ELECTRON MICROSCOPIC EXAMINATION
OF THE SITES OF NUCLEAR RNA SYNTHESIS
DURING AMPHIBIAN EMBRYOGENESIS

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ABSTRACT

The site of H^3-uridine incorporation and the fate of labeled RNA during early embryogenesis of the newt *Triturus pyrrhogaster* were studied with electron microscopic autoradiography. Isolated ectodermal and mesodermal tissues from the embryos were treated in H^3-uridine for 3 hours and cultured in cold solution for various periods before fixation with OsO_4 and embedding in Epon. At the blastula stage, the only structural component of the nucleus seen in electron micrographs is a mass of chromatin fibrils. At the early gastrula stage, the primary nucleoli originate as small dense fibrous bodies within the chromatin material. These dense fibrous nucleoli enlarge during successive developmental stages by the acquisition of granular components 150 A in diameter, which form a layer around them. Simultaneously larger granules (300 to 500 A) appear in the chromatin, and they fill the interchromatin spaces by the tail bud stage. Autoradiographic examination has demonstrated that nuclear RNA synthesis takes place in both the nucleolus and the chromatin, with the former consistently showing more label per unit area than the latter. When changes in the distribution pattern of radioactivity were studied 3 to 24 hours after immersion in isotope at each developmental stage, the following results were obtained. Labeled RNA is first localized in the fibrous region of the nucleolus and in the peripheral region of chromatin material. After longer culture in non-radioactive medium, labeled materials also appear in the granular region of the nucleolus and in the interchromatin areas. Further incubation gives labeling in cytoplasm.

INTRODUCTION

During the development of the amphibian embryo, the nucleoli first become visible at the time of gastrulation (11, 14, 24, 51) and gradually assume a typical reticular structure ("nucleolonema") (12, 24). However, no precise information has been obtained in regard to the mode of appearance and structural elaboration of the nucleolus in this cell type on a fine structure level. The formation of the nucleolus at a certain chromosomal region has been generally accepted by researchers using plant and mammalian cells (for review see Sirlin, references 42, 43). A number of electron microscope studies using various types of cells describe the behavior of the nucleolus during mitosis (30, 47), growth (20, 35), and differentiation (12, 24, 26). These studies also suggest a close topographical relation between nucleolar and chromosomal components. However, information on the manner in which the nucleolus originates is less detailed.

During gastrulation of the amphibian embryo,

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RNA synthesis is enhanced and ribosomal RNA synthesis becomes detectable (5–8). At the same time, the appearance and the increase in amount of many enzymes have been suggested (for review see Brachet, reference 3). A possible relationship between the nucleolus and ribosomes has also been suggested by several approaches, including electron microscopy (12, 15, 24, 26, 39, 46, 48), base composition analysis (13), and autoradiographic studies (38, 40, 43). The close correlation of the time of ribosome formation (5, 6, 8, 23) with the first appearance of a definitive nucleolus in the amphibian embryo (24) supports this hypothesis. More recently, a study by Brown and Gurdon (7) using an anucleolate mutant of *Xenopus* indicates that the presence of a nucleolus is necessary for synthesis of ribosomal RNA.

It has been repeatedly demonstrated in a wide variety of cell types that the initial labeling of RNA with radioactive precursors takes place in the nucleus (for review see Prescott, reference 40). Some autoradiographic studies (2, 4, 41, 44, 50, 52) on amphibian embryonic cells also implicate the nucleus itself as the main site of RNA synthesis throughout embryogenesis. A pattern of nuclear labeling with a heavy grain count over "nucleolus-associated chromatin" and nucleolus is reported, by Sirlin (41) and Sirlin and Elsdale (44), to occur in the larval stage when muscle differentiation is just beginning. However, no information has been

provided as to the precise localization of RNA-synthesizing sites in the nuclear components during amphibian embryogenesis. The recent development of electron microscopic autoradiography (9, 10, 17–19) permits the study of fine structural features of cellular synthetic activity which cannot be resolved with light microscopic autoradiography. In this study, labeling with H^3-uridine followed by high resolution autoradiography has been used to observe the sites and modes of nuclear RNA synthesis during embryogenesis of *Triturus pyrrhogaster*. Special attention has been given to the possible relation of the formation of the nucleolus and other RNA-containing components to the chromatin material in embryonic cells.

According to autoradiographic studies by Bieliavsky and Tencer (2), exogenous uridine is utilized preferentially for DNA synthesis during cleavage and for RNA synthesis during gastrulation and neurulation. Recently, Brahma and Yamada (4) have demonstrated that ectodermal cells of *Triturus pyrrhogaster* can for the most part incorporate tritiated uridine to synthesize RNA beginning in the early gastrula stage. The present autoradiographic analysis was performed from the gastrula stage on.

MATERIAL AND METHODS

Ectodermal and mesodermal cells of embryos of the newt *Triturus pyrrhogaster* were utilized in the present

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All figures are autoradiographs of thin sections of *Triturus* embryonic explants labeled with tritiated uridine. Duration of the treatment with tritiated uridine was 3 hours for all explants. After the treatment the explants were cultured in non-radioactive medium for various time intervals. Fixed with OsO₄ and embedded in Epon. Grains of Ilford L4 emulsion were developed in Microdol-X. Stained with alkaline lead.

Explanation of Figures

C, cytoplasm
CH, chromatin
F, fibrous component of nucleolus
G, granular component of nucleolus
IG, interchromatin granule
M, mitochondrion
N, nucleus

NA, nuclear annulus
NM, nuclear membrane
NU, nucleolus
P, pigment granule
S, developed silver grain
Y, yolk platelet

FIGURE 1   Portion of an ectodermal cell from the gastrula (stage 11), cultured for 3 hours after treatment with tritiated uridine. The chromatin components are found as strands and clumps. Within the chromatin clumps, nucleolus-like bodies can be found (arrows). Silver grains are found over the peripheries of chromatin clumps and the primary nucleoli. 21 days' exposure. × 12,000.
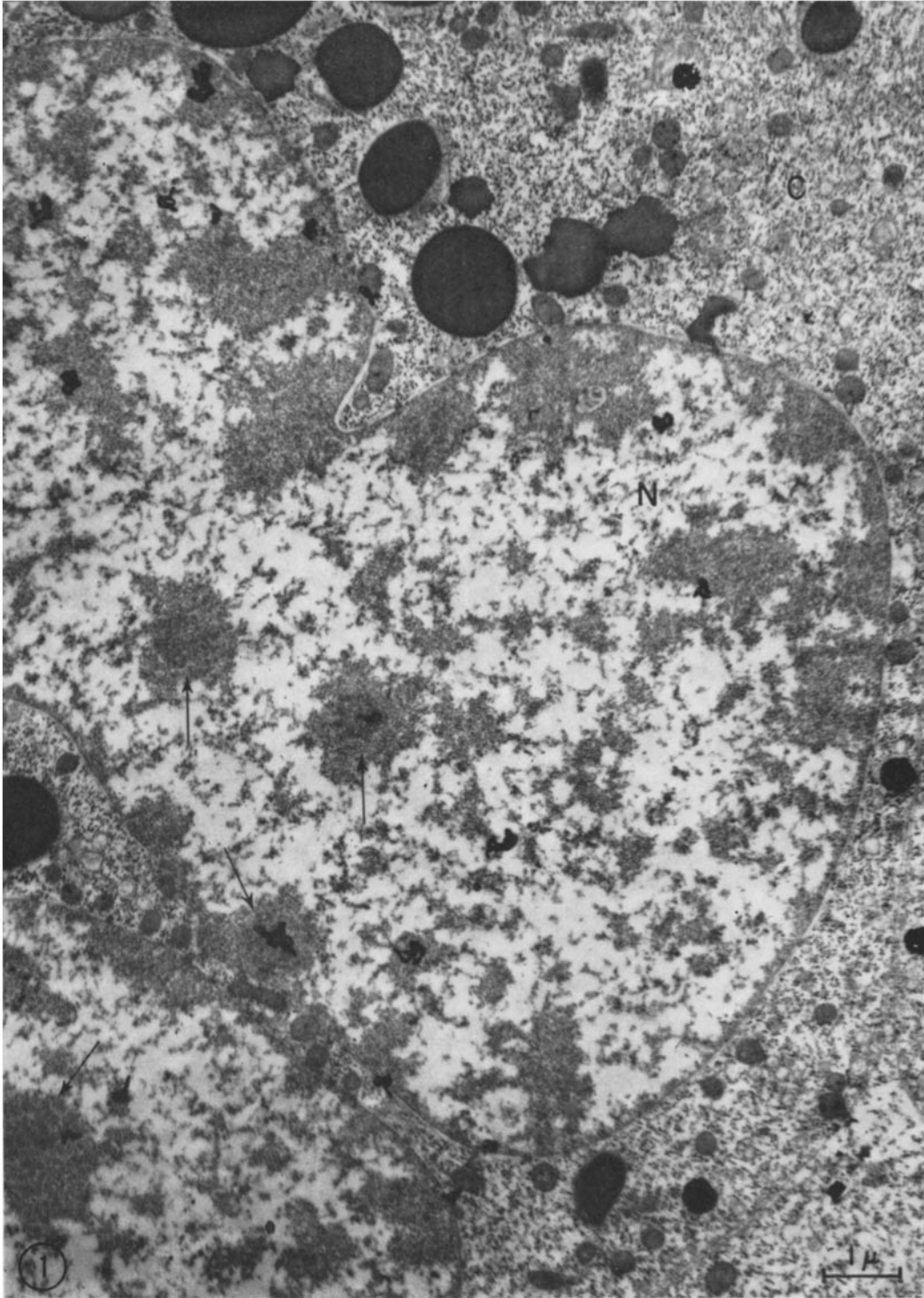
study. Experiments on the developing ectoderm were performed with the following series of tissues: presumptive ectoderm of the late blastula stage (Okada and Ichikawa (36), stage 10, 3-day embryo at 18°C); presumptive ectoderm of early gastrula stage (stage 11, 4-day embryo); optic vesicle of the tail bud stage (stage 26, 12-day embryo); and optic cup of forelimb bud stage (stage 35, 17-day embryo). The following series of mesodermal tissues was examined: dorsal lip (presumptive mesoderm) of stage 11, somites of stage 26, and trunk muscle of stage 35. In these two series of embryonic tissues, each tissue described is derived from the foregoing tissue of the earlier stage. Hence the study of each series should reveal progressive developmental changes of an ectodermal or mesodermal cell population, and comparison of the two series should indicate germ layer-specific and stage-specific changes. In earlier stages, each tissue was isolated and fixed; in the later stages a piece of embryo containing the tissue in question was isolated and fixed.

