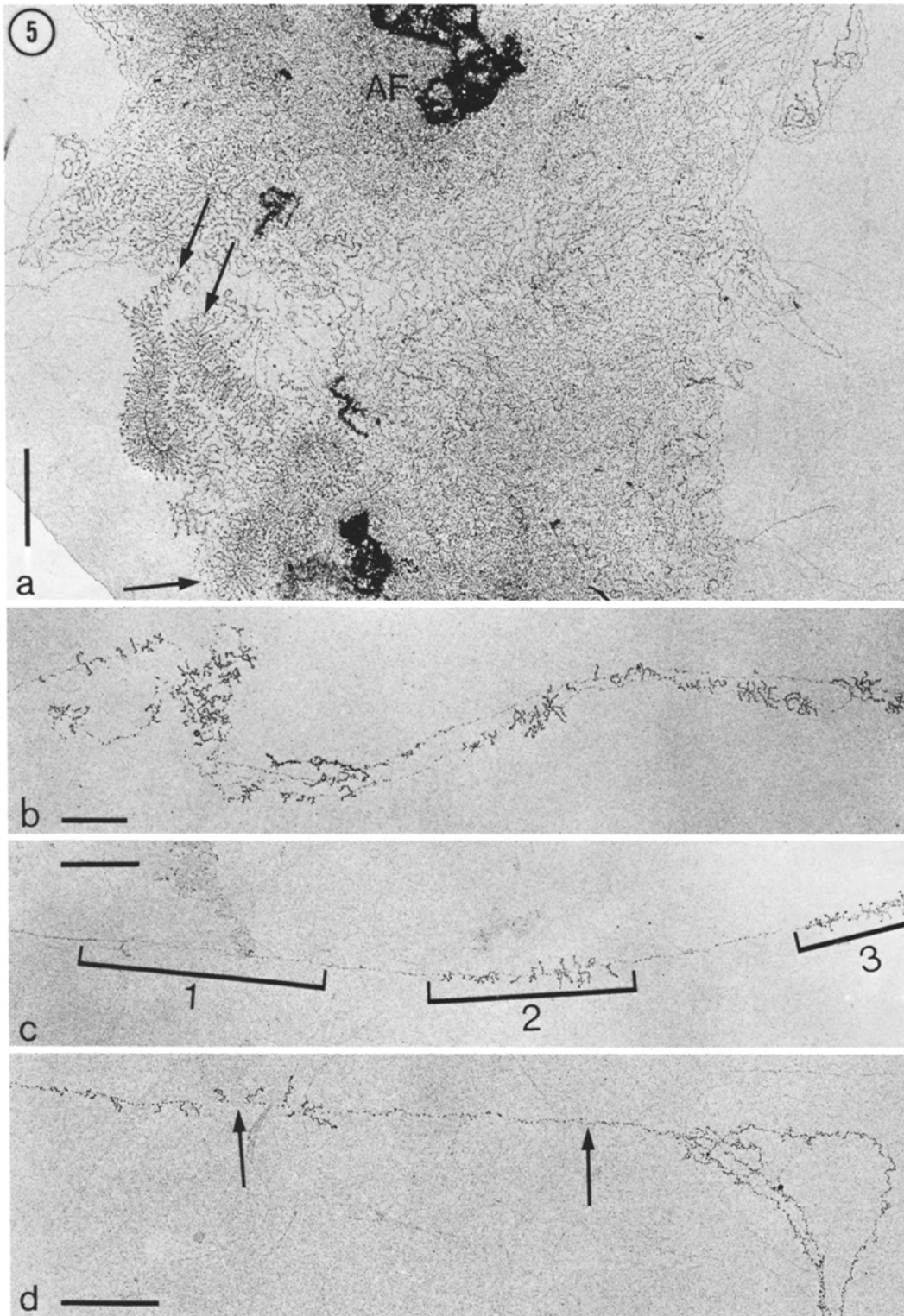


FIGURE 5 *a-d* Details of spread and positively stained nucleoli from previtellogenic *T. alpestris* oocytes. Different types in the pattern of the onset of rDNA transcription are demonstrated. Large fibrillar aggregates (*AF*) with only a few, but maximally active matrix units are recognized (arrows, *a*). This contrasts with the occurrence of many active adjacent matrix units which, however, are incomplete in their fibril package (*b*; see also cistrons nos. 2 and 3 in *c*, and the cistron denoted by the left arrow in *d*). Occasionally, one notes cascades of increasing transcriptional activity along one axis (see, for example, *c*). In such cases, untranscribed cistrons (e.g., gene no. 1 in *c* and that denoted by the right arrow in *d*) are clearly localized. (*a*) $\times 15,000$; (*b*) $\times 10,000$; (*c*) $\times 12,000$; (*d*) $\times 15,500$, scales indicate $1 \mu\text{m}$.