Isolated tissues or body parts were treated, with frequent agitation, in Holtfreter solution containing 50 μc/ml H³-uridine (New England Nuclear Corp., specific activity 3.89 c/mmole) for 3 hours at 18°C. Some of the explants were treated in the same solution for 30 minutes. They were then rinsed twice (each rinse lasting 2 minutes) and transferred in excess Holtfreter solution without tracer. After different periods (0, 1, 3, 6, 21 hours) of immersion in non-radioactive culture medium, a portion of each series was fixed for 40 minutes with 2 per cent osmium tetroxide in Holtfreter solution buffered with M/14 Veronal-acetate buffer at pH 7.4 (25). The fixed materials were rinsed in Holtfreter solution, passed through one change each of 50 and 70 per cent ethanol, and kept in cold 70 per cent ethanol overnight. After dehydration in an ethanol series, specimens were embedded in Epon 812 resin (33).

Ultrathin sections (0.03 to 0.05 μ) for electron microscopy were cut out with a diamond knife on a Porter-Blum microtome. Thin sections were mounted on Athene type 150-mesh grids coated with a collodion-carbon layer.

For high resolution autoradiography, the emulsion should form a monolayer of silver halide crystals, closely and uniformly packed over the specimens (9). An "expandable loop" method, developed in collaboration with O. L. Miller, Jr., was used to make the thin emulsion films. Two to five grids were affixed to a microscope slide by their edges with "double stick" Scotch tape. The expandable loop is made simply by attaching one end of a piece of nickel-chrome alloy wire to the outside of the tip of a disposable syringe and by pulling the other end through the tube. The diameter of the small loop thus formed can be increased up to 30 to 50 times by manipulating the free end of the wire. Subsequent steps are performed in a darkroom with a Kodak Wratten series OC safelight. Ilford L4 nuclear track emulsion is melted in a bath at 45°C and cooled to near 30°C. The loop, drawn to its minimum diameter, is dipped into the emulsion, withdrawn, and slowly expanded until the emulsion film in the wire loop displays

FIGURE 2 Small portion of an interphase nucleus in the presumptive ectoderm from the gastrula (stage 11). Fixed immediately after isotope treatment. The primary nucleolus seems to be a compact mass of fibrous material. The chromatin fibrils are connected with the primary nucleolus (arrows). No silver grains are seen over the primary nucleolus. 14 days' exposure. × 30,000.

FIGURE 3 Organized nucleolus in the explant from the tail bud embryo (stage 26). Fixed after 21 hours' culture. The nucleolus consists of a central fibrous part surrounded by a granular part. The material of the fibrous part has a close similarity to the main component of the primary nucleolus shown in Fig. 2. The granular part is obviously made up of uniform particles 150 A in diameter. Many interchromatin granules (arrows) 300 to 500 A in diameter are intermingled with the chromatin fibrils. Three silver grains over the granular area. 14 days' exposure. × 30,000.

FIGURE 4 Reticulated nucleolus in the explant from the forelimb bud embryo (stage 35). Fixed after 6 hours' culture. The nucleolus exhibits two distinct parts: a dense fibrous and a granular part. The central part of the nucleolus is composed exclusively of fine-textured material. The granular components of the nucleolus are opaque particles having an average diameter of 150 A. Numerous clusters of interchromatin granules (300 to 500 A) are distributed throughout the nuclear area (arrows). Silver grains occur on boundaries between the fibrous and granular parts. 14 days' exposure. × 30,000.
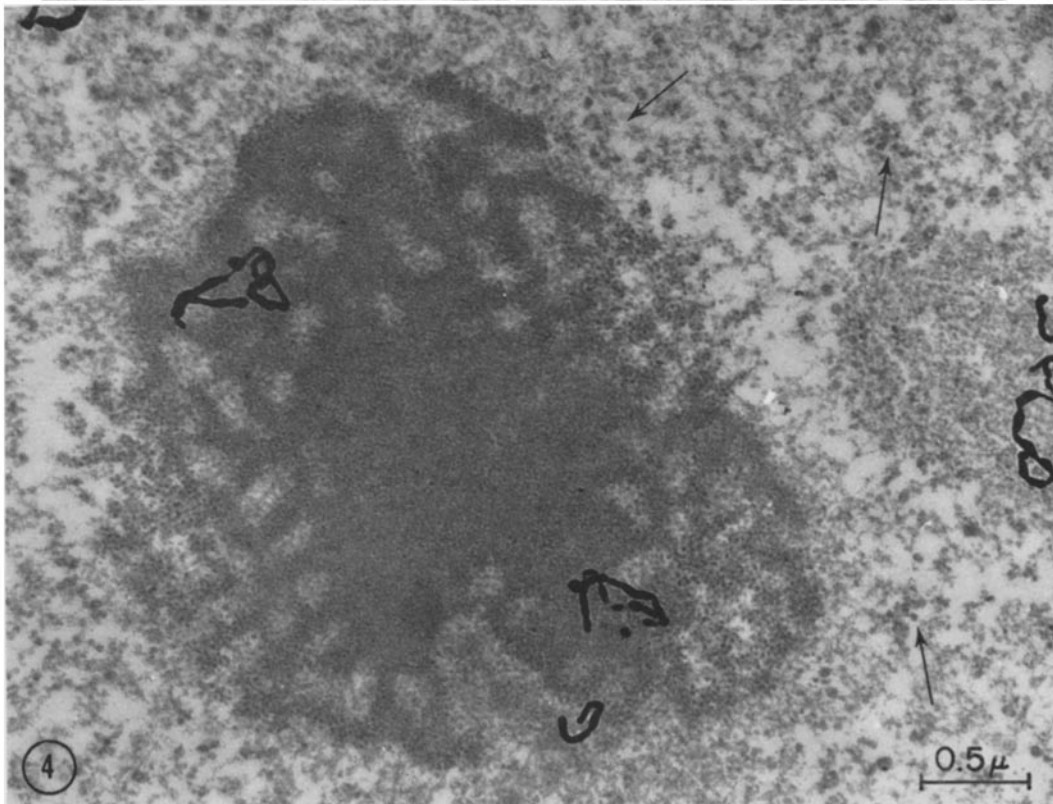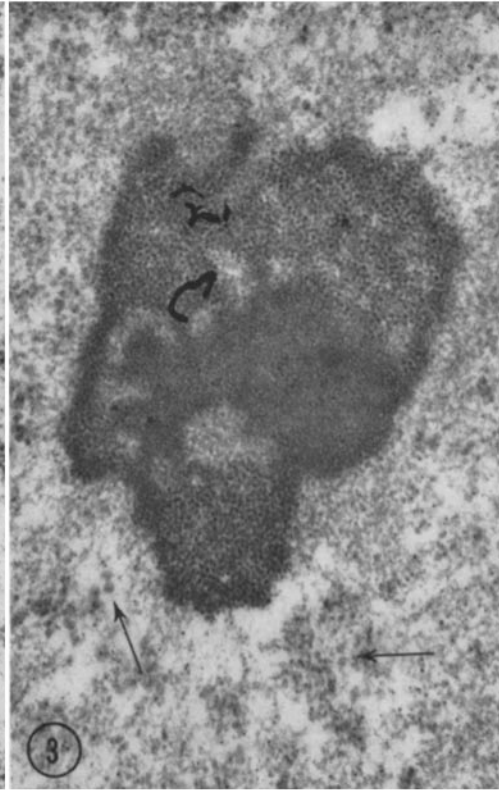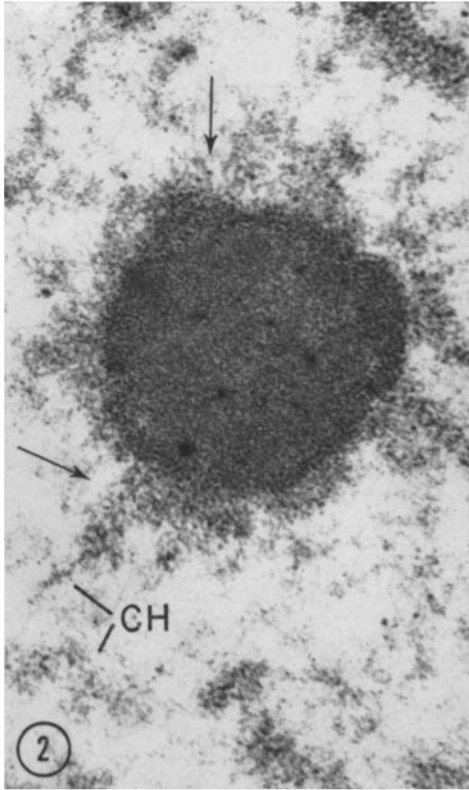
low-order interference colors. Within a few seconds the interference colors suddenly brighten, indicating that the film has gelled. A portion of the film showing light gold interference color (indicating about 0.1 μ thickness) is applied to the surface of the grids. This technique regularly produces film of even thickness and even grain distribution. The grids were stored in light-tight slide boxes containing a small pack of Drierite and exposed for 2 to 8 weeks. The grids were then removed from the adhesive and the autoradiographs were photographically processed by floating upside down on drops of developer (Kodak Microdol-X solution for 3 minutes) and fixing (Kodak Acid Fixer, 3 minutes), followed by rinsing in distilled water. Sections were usually stained with alkaline lead in 0.03 N NaOH for 15 minutes (29), then washed with "CO_2-free" distilled water.