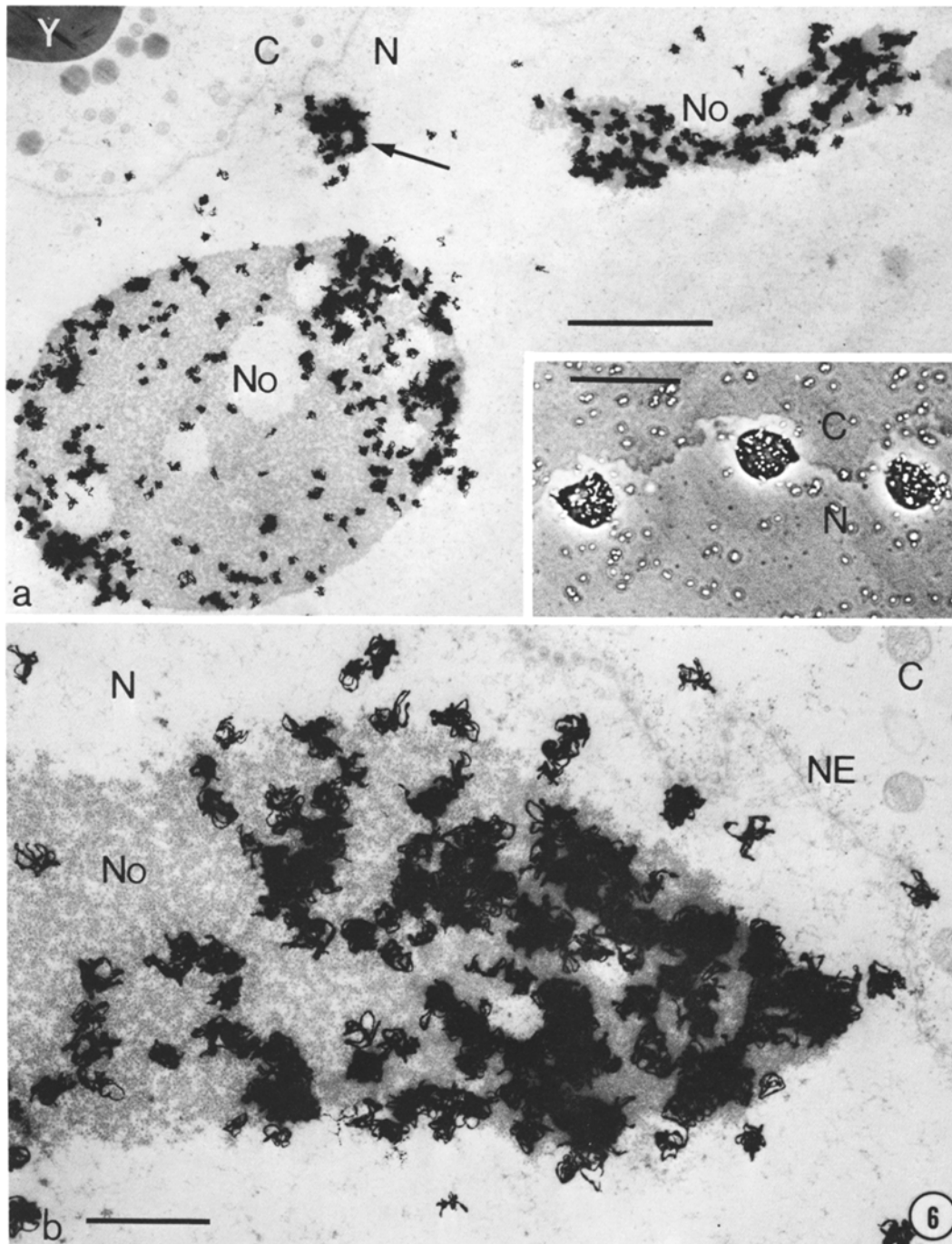


FIGURE 6 *a, b* Light and electron microscope autoradiography of *T. alpestris* oocyte nuclei (*N*) at mid-lamprush stage (oocyte diameters, 0.8–1.0 mm) after incorporation of [^3H]uridine *in vivo* for 4 h. Preferential labeling of the nucleoli (*No*) is demonstrated by autoradiography in the light (*inset, a*) and electron microscope. Grains are largely enriched over caplike regions of the nucleolar periphery (*a, b*). Such highly labeled “caps” are frequently but not always oriented toward the nuclear envelope (*NE, b*). Some of the highly labeled smaller aggregates (arrow, *a*) might also represent “micronucleoli.” *C*, cytoplasm; *Y*, yolk platelet. (*a*) $\times 4,200$, scale indicates 5 μm ; *inset (a)* $\times 800$, scale indicates 20 μm ; (*b*) $\times 18,000$. Scale indicates 1 μm .

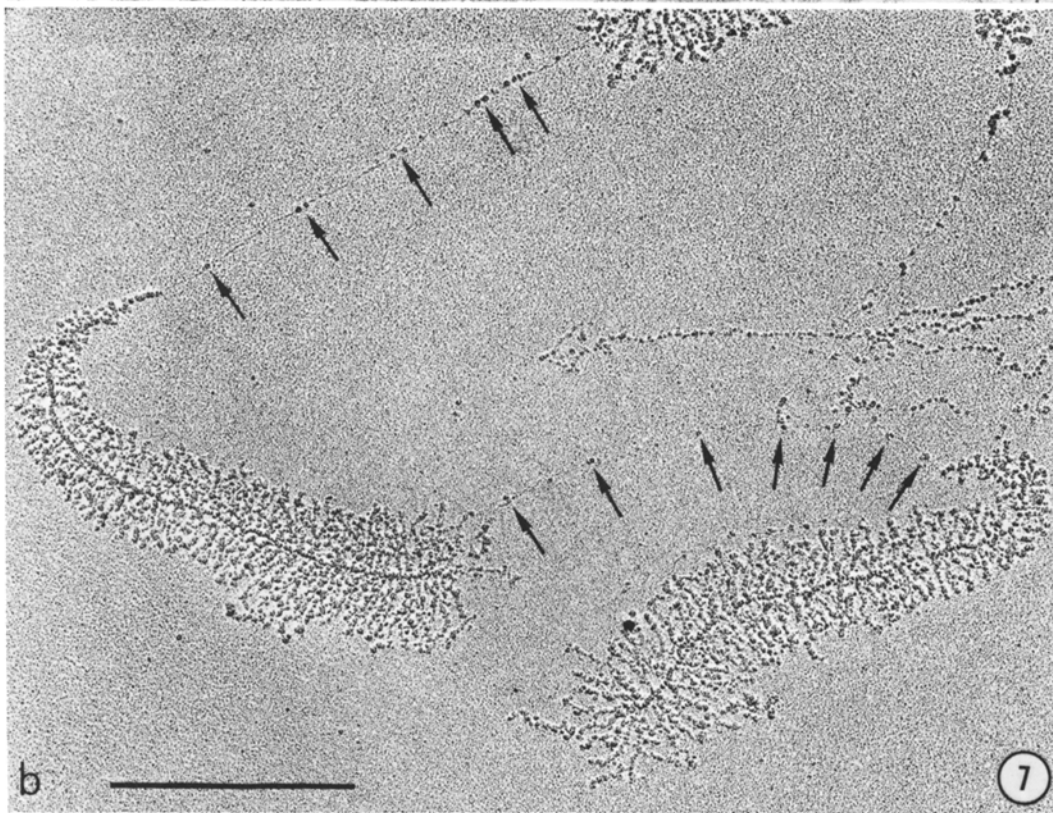
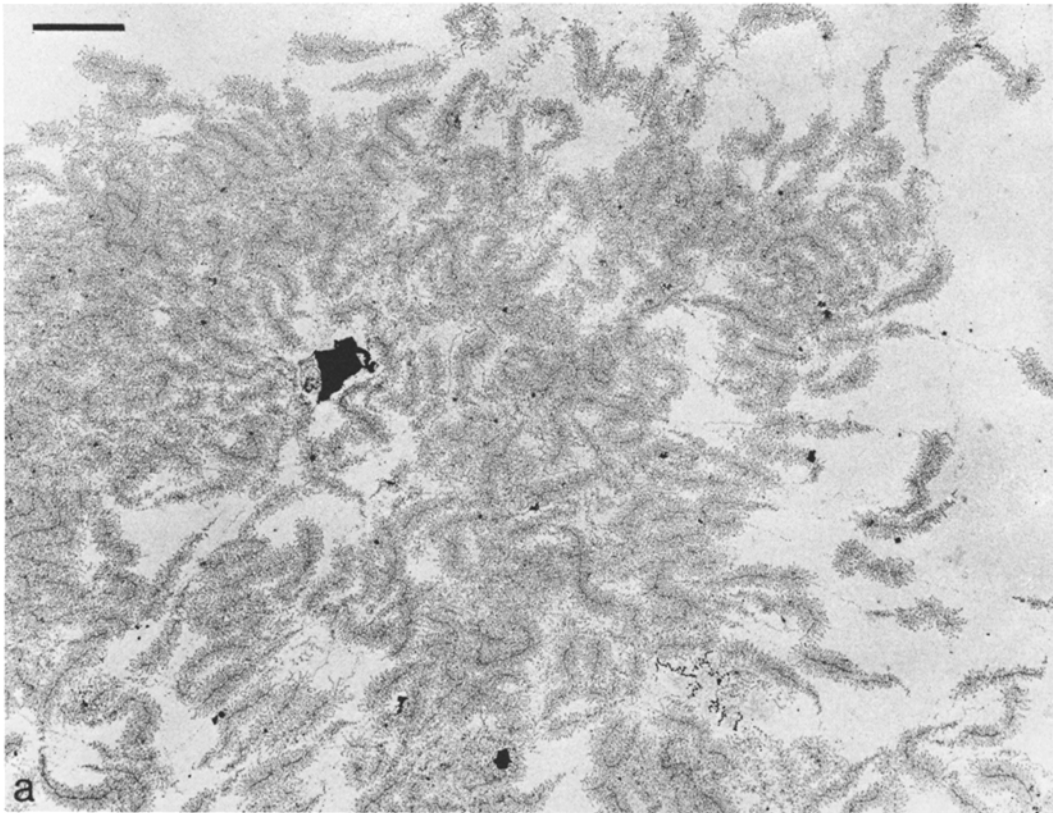
units containing from ca. 10 to 80 lateral fibrils often covered a total of 2.0–2.8 μm on the axes, i.e. lengths which correspond to “normal” matrix units (53–55, 73). Transcriptional activity varied considerably along a given nucleolar chromatin axis. Figs. 5 *c* and *d* present examples of axes in which “naked” (nontranscribed) stretches alternate with cistrons that are transcribed, though with increased spacing of the nascent RNP fibrils. A quantitative evaluation of the mean number of lateral fibrils per axial length is presented in Table II. Fibril-free nucleolar chromatin strands appeared frequently studded with dense, 80–150-Å large particles which possibly represent RNA polymerase molecules (for similar interpretation see references 50, 54, and 55). The occurrence of such particles, however, was not restricted to axial segments within matrix units (e.g., Fig. 5 *d*; see also below). The alternative interpretation of such particles as representing “nucleosomes” (for the diversity of sizes for nucleosomes see, e.g., references 56, 57) seems rather unlikely to us because of their rather irregular distribution in spacer intercepts and the lack of an inverse correlation of spacer intercept lengths with the number of associated particles (a detailed discussion is presented in W. W. Franke et al., manuscript in preparation).

VITELLOGENIC OOCYTES: Light and electron microscope autoradiographs of sections through “semi-isolated” nuclei (i.e. isolated but not completely freed from adherent cytoplasmic material) from [^3H]uridine-labeled vitellogenic oocytes revealed strong and preferential labeling of the peripherally located nucleoli (Fig. 6; cf. also references 43 and 48). When the fine structure of such nucleoli was examined in ultrathin sections, a progressive enlargement of the whole nucleolus as well as an increase in the relative proportion of fibrils contained in a loose package was noted, especially in the early phase of vitellogenesis,

together with the appearance of typical nucleolar granules. For detailed descriptions of the nucleolar ultrastructure in this well-studied stage of oogenesis, the reader is referred to the literature (e.g., references 43, 48, 52, 87, and 89). This oogenic stage apparently represents maximal transcriptional activity of cistrons for pre-rRNA (see above; cf. also references 16, 17, 19, 28, 49, and 73), a conclusion which is in accord with the observations in spread preparations of a maximal number of matrix units, all with maximal packing of lateral fibrils (Fig. 7; Table II), and which agrees with the findings of other authors (53–55; cf. also reference 73). While the vast majority of spread nucleoli of this stage showed maximal density of the matrix units, we occasionally noted “naked” intercepts on otherwise active nucleolar chromatin axes, the length of which suggested that an individual cistron was left untranscribed (cf. also reference 73). From evaluations of spread preparations of a very high number of oocytes (more than 300 oocytes 0.5–1.5 mm in diameter from different animals, all collected during the early breeding season, totalling more than 1,000 observed nucleolar units) we estimated that consistently 90%–95% of the possible cistronic regions appeared as complete matrix units (see also Table II). The rarely observed untranscribed cistronic regions, however, also showed associated granular particles similar to those tentatively interpreted as RNA polymerase molecules (for references, see above), and similar granules were also routinely observed in spacer intercepts (e.g., Fig. 7 *b*). The pattern of arrangement of such particles in both types of untranscribed regions of nucleolar chromatin, however, was somewhat irregular, although a tendency to local grouping was sometimes indicated (e.g., in the upper part of Fig. 7 *b*).

MATURE OOCYTES: The nuclear cytology of the late phase of amphibian oogenesis is char-

FIGURE 7 *a, b* Typical appearance of a fully transcribed nucleolus isolated from a mid-lampbrush stage oocyte (diameter 0.8 mm) of a newt collected during the breeding season, as revealed after spreading and positive staining. The abundance of complete matrix units (up to 400 in this specific nucleolar unit) is demonstrated (*a*). All genes seem to be transcribed. The particles containing the RNA polymerases are located at the bases of the tightly packed lateral fibrils and are seen with particular clarity after metal shadowing of the spread preparation (*b*). Similar particles are also recognized in the adjacent “spacer” intercepts of the axes, though at larger distances and much less regularly spaced (arrows). This technique, which facilitates the identification of the particles, results in a coarsening of the structures so that the particle dimensions are somewhat overestimated (apparent particle sizes from 160 to 250 Å). (*a*) $\times 6,000$; (*b*) $\times 32,000$. Scales indicate 2 μm (*a*) or 1 μm (*b*).



acterized by the formation of a central, spherical aggregate which contains the now contracted lampbrush chromosomes and two size classes of nucleoli—those with a diameter of about 2.5 μm , and larger nucleoli which in size and substructural aspects correspond to the few that remained in the periphery (Fig. 8). The large nucleoli incorporated tritiated uridine into RNA irrespective of their localization in the center or periphery of the nucleus (Fig. 8 *a-c*). Such uridine incorporation was noted even in fully mature oocytes approximately 2 days before ovulation (for similar statements in *X. laevis* see references 11, 42, 45, and 95). These autoradiographic results are compatible with the biochemical determinations of rRNA synthesis described above. On the other hand, the ultrastructure of such nucleoli, either peripherally or centrally located, was dominated by distinct zones of an extremely dense aggregation of nucleolar fibrils, indicative of far-reaching inactivation (*AF* in Fig. 8 *d*). Some vacuolizations, however, remained, and granular particles could still be encountered, albeit rarely (Fig. 8 *d*). In addition, we observed the formation of paracrystalline arrays of nucleolar material in fully mature oocytes (Fig. 8 *e*). It should be noted that this ultrastructural aspect differs in detail from that of the actinomycin D-inactivated nucleoli of lampbrush-stage oocytes (74).