Observation and photographic recording of the thin sections was done with an RCA EMU-3E microscope and a Siemens Elmiskop I. Most of the micrographs were taken at an initial magnification of X4000 and were enlarged to X12,000 on Kodabromide photographic paper (8 by 10 inches). These electron micrographs were used for quantitative analyses of the sites of incorporation into RNA as well as for observation of fine structure. Measurements of nuclear and nucleolar areas were made from the same electron micrographs with the aid of a planimeter. The developed grains of the autoradiographs appear as single filaments of metallic silver in the electron micrographs. However, it is not possible to determine which end of a filament was the starting point in the original latent image produced by the tritium-emitted beta particle. In this study, the cytological site of a decayed tritium has been arbitrarily designated as the median point on a line drawn between the two ends of a silver grain filament.

In order to determine the specificity of labeling with tritiated uridine in this system, some control experiments were performed. To remove unincorporated precursors, some of the OsO_4-fixed explants were subjected to extraction with 5 per cent trichloroacetic acid at 3°C for 10 minutes. After the routine procedures of preparation, thick sections were studied with light microscopic autoradiography. Comparison of acid-treated samples with those not subjected to the treatment has confirmed that there

is no significant difference in number and distribution of the silver grains. Hence, it is assumed that labeled compounds in the present autoradiographs are nucleic acids.

During the early stages of amphibian development, exogenous uridine is incorporated into DNA (2). Hence, it is necessary to decide the extent of incorporation of radioactivity into RNA and DNA in the present study. The ectodermal and mesodermal explants at each developmental stage were exposed to H^3-uridine for 3 hours, immediately fixed in ethanol–acetic acid, and sectioned in methacrylate. After removal of the resin, thick sections were treated with 0.01 per cent ribonuclease (pH 7.0) for 2 hours and covered with Ilford L4 liquid emulsion. The autoradiographs were examined with the light microscope. At embryonic stage 10, only weak reduction of grain density was detected during digestion by RNase. At stage 11, treatment with RNase caused more than 70 per cent reduction of grains in most explants. At stages 26 and 35, almost complete removal of grains could be attained with RNase. The present autoradiographic examination with the electron microscope was performed on the ectodermal and mesodermal cells of embryonic stages 11, 26, and 35. It is confirmed, therefore, that at these stages the distribution of radioactivity may reflect mostly that of synthesized RNA.

RESULTS

Morphology

The fine structure of the interphase nucleus during successive stages of development in the *Triturus* embryo after osmium tetroxide fixation and methacrylate embedding has been described previously (24). Epon embedding allows a better preservation of the fine structure of nuclear components in the same material.

In presumptive ectoderm and mesoderm cells of embryonic stages 10 and 11, the chromatin material is rather unevenly and sparsely distributed throughout the interphase nucleus (Figs. 1 and 5). As shown in Figs. 2 and 6, the clumped chromatin consists predominantly of a fine-tex-

FIGURE 5 Portion of a mesodermal cell from the dorsal lip of the gastrula (stage 11). Fixed after 3 hours' culture. Chromatin components are unevenly and sparsely distributed. A primary nucleolus (*NU*) is surrounded by the chromatin components. Primary nucleoli and chromatin clumps are labeled. 21 days' exposure. X 15,000.

FIGURE 6 Enlargement of a part of Fig. 5. Four silver grains lie over the primary nucleolus (*NU*). Note the chromatin fibrils connected with the nucleolus (arrows). X 50,000.
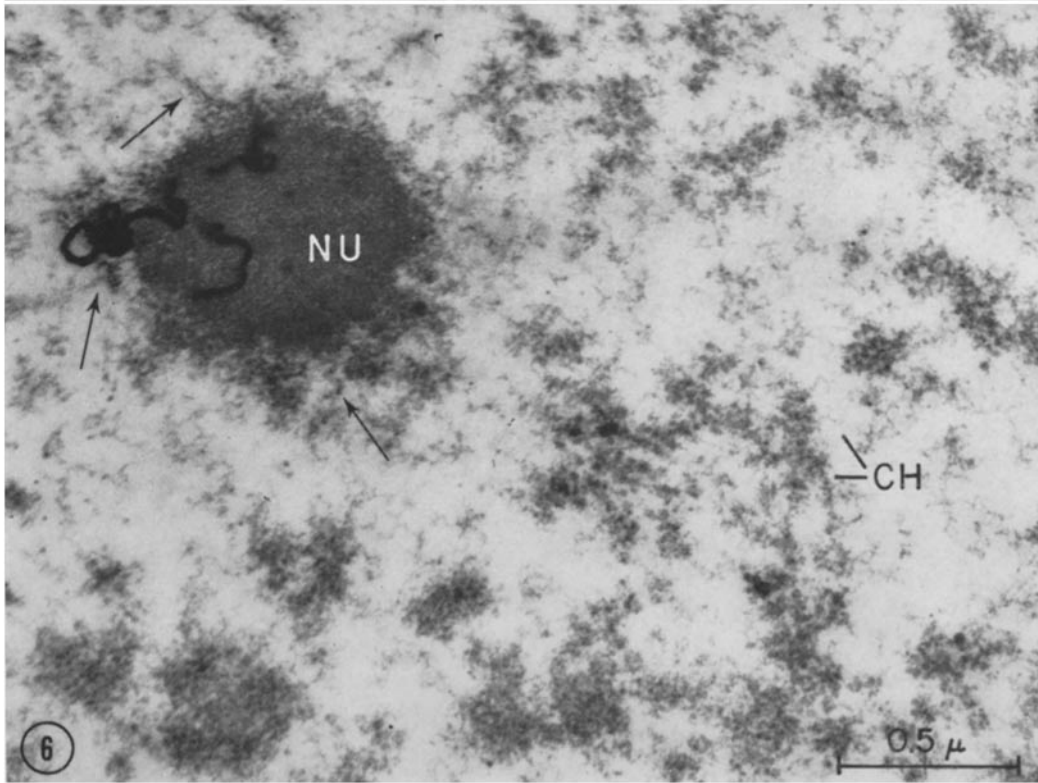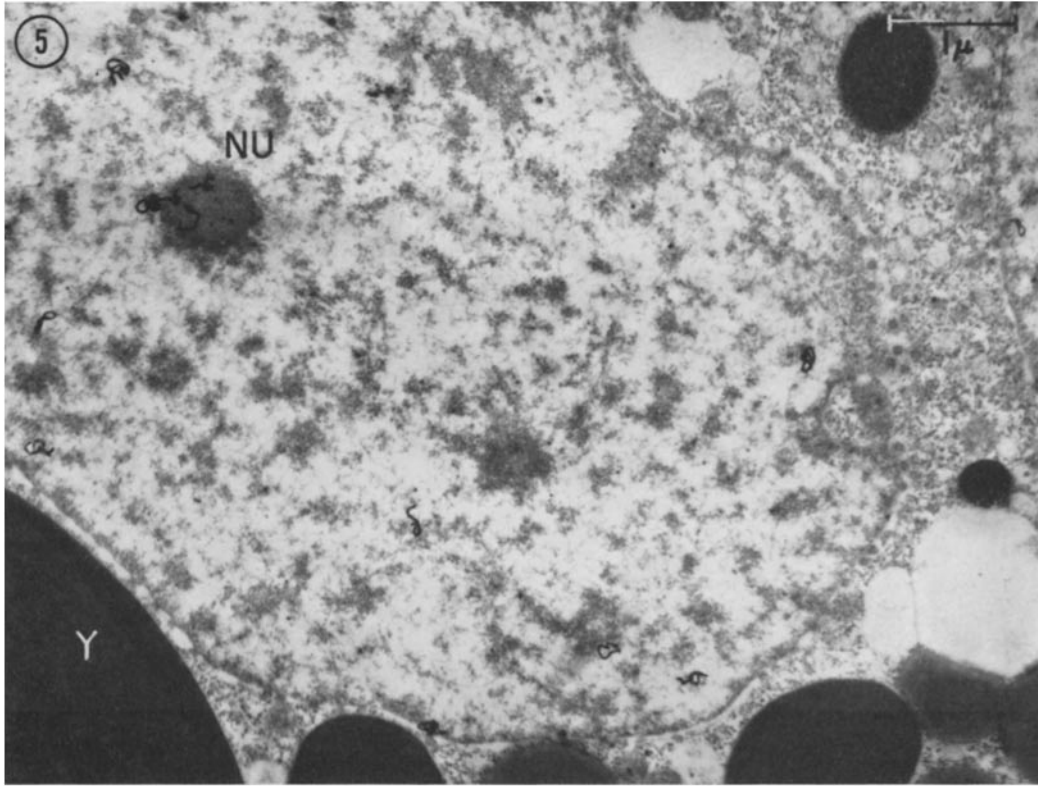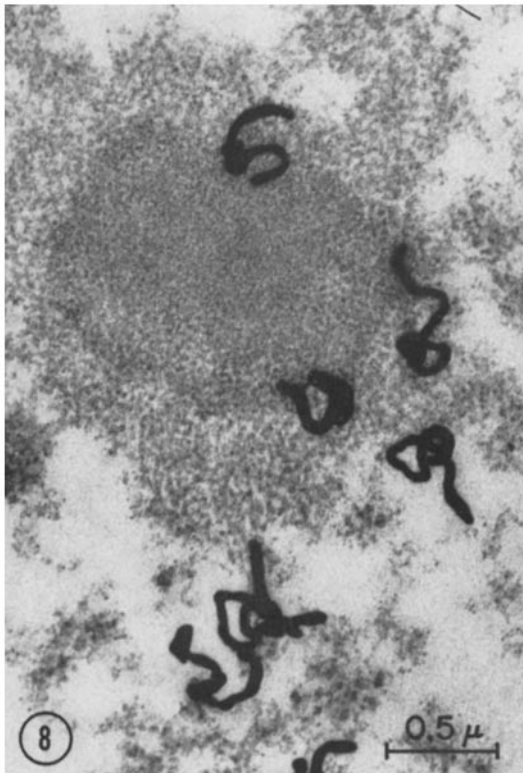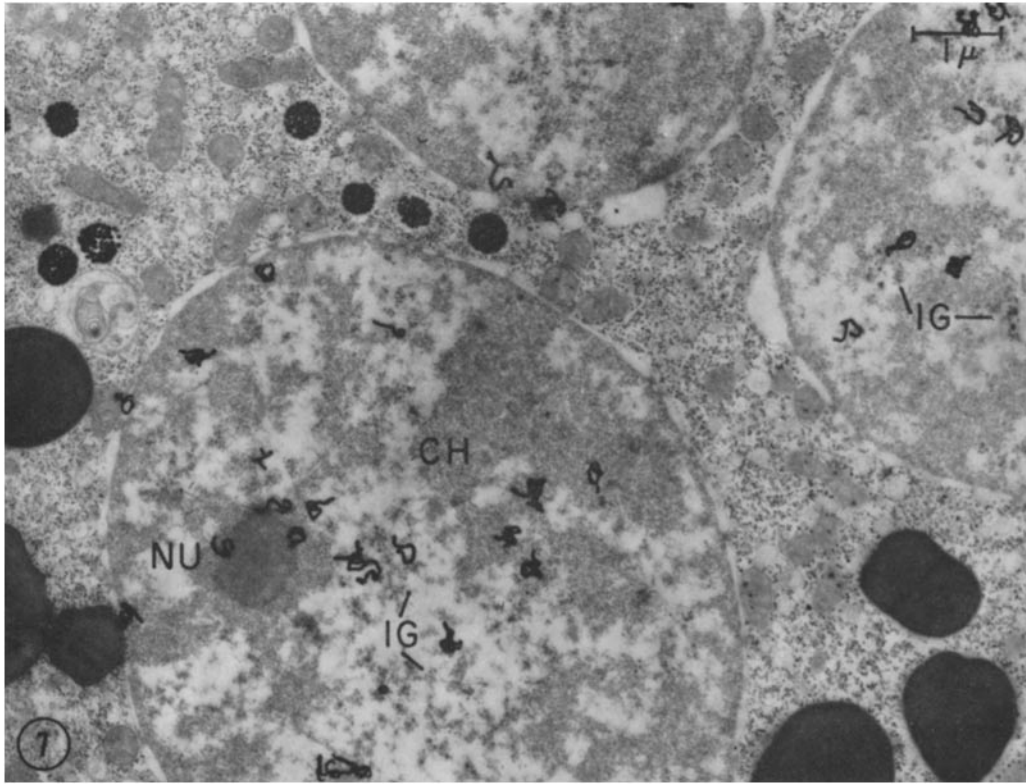
FIGURE 7 Portion of an ectodermal cell from the gastrula (stage 11), cultured for 21 hours after isotope treatment. The chromatin component (*CH*) is disposed in distinct clumps. Within the interchromatin regions, opaque interchromatin granules (*IG*) are dispersed. Silver grains are observed over the nucleolus, periphery of chromatin clumps, and interchromatin spaces. 21 days' exposure. × 12,000.