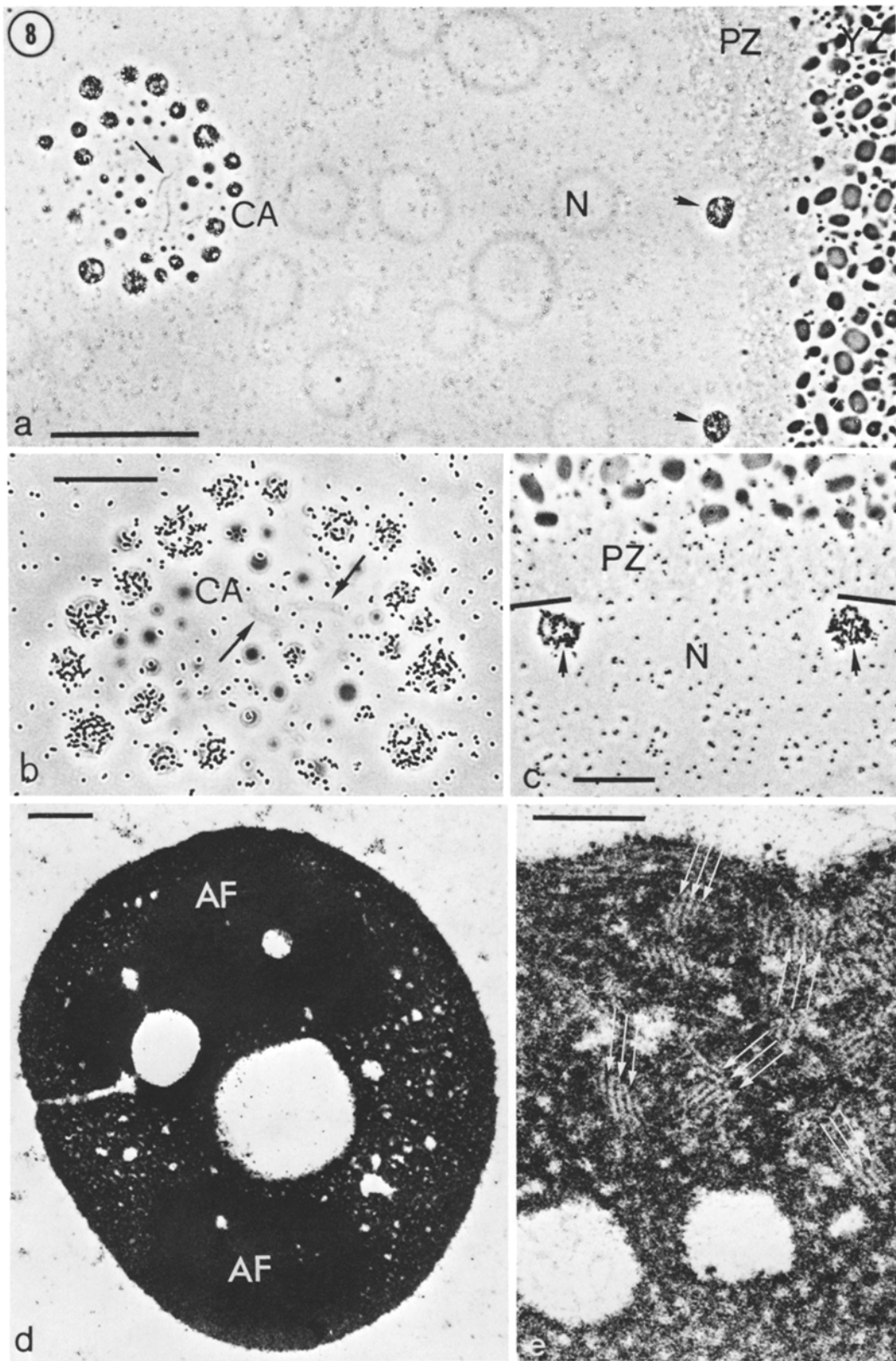
Spread preparations of nucleoli from mature oocytes revealed a widely heterogeneous pattern of transcriptional activity. The appearance of a moderate proportion of the nucleoli closely resembled that of nucleoli commonly found in the vitellogenic stage, i.e. uninterrupted series of complete or almost complete matrix units (e.g., Figs. 9, 10 *a*). This pattern was particularly common for the smallest identifiable molecular rDNA units of

nucleolar chromatin, i.e. distinct rings² which contained distinct numbers of cistrons (Fig. 9 *a* and *b* gives examples of circular units of four and seven repeating units, respectively). It is worth emphasizing that the degree of lateral fibril packing can be variable in the individual cistrons, even in a given nucleolar chromatin ring (Fig. 9).

Most of the nucleoli from mature oocytes showed the coincident occurrence of extended "naked" nucleolar chromatin axes and complete or almost complete matrix units in variable proportions (Figs. 10, 11). Situations such as those, for example, shown in the survey micrograph of Fig. 10 *b* are reminiscent of what has been described above for the nucleoli from previtellogenic stages and are especially interesting in the context of discussions of regulatory mechanisms of rDNA transcription, since they clearly demonstrate that in one and the same nucleolus some genes can be fully transcribed whereas others show no activity at all. In addition, a variety of matrix unit structures with diluted coverage of lateral fibrils (Figs. 11, 12) was noted, all indicative of a continuing but reduced transcriptional activity (for quantitative data on lateral fibril density, see Table II). The details of the widely

²The existence of similar ringlike rDNA units of variable lengths, which seems to be a general feature of amplified rDNA, has hitherto been demonstrated in preparations of isolated DNA in a variety of cell types such as oocytes of *X. laevis* (38, 64) and some Dytiscid beetles (33), the macronuclei of the ciliate *Tetrahymena pyriformis* (32), and in the slime mould *Physarum polycephalum* (71; R. M. Grainger, Yale University, personal communication). Corresponding rings of axes of nucleolar chromatin have also been observed in vivo in a transcriptionally active state in spread preparations of oocytes of *Xenopus laevis* (55) and the water beetle *Dytiscus marginalis* (83).

FIGURE 8 *a-e* Light microscope autoradiographs of nuclei of mature *T. alpestris* oocytes as revealed after labeling with [³H]uridine in vivo for 6 days (*a-c*), and electron micrographs of sectioned nucleoli of the same stage (*d, e*). One can distinguish peripheral large nucleoli (arrowheads in *a* and *c*), usually few in number, and a centrally located aggregate (*CA*; for higher magnification, see *b*) that consists of two distinct classes of nucleoli, large and small, and the now largely inactive lampbrush chromosomes (e.g., as denoted by the arrows in *a* and *b*). The silver grain distribution shows a significant enrichment over both sorts of large nucleoli, the peripheral and the central ones, in contrast to the absence of label in the small (central) nucleoli and the chromosome strands. The fine structure of the large central nucleoli in mature oocytes is demonstrated in *d* and *e*. Besides the densely aggregated fibrillar region (*AF*), small areas with paracrystalline arrays (arrows in *e*) are noted. *YZ*, yolk-containing zone of the cytoplasm; *PZ*, perinuclear zone of the cytoplasm; *N*, nucleoplasm. (*a*) \times 460; scale indicates 50 μm . (*b*) \times 800; (*c*) \times 630; scales indicate 20 μm . (*d*) \times 10,000, scale indicates 1 μm . (*e*) \times 35,000; scale indicates 0.5 μm .



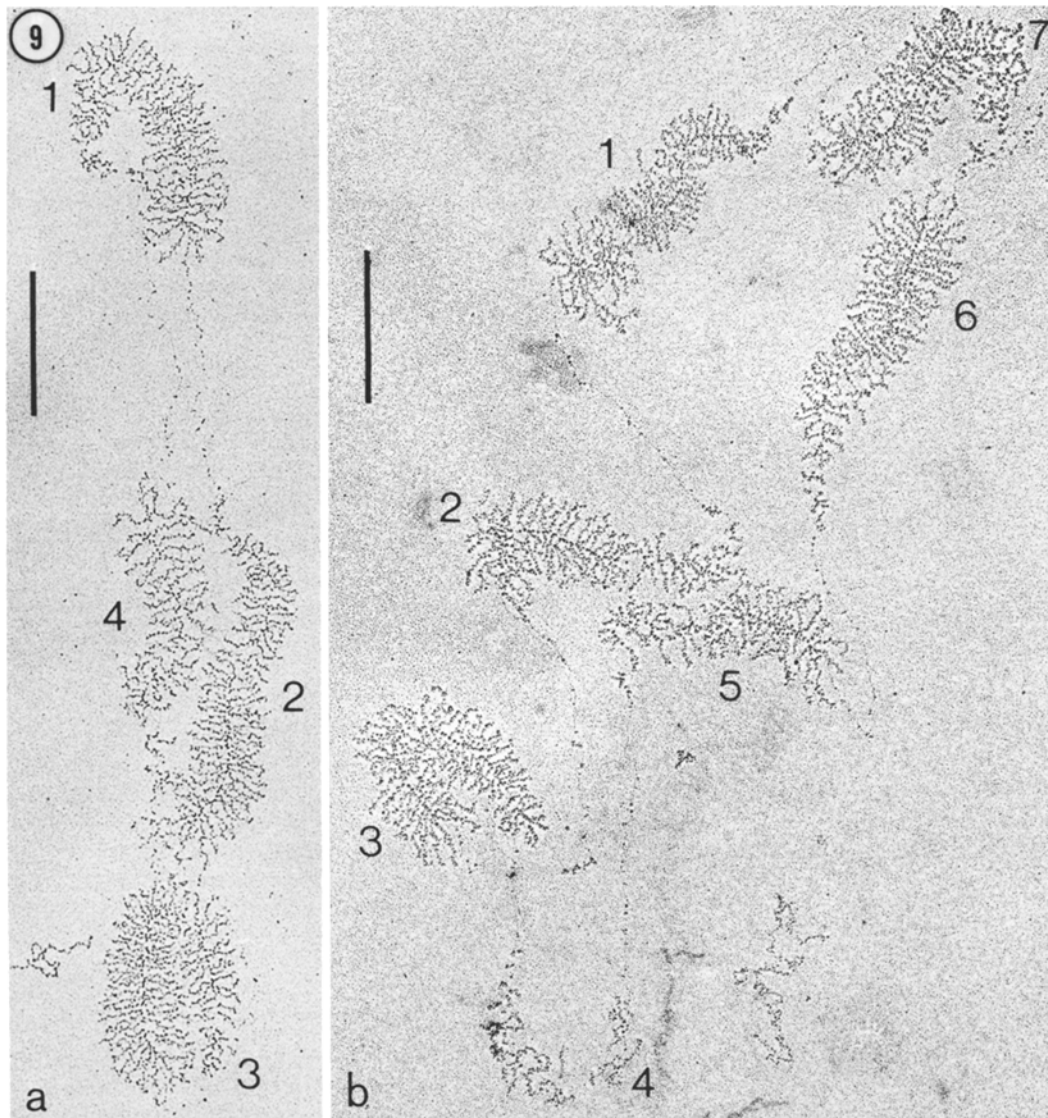


FIGURE 9 *a, b* Spread preparations of rings of pre-rRNA cistrons contained in nucleoli isolated from mature *T. alpestris* oocytes (1.6 mm in diameter). Four (in *a*) and seven matrix units (in *b*) are recognized, and all appear to be fully active, except for gene no. 4 in (*b*). (*a*) shows the normal appearance as revealed with the "Miller technique" whereas (*b*) presents the enhancement of some structural details due to shadow cast. Note the association of distinct granular particles with the "spacer" regions in (*b*). Numbers denote sequences of cistrons. (*a*) $\times 19,000$; (*b*) $\times 20,000$; scales indicate 1 μm .

varying pattern of the lateral fibrils within such incomplete matrix units are as described for previtellogenic oocytes (see above) and for actinomycin D-treated nucleoli of lampbrush-stage oocytes (74). The RNA polymerase-like granular particles (see above) were noted again in all three kinds of untranscribed regions: (*a*) the spacer intercepts; (*b*) the completely inactive ("silent") cistronic sec-

tions; and (*c*) in between the lateral fibrils of matrix units that show reduced activity (Fig. 12).

Measurements of the distances between adjacent RNA polymerase granules in diluted matrix units, including both free granules and those anchoring lateral fibrils, showed a clear tendency toward larger spacings as compared with the fully active state of the vitellogenic phase. They also indicated

a reduction in the total number of "particles" per cistron intercept, which perhaps reflects a dilution of the attached RNA polymerases (for related biochemical findings in *E. coli* cf. reference 20).

DISCUSSION

The present study illustrates that natural changes in transcriptional activity of nucleolar chromatin can be quantitatively assessed in the electron