FIGURE 8 Enlargement of a part of Fig. 7. Three silver grains are found over the peripheral granular region of the nucleolus. × 30,000.
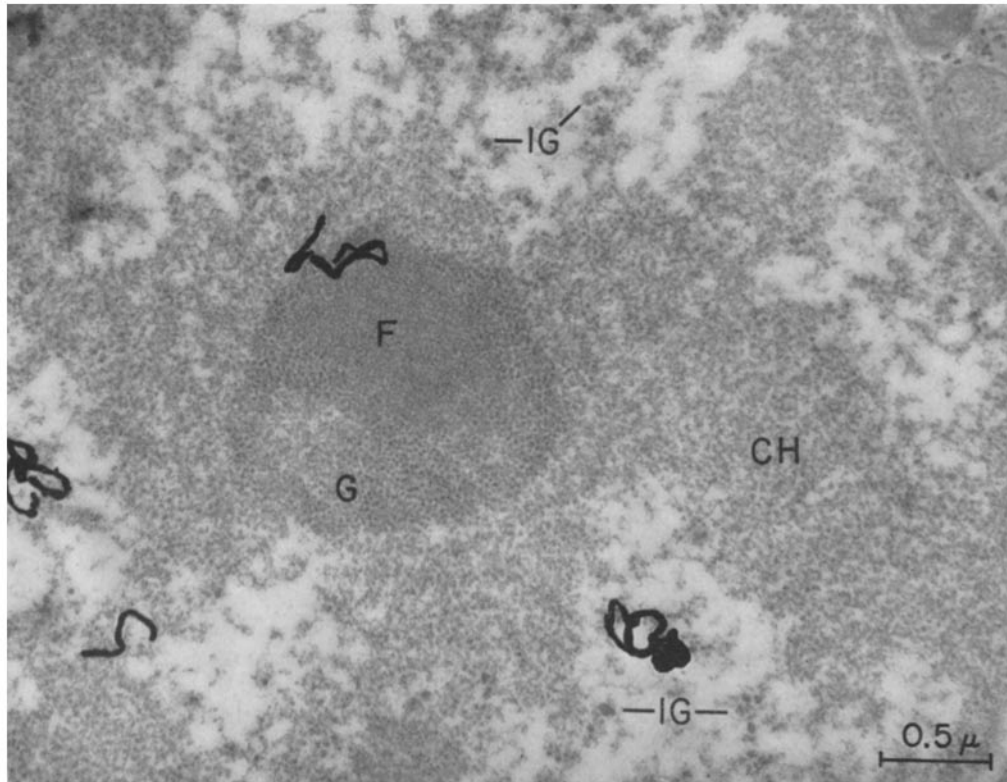
FIGURE 9 Portion of an interphase nucleus in the ectodermal explant from the gastrula (stage 11). Fixed after 21 hours' culture. The nucleolus, which is surrounded by associated chromatin (*CH*), consists of a fibrous region (*F*) and a granular region (*G*). Within the nucleoplasm, some interchromatin granules (*IG*) can be seen. 21 days' exposure. × 30,000.

tured component. The fine-textured component probably corresponds to convoluted fibrils 30 to 100 A in diameter. It will be referred to here as *chromatin fibrils*. This material appears in general to be similar to the component observable within metaphase chromosomes (Figs. 10 and 11). In the clumps of chromatin, a few dense particles larger than 200 A (interchromatin granules?) make intimate contact with the chromatin fibrils (Figs. 2 and 6). As for the interchromatin area, it contains loosely distributed convoluted fibrils which can hardly be distinguished from those observed within the chromatin area.

The nucleolus-like bodies rarely occur within clumps of chromatin material in cells of the presumptive ectoderm and mesoderm at stage 10. At stage 11, they become easily detectable in nuclear sections especially of presumptive mesoderm. Each body is less than 1.5 μ in diameter and

more or less spherical. It appears as an aggregation of fibrils much below 100 A in diameter and is more compact than the surrounding chromatin material (Figs. 2 and 6). The fibrillar material is very similar to that observed in the central region of a typical nucleolus (Figs. 3, 4, 8, 9, 14, 16, 17, and 20). To distinguish this stage of the nucleolus from the later stage, it is referred to as the *primary nucleolus*. This type of nucleolus-like body in the embryonic cells is very similar both to that of iris cells during early lens regeneration in the adult *Triturus* (26) and to that seen in the oocyte during early oogenesis of *Triturus* (35).

When ectodermal and mesodermal explants from early gastrulae are cultured for 24 hours, some modifications are observed in the nuclear structure (Figs. 7, 8, 9, and 12). A comparable modification can be detected in mesodermal and ectodermal cells of intact embryos during gastrula-

tion (24), when the early gastrulae develop to late gastrulae after 24 hours. In the area of low electron opacity, the interchromatin spaces, coarse-textured dense granules appear. These are occasionally in intimate contact with one another and with the fine-textured chromatin fibrils. They are larger (300 to 500 A) and more opaque than cytoplasmic ribosomes and are often irregular in shape (Figs. 8 and 9). These structures will be referred to as *interchromatin granules* according to Swift (49) and Bernhard and Granboulan (1). Similar larger particles have been detected in the chromosomes or nucleoplasm of various types of amphibian cells (12, 15, 18, 24, 25, 26, 35, 39, 49). The number of nucleoli per nuclear section is found to be less than that seen in the earlier stage. The maximum diameter of the nucleoli observed is 2 μ. In some cases, a granular component appears within the fibrous material of the primary nucleolus. These granular components are *ca.* 150 A in diameter and closely resemble the cytoplasmic ribosomes. In other cases, the granules are also seen in the peripheral region which surrounds the mass of the fibrous material (Figs. 8 and 9). At this stage, the nucleolus has attained its typical structural organization comprising fibrous and granular components, as reported by many authors (1, 12, 17–19, 26, 30, 35, 39, 46–49). These nucleoli are always in intimate contact with the chromatin fibrils.

In both optic vesicle and somite of stage 26, the nuclei become distinctly smaller and denser. The chromatin is rather uniformly distributed and not so sharply segregated from the chromatin granules as during the previous stage (Figs. 3, 13 to 17). Interchromatin granules are significantly increased in number and diffusely distributed. Typically there are two definitive nucleoli in each nucleus. The nucleoli are large, up to 3 μ in

diameter in section. They vary in compactness and shape, but all exhibit two distinct morphological parts, a fibrous component and a granular component (Figs. 3, 14, 16, and 17). The former component consists of tightly packed convoluted fibrils much below 100 A in diameter. It tends to occupy the central position of the nucleolus. The latter component consists mainly of granules *ca.* 150 A in diameter, and tends to surround the former, or to fill up the space between the fibrous components. The presence of these two regions having different densities and staining properties has also been demonstrated by light and electron microscopy in various other materials (1, 34, 39, 48). At this stage, nucleoli also show some modification resulting in the formation of a reticulated structure, the "nucleolonema."