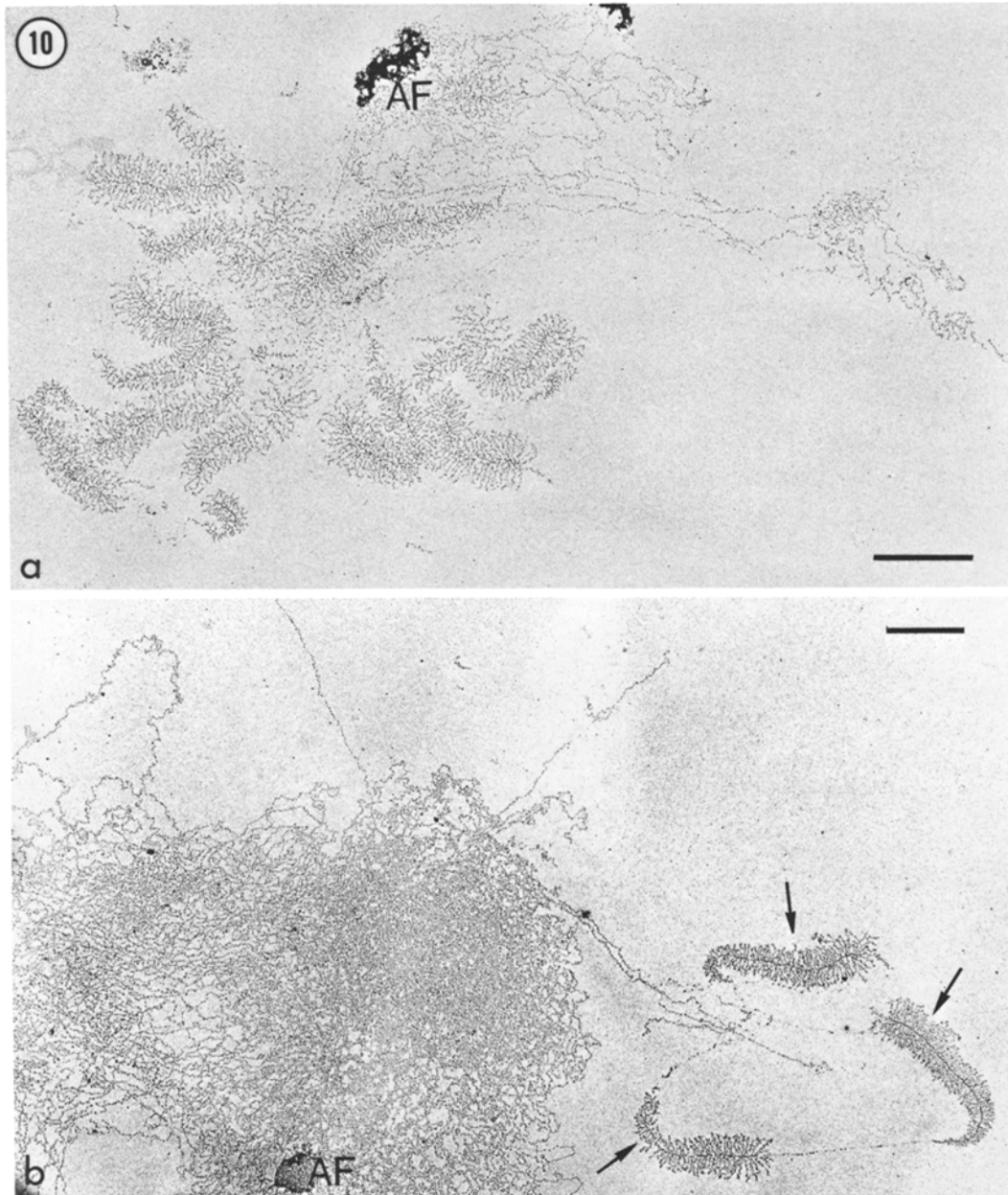


FIGURE 10 *a, b* Spread preparations of nucleoli from mature oocytes of *T. alpestris* (1.7 mm in diameter) demonstrating different degrees and patterns of reduction of transcriptional activity. In both figures, a large part of rDNA is not at all transcribed and appears in the form of "naked" axes which tend to occur as aggregated fibrils (AF in *a* and *b*). Some transcribed cistrons show a "dilution" of associated lateral fibrils (e.g., in *a*) whereas others (e.g., those denoted by the arrows in *b*) show still normal dense packing. (*a*) $\times 13,500$; (*b*) $\times 10,000$; scales indicate 1 μm .

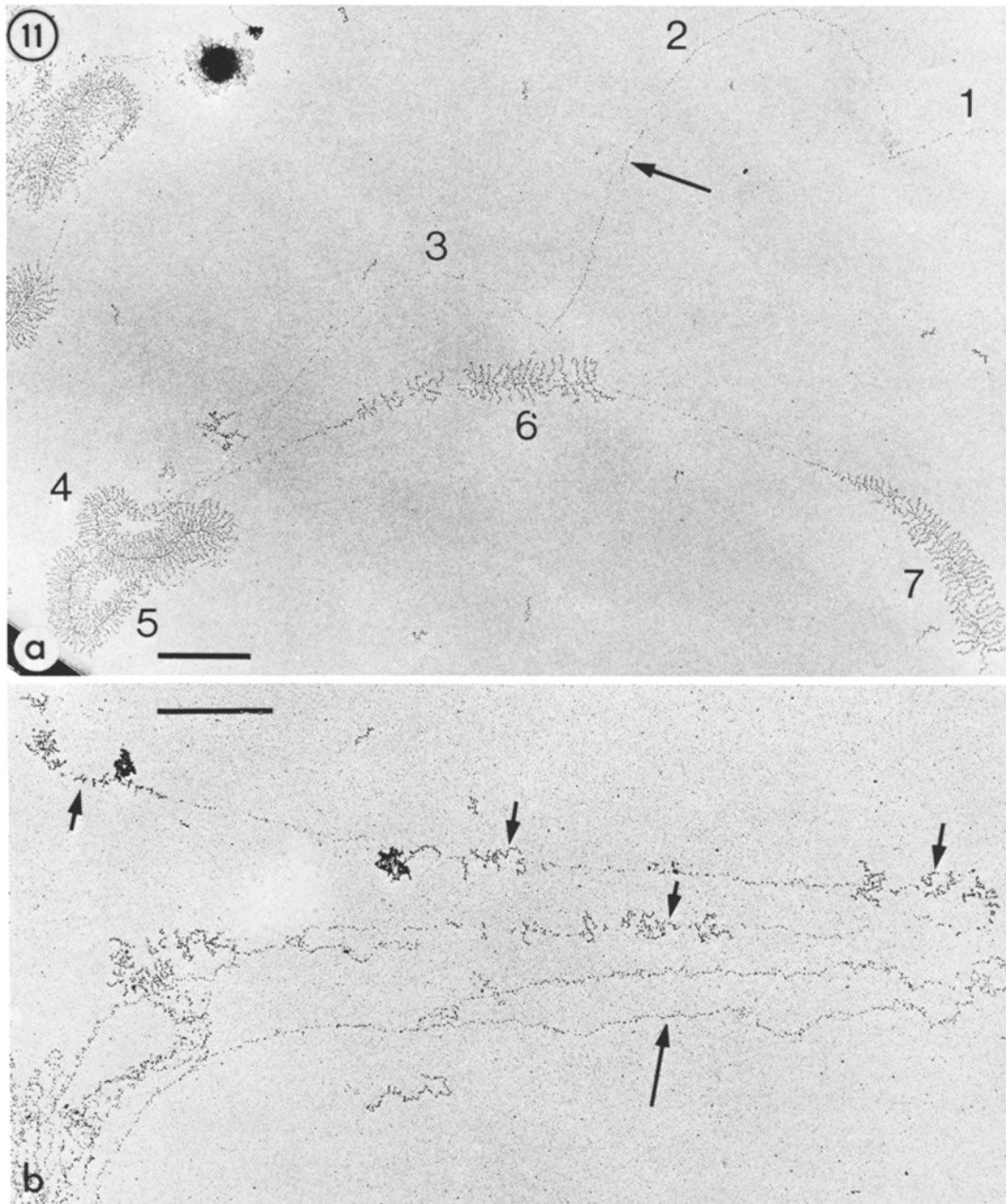


FIGURE 11 *a, b* Coincident occurrence of fully (*a*) and partially (*b*) transcribed cistrons of mature *T. alpestris* oocyte and inactive cistrons in adjacent stretches of nucleolar fibrils as revealed in optimally spread regions. The arrow in (*a*) denotes an axial strand with a series of "dormant" (nontranscribed) genes (nos. 1-3) in sequence with four almost fully transcribed genes (nos. 4-7). The arrow in (*b*) denotes strands with no transcriptional activity adjacent to many cistrons with incomplete matrix units (arrows) (*a*) $\times 13,000$; (*b*) $\times 16,000$; scales indicate 1 μm .