In both optic cup and muscle of stage 35, the nucleoli become larger and more reticulated than in the earlier stage (Figs. 4 and 20). The reticulated nucleolus is made up of granular and fibrous components which are segregated into irregularly shaped regions. As shown in Fig. 4, the reticulum of the nucleolus is denser in regions where it consists mainly of closely packed fibrillar material. In most cells, the chromatin is homogeneously distributed except for occasional clumping along the nuclear envelope, and fine chromatin fibrils are intermingled with a large number of interchromatin granules (Fig. 18). In some cases in both tissues, the chromatin fibrils are randomly distributed in strands and clumps which remain fairly well delimited from the large areas containing interchromatin granules (Fig. 19).

Autoradiography

When the embryonic explants at each developmental stage were exposed to H³-uridine for 30 minutes, immediately fixed, and then studied in

FIGURE 10 A metaphase figure in an ectodermal explant from the gastrula (stage 11). Fixed after 21 hours' culture. Several chromosomes, which appear homogeneous, can be seen. A number of mitochondria (*M*) and a few vesicles can be identified among the chromosomes. Numerous scattered opaque granules are also observed. Some chromosomes are labeled. 21 days' exposure. × 10,000.

FIGURE 11 Enlargement of a part of Fig. 10. At this magnification the bulk of the chromosomes is seen to be made up of a fine filamentous material, like the chromatin of interphase nuclei. The interchromatin granules (*IG*) seen in interphase nuclei are completely segregated from the fibrils of the chromosomes. A yolk platelet (*Y*) shows a crystalline structure (25) with a spacing of 80 A. × 50,000.
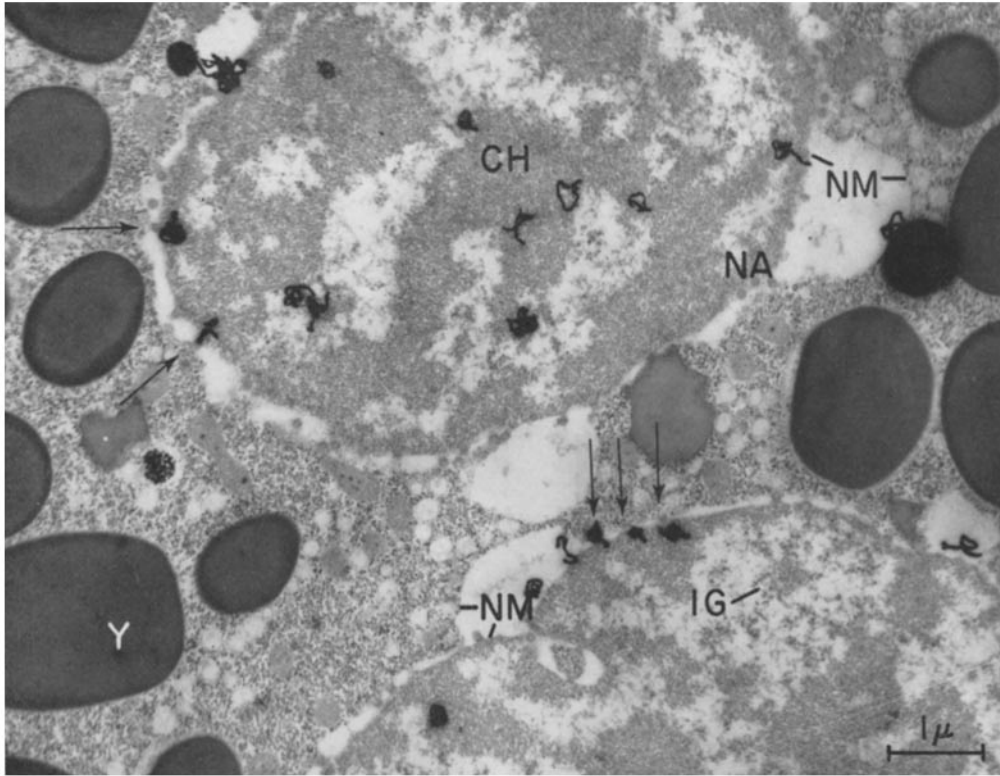
10 Y M— P— CH 1 μ

11 S —IG IG Y CH 0.5 μ
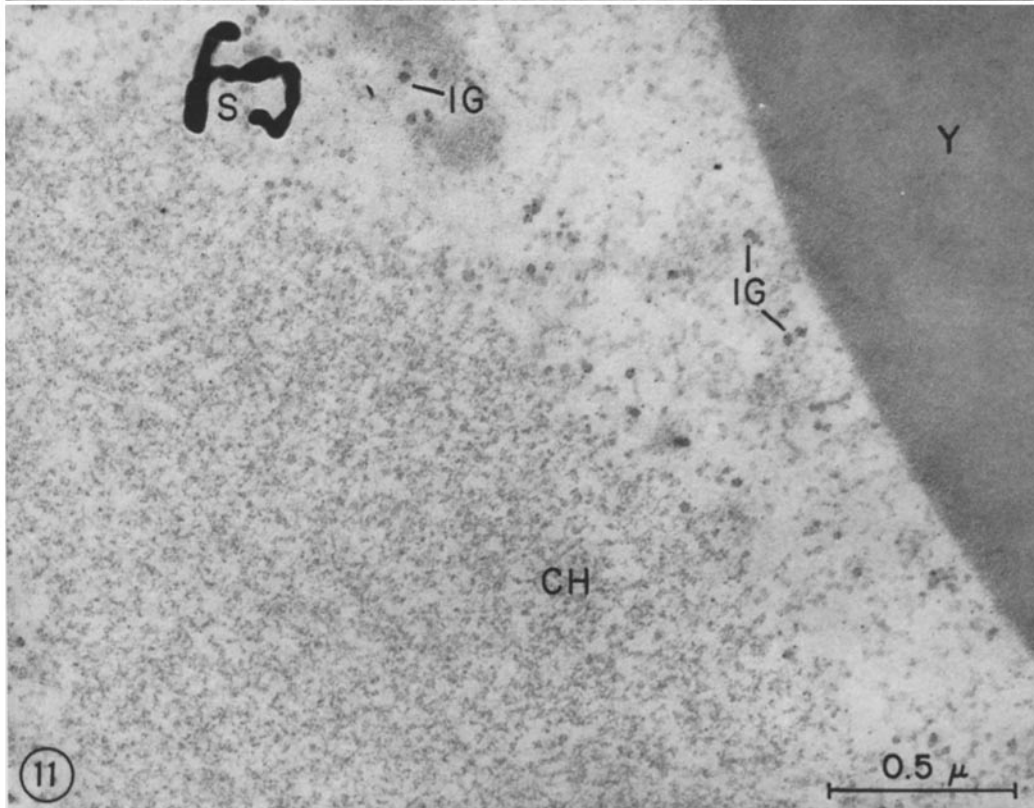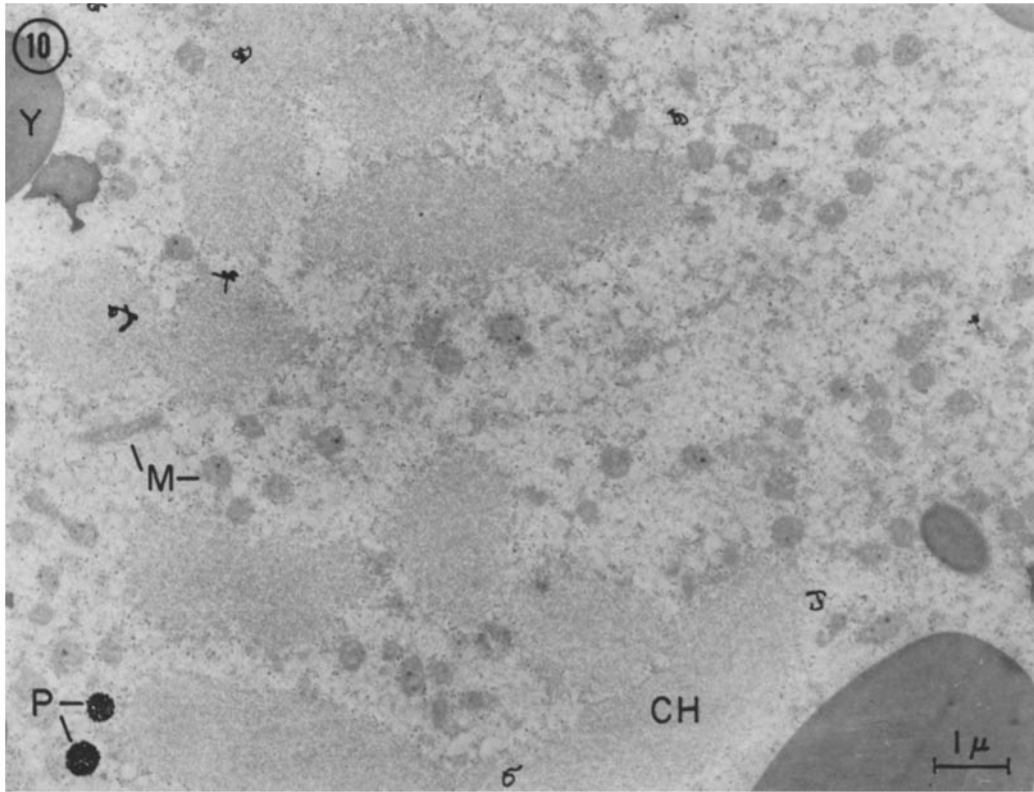
FIGURE 12 Portion of an ectodermal cell from the gastrula (stage 11). Fixed after 21 hours' culture. The external membranes of the nuclear envelope (NM) are undulatory and at some positions protrude far into the cytoplasm. The envelope is provided with many pores, where the inner and outer nuclear membranes are joined. Annuli (NA) are observed surrounding the pores in the envelope, which is sectioned tangentially. Boundaries between chromatin and interchromatin regions are sharply defined. Silver grains are detected over the extruded parts of the nuclear envelope and the pores (arrows). The chromatin regions are labeled. 21 days' exposure. × 12,000.

autoradiographs, a few silver grains occurred over the interphase nucleus. The amount of radioactivity was too small to determine the intranuclear localization.