microscope by means of the spreading technique. In a previous publication, we discussed the possibility that "diluted" matrix units or the total absence of lateral fibrils in some individual cistron

regions as occasionally noted in lampbrush-stage oocytes from various amphibia (73) are due to the artificial removal of lateral fibrils during preparation. Although our present study does not rule out

that such artifacts may contribute to changes in the appearance of the matrix unit, the highly significant morphological changes occurring during amphibian oogenesis, which were well correlated with the biochemical results (see below; cf. also reference 74) and well reproducible using various modifications of the isolation media and the preparation procedures (for details see also refer-

ence 76), favor the notion that changes in the morphology of the matrix unit primarily reflect true changes in transcriptional activity.

From the combined biochemical, autoradiographic, and morphological data, we draw the following conclusions. (a) A marked modulation of the rate of rRNA synthesis occurs during oocyte development in *T. alpestris*. (b) Neither in previtel-

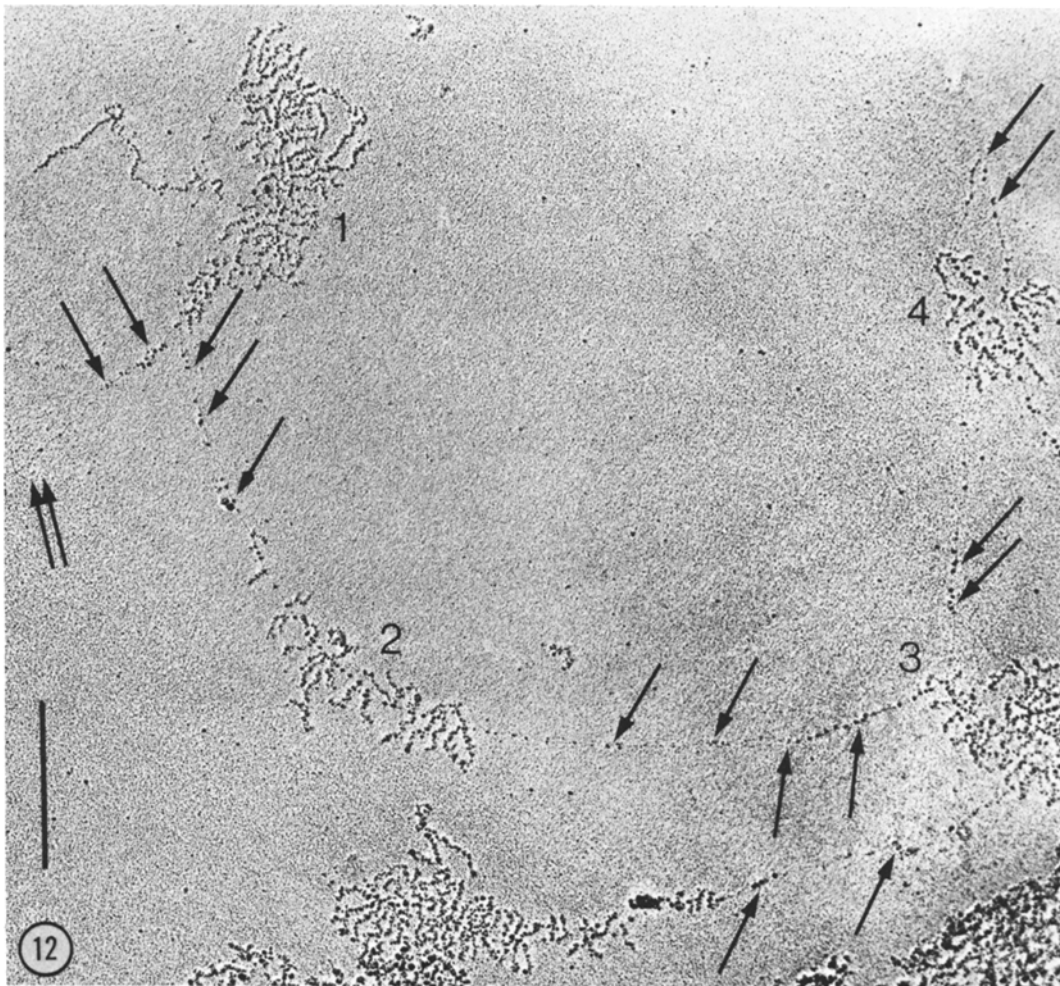


FIGURE 12 A series of rDNA repeating units (the beginning of one strand is denoted by the double arrow in the very left) as revealed in the electron microscope after isolating, spreading, and metal shadowing of nucleoli from mature *T. alpestris* oocytes (1.7 mm in diameter). On the axial fibril shown in the upper part, four successive pre-rRNA cistrons exhibit different morphology; cistron no. 1 appears normal, i.e. fully active; cistron no. 2 shows diluted associations of lateral fibrils; cistron no. 3 seems to be not transcribed at all; and cistron no. 4 is visualized as a matrix unit with a central "gap," i.e. absence of many lateral fibrils within the matrix unit. Note again that particles similar to those within the matrix units which probably contain the RNA polymerases are also existent in spacer regions and on untranscribed cistrons (arrows) without associated fibrils, although at lesser density and rather irregularly arranged. However, small groups of such particles with closest spacing are also frequently recognized in nontranscribed sections. $\times 22,000$; scale indicates 1 μm .