If, on the other hand, the time of H^3-uridine treatment was extended to 3 hours, many silver grains were obtained in electron micrographs at each developmental stage. At stage 11, silver

FIGURE 13 A cell of the optic vesicle isolated from the tail bud embryo (stage 26). Fixed after 3 hours' culture. The nucleus reveals a nucleoplasm much denser than that of the gastrula, and there is close intermingling of granular components with the chromatin fibrils. A round nucleolus (NU) is at upper left. 21 days' exposure. × 10,000.

FIGURE 14 Enlargement of a part of Fig. 13. The nucleolus shows three fibrous areas surrounded by granular material. The granular area (G) of the nucleolus is mainly made of uniform particles 150 A in diameter. Silver grains are associated with the fibrous areas. In the nucleoplasm, chromatin fibrils are closely intermingled with interchromatin granules. × 30,000.
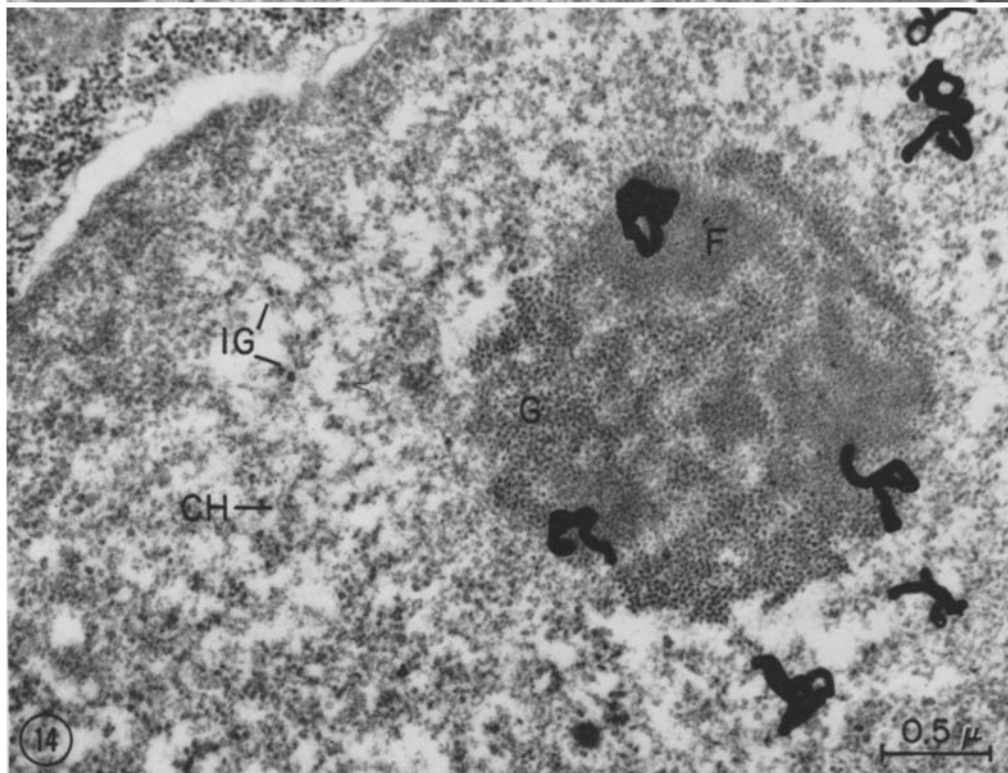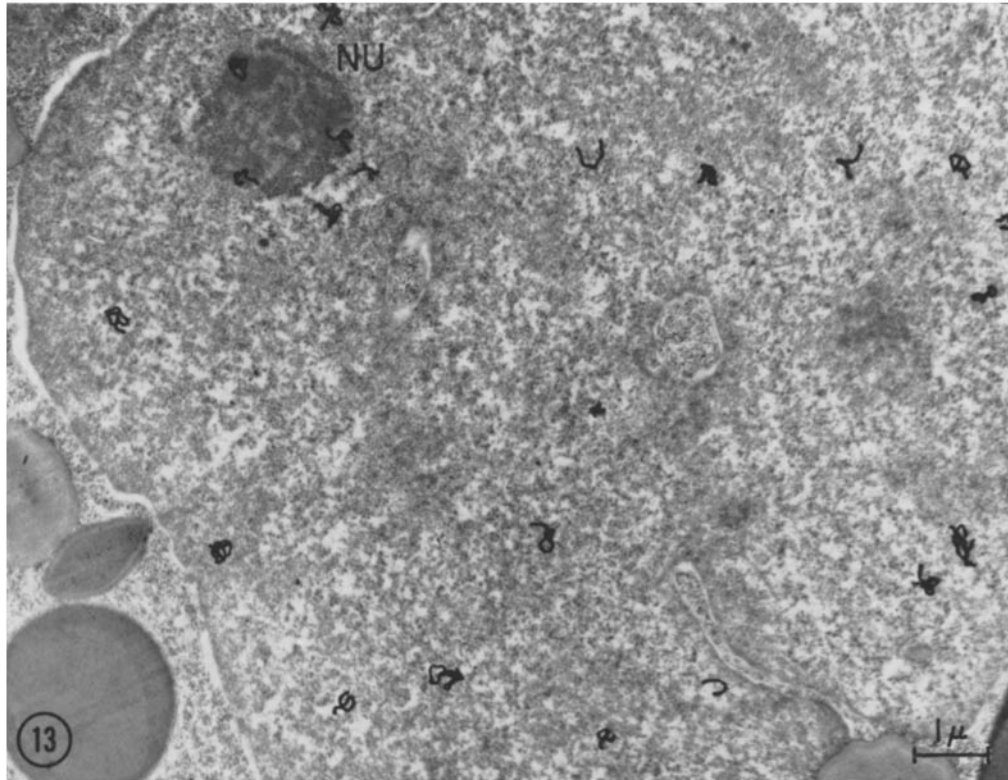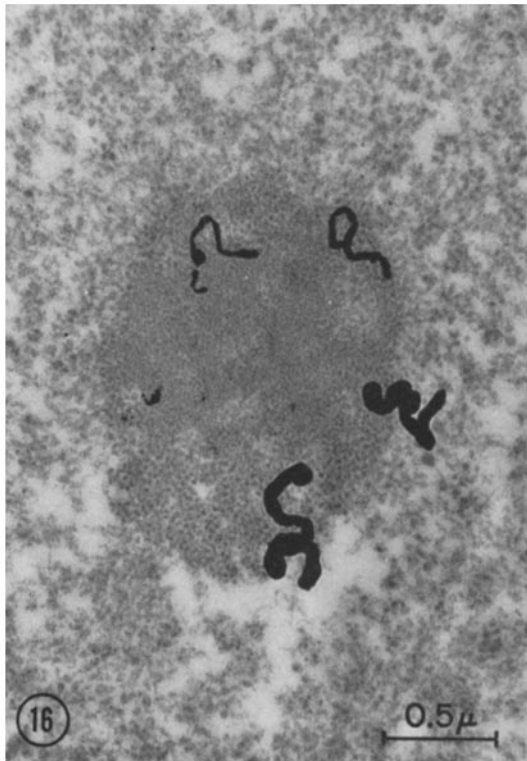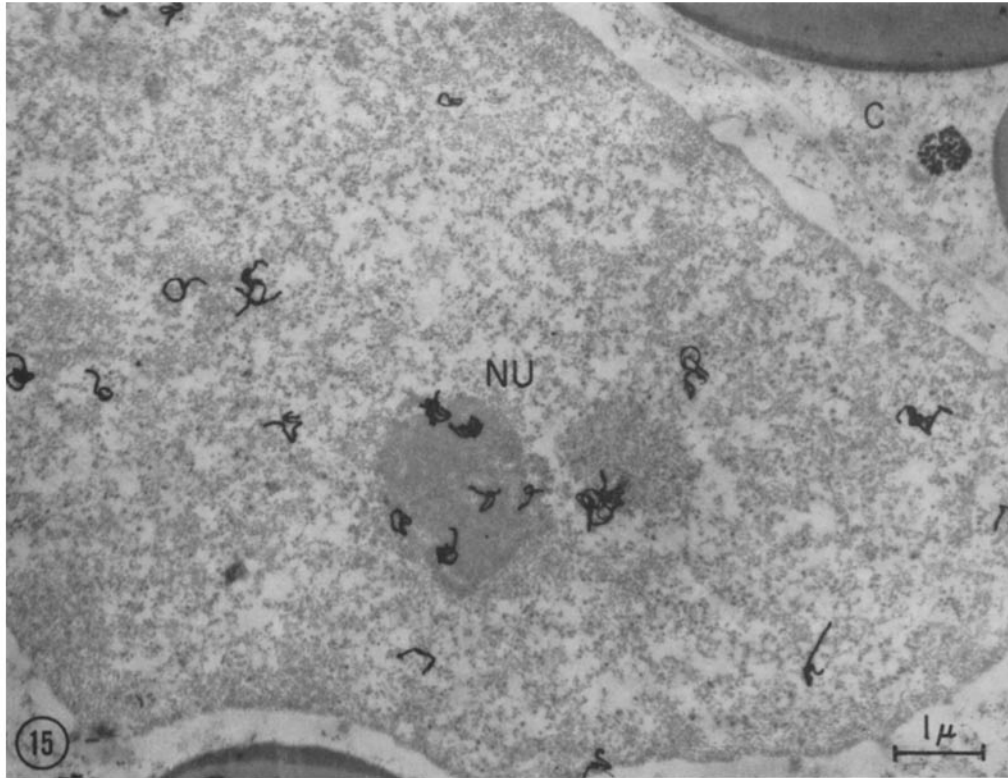
FIGURE 15 A cell in the somite explant from the tail bud embryo (stage 26). Fixed after 21 hours' culture. In the nucleus, the chromatin fibrils form small clumps and are associated with granular components. The nucleolus consists of fibrous and granular regions. Many silver grains occur over the chromatin. In the nucleolus, silver grains are associated with both the fibrous and the granular regions. 21 days' exposure. × 12,000.

FIGURE 16 A nucleolus in an optic vesicle explant from the tail bud embryo (stage 26). Fixed after 21 hours' culture. Silver grains are found over the granular region of the nucleolus. 21 days' exposure. × 30,000.
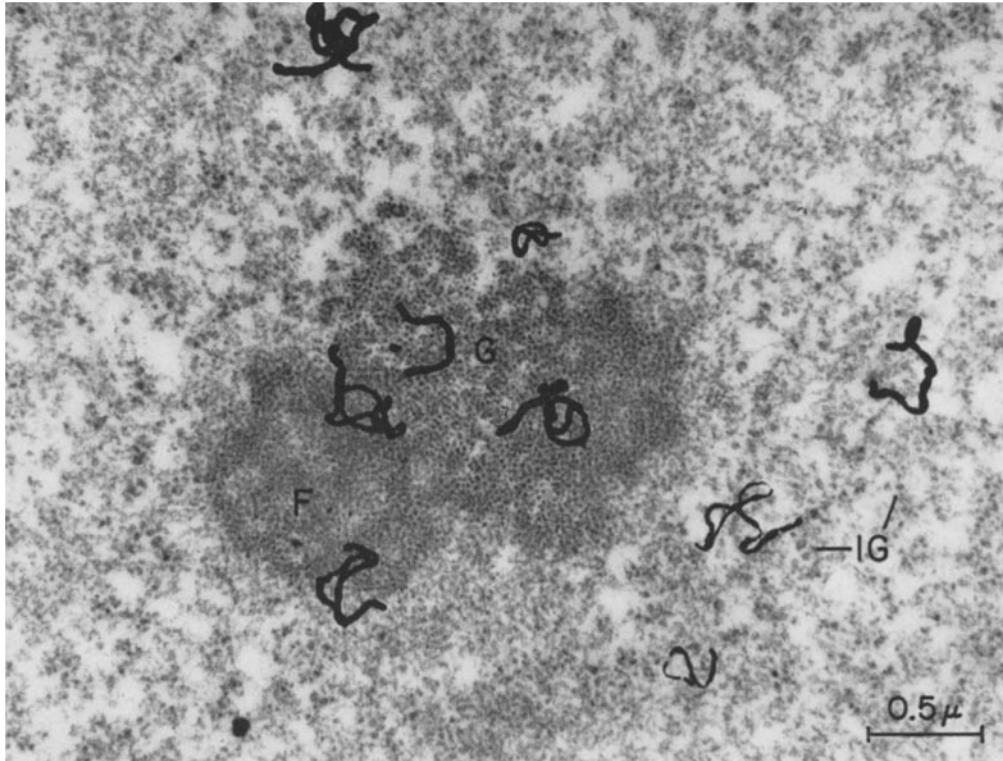
FIGURE 17 A high magnification of an interphase nucleus in a somite explant from the tail bud embryo (stage 26). After 21 hours' culture, only the granular part of the nucleolus seems to be labeled. The granular part (G) of the nucleolus shows a reticulated appearance and is made up of uniform particles 150 A in diameter. The nucleoplasm reveals chromatin fibrils associated with interchromatin granules (IG). 21 days' exposure. × 30,000.

grains are localized over interphase nuclei in explants of the 3-hour period of culture in Holt-freter solution after a 3-hour period of incubation in H^3-uridine (Figs. 1 and 5). (In future references to such incubation, only the treatment time in Holtfreter solution will be specified, since the time in radioactive medium was always 3 hours). In gastrular explants, more nuclei become labeled and the number of grains per nucleus increases, as the culture time is prolonged up to 21 hours (Table I). At 3 hours there is no significant difference in labeling between the presumptive ectoderm and mesoderm (Table I). At 21 hours, the former becomes more highly labeled than the latter (Table I).

At stages 26 and 35, most of the silver grains are located over the nucleus after a 1- or 3-hour period in non-radioactive medium (Figs. 13, 15, 18, and 19). When the incubation in non-radioactive

medium is extended to 21 hours, the cytoplasmic grain fraction increases at the expense of the nuclear fraction. At both stages, the nuclear counts in muscle and optic tissues do not differ significantly from each other.

For each electron micrograph of the developing ectodermal and mesodermal series, the number of silver grains was scored for (a) chromatin area, (b) interchromatin area, (c) granular area of the nucleolus, and (d) fibrous area of the nucleolus.

Throughout the developmental stages studied, in the majority of the cells the nucleolus is labeled. The nucleolus and the nucleoplasm were compared with respect to grain count per unit area. Table I shows that the average grain count per unit area of nucleolus is distinctly higher than that per unit area of other regions of the nucleus in the ectoderm and mesoderm cells at all developmental stages. These data show no significant

change in the ratio during a 21-hour period of culture in Holtfreter solution after an isotope treatment.

In many nuclei throughout stages 11 to 35, particularly those exhibiting significant incorporation of uridine, the chromatin material and the granular component of the nucleoplasm are so intermingled that it is difficult to distinguish them (Figs. 13, 15, and 18). In other nuclei throughout the stages studied, the boundaries of the chromatin are sufficiently sharply defined, but lower numbers of grains are present in general. In such nuclei, silver grains are preferentially associated with the interchromatin space and the periphery of the chromatin clumps (Figs. 7, 12, and 19). Although large areas of condensed chromatin are present, few grains occur over them. Some silver grains are found over the mitotic chromosomes, although the frequency of the grains is low (Fig. 10).

When possible the grains were counted separately over the fibrous and granular areas of the nucleolus throughout development. In selected nucleoli showing larger size, throughout stages 11 to 35, the percentage values of grain counts for the fibrous and the granular areas were computed separately (Table II). In the stage 11 explant after 3-hour culture, the grains are localized over the primary nucleolus, which consists mainly of fibrous material (Fig. 6). Labeling over the primary nucleolus is significantly greater in the mesoderm than in the ectoderm (Table II). After 21-hour culture of stage 11 explants, labeling occurs also in a peripheral region of the nucleolus, which now contains granular components (Fig. 8). At stages 26 and 35 after 3-hour culture, silver grains are preferentially associated with the fibrous portions of the organized nucleolus (Figs.

14 and 20). If, on the other hand, the time of treatment in non-radioactive medium is extended to 21 hours, the grains become preferentially distributed over the granular region of the nucleolus (Figs. 16 and 17).

Labeling over the cytoplasmic region increases with extension of the culture period. In autoradiographs of gastrular explants after 21 hours of incubation, the perinuclear cytoplasm has a grain count slightly higher than the background level. In Fig. 12, silver grains are located over nuclear pores, and they are also found in the expanded interspaces within the nuclear envelope, the outer layer of which protrudes extensively into the cytoplasm. In explants from stage 26 embryos after 21 hours' culture, the cytoplasmic grain fraction increases at the expense of the nuclear fraction. In autoradiographs of explants from stage 35 embryos fixed after 1-hour and 3-hour culture, a number of grains appear over the cytoplasm (Fig. 18).

DISCUSSION

In agreement with most other investigations, the present studies confirm the nucleus as the almost exclusive site of RNA synthesis during amphibian embryogenesis. The high resolution autoradiography also provides further information as to the fine structure of nuclei in active phases of RNA synthesis and the possible sites of uridine incorporation in such nuclei.

At the cleavage and blastula stages of amphibian development, it has been reported that exogenous uridine is mostly incorporated into DNA (2) and that the extent of RNA synthesis is limited (5, 6, 8). In *Triturus* embryonic cells at these stages, the interphase nucleus lacks a nucleolus and inter-

FIGURE 18 Cells in an optic cup explant from the forelimb bud embryo (stage 35). Fixed after 1 hour's culture. The chromatin fibrils are intermingled with interchromatin granules. Labeling occurs throughout the nucleus proper and is concentrated over the nucleolus. The heavily labeled part is the fibrous area. Some silver grains are found over the cytoplasm. 21 days' exposure. × 8000.