logenic (0.2–0.35 mm cell diameter) nor in mature stages is the synthesis of 28S and 18S rRNAs completely suppressed. It can be detected, though at reduced levels. We cannot exclude, however, that our data obtained for the mature oocytes represent maximal figures for this stage; seasonal changes may occur in all oogenic stages, and the specific activities may, for example, be much lower during hibernation. (c) The formation of 28S and 18S rRNA is regulated primarily at the transcriptional level. (d) The morphological manifestation of reduced transcriptional activities of cistrons of the pre-rRNA is similar in oogenic stages characterized by increasing or decreasing rRNA synthesis. From the data obtained with the different techniques (Table II), we further conclude that the overall transcriptional activity of the genes of the pre-rRNAs is correlated with the total number of RNP fibrils attached to nucleolar chromatin axes in the steady-state situation, provided the measurements included repeating units of different nucleoli in order to minimize possible errors introduced by the internucleolar heterogeneity and the demonstrated variations in lateral fibril packing. While this correlation is close in the later oogenic stages, the transcriptional activity calculated from spread preparations of nucleoli of previtellogenic oocytes is markedly higher than the corresponding value determined from biochemical experiments (Table II). This deviation may be due to an overestimation of axial intercepts covered by lateral fibrils relative to the "naked" regions since the latter tend to aggregate and thus are frequently difficult to trace, or to possible transcriptional activity in the spacers of the pre-rRNA genes (for transcriptional activity in such rDNA "spacer" intercepts see reference 73; cf. also references 76 and 84). On the other hand, this difference could also be explained by the assumption of some defective processing of the rRNA precursor molecules at this stage, so that a certain proportion of the pre-rRNA synthesized would not be incorporated into ribosomes (for an example of such post-transcriptional regulatory mechanisms see the introductory paragraphs).

The correlation between the mean number of nascent RNP fibrils in nucleolar chromatin and the rate of synthesis of 28S and 18S rRNA rules out the possibility that, at least during the later stages of amphibian oogenesis, ribosome biogenesis is regulated exclusively at a post-transcriptional level. Continuous production of pre-

rRNAs coupled with subsequent degradation of rRNA precursor molecules ("wastage"; cf. reference 12; for further references see introductory paragraphs) and/or enhanced turnover rates of cytoplasmic rRNA (for an example of increased half-life times of cytoplasmic rRNA in resting stages of cultured fibroblasts see references 1, 41, and 92) should lead to the appearance of the whole set of complete matrix units in stages of reduced rRNA accumulation. The results (Table II) also do not support the concept that the formation of ribosomal RNAs is exclusively regulated by changes in the rate of elongation of the nascent pre-rRNA chain (e.g., reference 30), with all potential genes and polymerases being permanently in a state of transcription. Marked differences in the rate of elongation among different matrix units are also unlikely since we found a rather homogeneous distribution of radioactivity in the matrix units as revealed by electron microscope autoradiographs of such spread preparations (preparative details are presented in Trendelenburg et al., manuscript in preparation).

rRNA cistrons in oogenic stages of low transcriptional activity typically display a striking morphological heterogeneity similar to that described for actinomycin D-inactivated nucleoli of this cell type (74). A schematic collection of the various possible basic alternatives in the appearance of partially inactivated matrix units is presented in Fig. 13. Occasionally, complete or almost complete matrix units are followed by totally untranscribed cistrons (case B in Fig. 13; the "normal" situation of fully active matrix units arranged in tandem as found in vitellogenic oocytes is sketched under A). A reduction in the number of lateral fibrils simultaneously present on a pre-rRNA gene results either in a nearly homogeneous dilution (see gene no. 1 in panel C of Fig. 13; for reports of similar observations in the rRNA loci of chromosomes of *E. coli* grown under suboptimal conditions, see reference 36) or in distinct "gaps" within matrix units otherwise well covered with fibrils (case C, gene no. 2 in Fig. 13). A high degree of variability in the transcriptional activity among adjacent genes, i.e. that complete and dilute matrix units are found on the same axis (Fig. 13, case D), is another alternative in structural change. All of these various aspects were observed in clear examples in previtellogenic and mature oocytes of the alpine newt. In our opinion, the demonstration of structures corresponding to those cases sketched

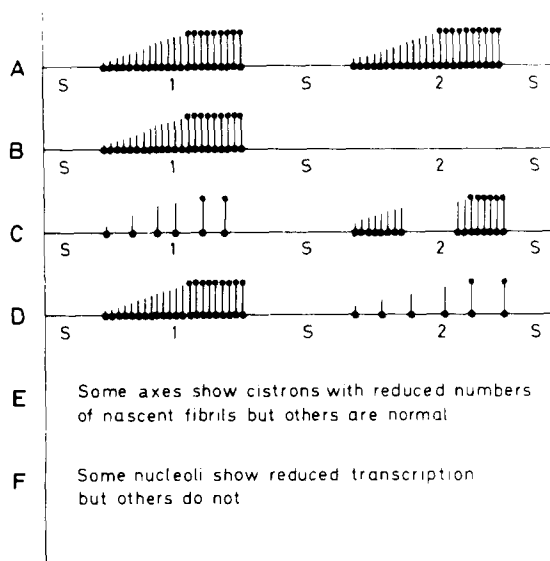


FIGURE 13 Schematic presentation of various alternatives in morphological changes correlated with reduced transcriptions of rDNA. Cistron regions are indicated by the numbers and are separated from each other by spacer intercepts (S). For explanation and discussion, see text.

in B and D in Fig. 13 is particularly important in that it shows that the very type of regulation of the transcription of genes of pre-rRNA is specific for the individual genes but not principally general for the given species, cell type, nucleus, nucleolus, and chromatin strand (see also below). The possibility sketched in E (Fig. 13), namely, that the reduction is restricted to some axial strands of nucleolar chromatin within a specific nucleolus, was only rarely suggested in our spread preparations (see, however, reference 50). The pattern illustrated in F (Fig. 13) was occasionally indicated in previtellogenic and mature oocytes, suggesting some, albeit not absolute (see above) nucleolar autarchy of transcriptional regulation, but was not a dominant one as demonstrable by the rather homogeneous labeling of the nucleoli in the autoradiographs.

The activation and inactivation of the pre-rRNA genes is apparently not an "all or none" process but is regulated by the frequency of initiation events by the RNA polymerases in a mode that is specific for the individual gene. Our data are not in accord with the conclusions of Perry and Kelley (60; see also, however, reference 59) who interpreted the high sensitivity of eucaryotic rRNA genes to low doses of actinomycin D as being due to a common "promotor-like" region

that governs the regulation of a whole set of genes. Our data rather are in accord with the report of Hackett and Sauerbier (35) that each rRNA gene in mouse L cells has its own "promotor" locus. Provided the axis-attached particles do represent RNA polymerase A molecules (for nomenclature and biochemical properties of this enzyme see references 2, 3, 65, 66, 81, 90, and 93), it might further be concluded that not all polymerase particles attached to the axes of diluted matrix units are associated with lateral fibrils. The distribution of these particles, then, would further indicate that the binding of polymerase molecules neither is specific for template regions nor results necessarily in the transcription of template sequences.

It is evident from the investigations of Roeder (66) that the levels of the specific RNA polymerase activities are not correlated with the specific degrees of transcriptional activity but that the enzymes are present in excess during all stages of amphibian oogenesis. Thus, the quantitative regulation of transcription of rRNA genes is presumably controlled by regulatory factors, probably of proteinaceous nature (14), which interact either with RNA polymerases or directly with the template (for an example of factors presumed to regulate gene activity in eucaryotes, see references 5, and 68).

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