FIGURE 19 A myoblast in a muscle explant from the forelimb bud embryo (stage 35). Fixed after 1 hour's culture. The chromatin region is disposed in strands which are relatively sharply delimited from the granular regions. A large nucleolus appears reticulated and is surrounded by associated chromatin. Most of the silver grains lie within boundaries of chromatin clumps. The opaque part of the nucleolus is also labeled. 21 days' exposure. × 10,000.
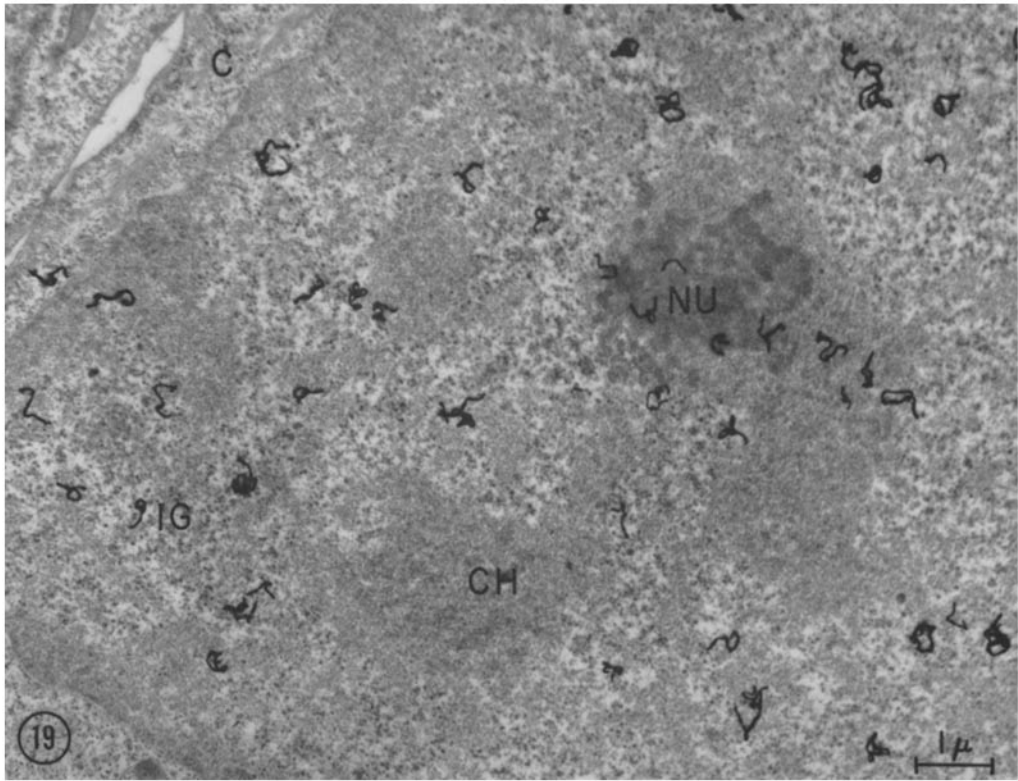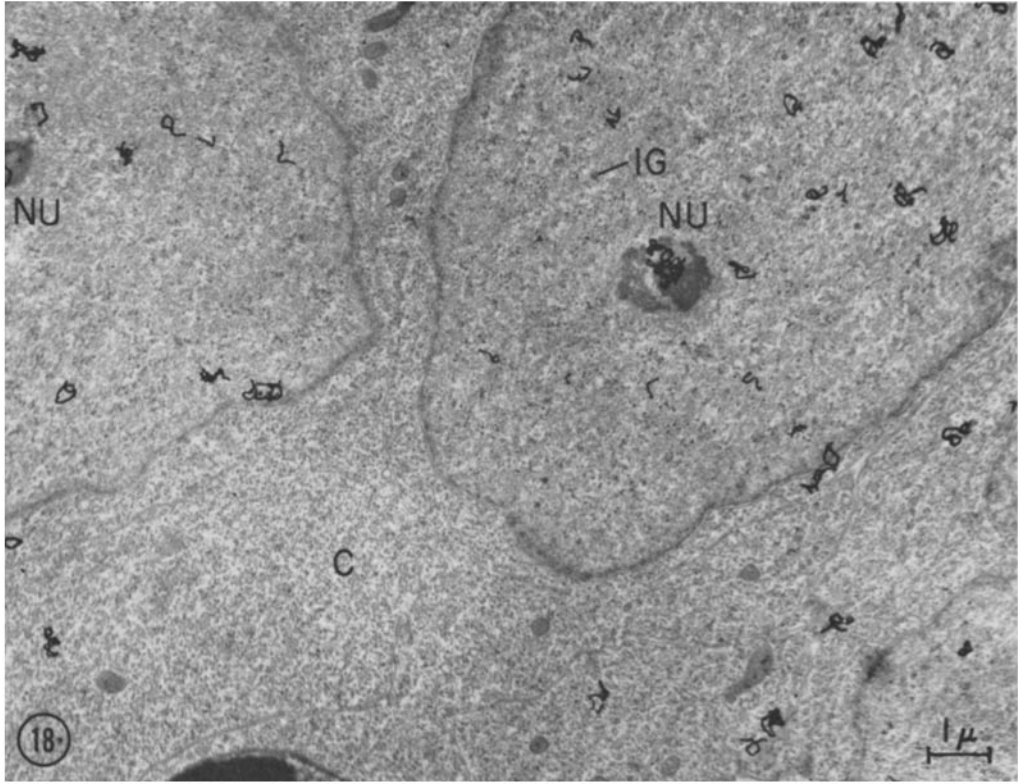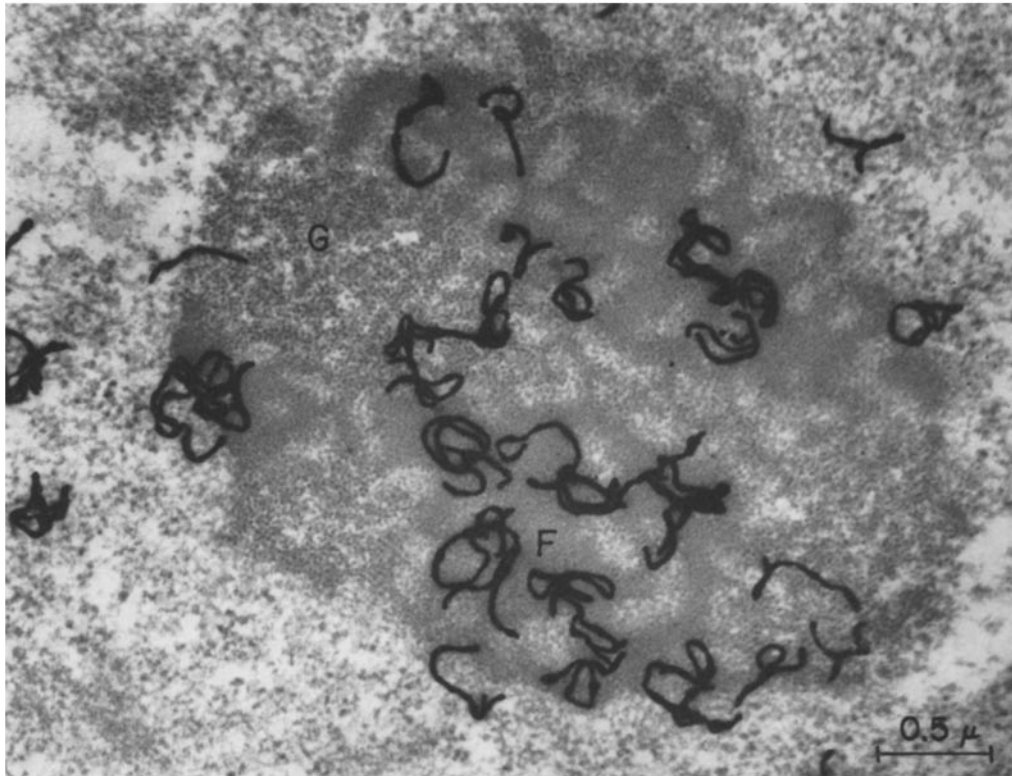
FIGURE 20 A high magnification of an interphase nucleus in a muscle explant from the forelimb bud embryo (stage 35). Fixed after 3 hours' culture. A large part of the grains is located over the fibrous regions of the reticulated nucleolus. 21 days' exposure. × 30,000.

chromatin granules. The only structural component of the nucleus observable with routine electron microscopy is a mass of chromatin fibrils. During gastrulation, mitotic activity slows down considerably, and the nucleus becomes capable of utilizing more uridine to synthesize RNA (2, 4, 52). During this phase, ribosomal RNA synthesis is initiated and synthesis of DNA-like RNA is increased (5, 8). The present morphological observations demonstrate the first appearance of definitive nucleoli and interchromatin granules at gastrulation.

Throughout embryogenesis, the major components of the interphase nuclei which incorporate tritiated uridine appear, in electron micrographs of OsO₄-fixed material, as chromatin fibrils finely textured throughout the nucleus. In many nuclei, particularly those exhibiting intense incorporation of tritiated uridine, the chromatin fibrils are sparsely distributed. In nuclei showing clumped strands of dense chromatin, silver grains occur

more abundantly over the periphery of chromatin strands or the interchromatin space than over the dense chromatin area. The interchromatin space also contains highly dispersed chromatin fibrils. These observations agree with the autoradiographic study by Littau et al. (31) of tritiated uridine incorporation into the isolated thymus nuclei, which indicated that the DNA active in RNA synthesis is mainly in the diffuse, extended chromatin rather than in the condensed, compact masses of chromatin. Concomitantly with increase of the nuclear RNA synthesis in amphibian embryonic cells, interchromatin granules appear within chromatin strands and later fill up the spaces between the chromatin fibrils. The chemical composition of the interchromatin granules has not yet been elucidated. However, Swift (49) suggests that the interchromatin granules contain RNA of a particular type, resistant to RNase treatment and perchloric acid extraction. According to the recent microelectrophoretic measure-

ments of the nuclear components of *Triturus* oocytes by Edström and Gall (13), the nucleoplasmic RNA is quite unlike the nucleolar and cytoplasmic RNA with respect to the base ratio. To determine the formation site of interchromatin granules, the pattern of labeling was investigated. Some of the present data indicate that the peripheral region of the chromatin strands is labeled first, and that later the interchromatin space is labeled. This sequential labeling is in conformity with the hypothesis that the synthesis of RNA of interchromatin granules is dependent on the chromatin fibrils. A probable origin of a similar type of granule on the loops of the lampbrush chromosomes of the amphibian oocyte has been suggested by Gall (15).

The formation of nucleoli at a specific genetic locus has been assumed in amphibian embryonic cells by Elsdale *et al.* (14), who established a mutant strain of *Xenopus laevis* lacking typical nucleoli. Studying the one-nucleolate heterozy-

gotes of *Xenopus laevis*, Kahn (22) obtained evidence for the idea that the secondary constriction of a particular chromosome forms the site of the nucleolar organizer. In *Triturus* embryonic cells, the diploid nucleus has the potentiality of containing two definitive nucleoli, one formed presumably in conjunction with each nucleolar organizer. As an initial step in nucleolus formation, the present study has suggested a gradual accumulation of fine-textured fibrils within chromatin strands at the gastrula stage. This body has been called the primary nucleolus. During gastrulation, granules first appear within the mass of fibrils, and later accumulate in the peripheral region. This new component of the nucleolus closely resembles cytoplasmic ribosomes in size and density. During successive developmental stages the nucleoli enlarge by acquisition of ribosome-like particles, which form a layer around the dense fibrous mass. The gross features of the two nucleolar components, ribosome-like particles and fine-text-

TABLE I

Ratio of Grain Counts per Unit Area of Nucleolus and Other Parts of Nucleus at Different Incubation Times after 3 Hours' H³-Uridine Treatment

Ectodermal and mesodermal tissues at stages 11, 26, and 35 were studied. If, in each section observed, the grain count over the nucleolus is X, the nucleolar area is Ax (μ^2), the grain count over the other parts of the nucleus is Y, and the area is Ay (μ^2), the ratio between the nucleolus and the other parts of the nucleus per unit area can be estimated by $R = X Ay/Y Ax$. A confidence interval was computed from the formulas: $R_l = Ay(T - S)/Ax(1 + S)$ and $R_u = Ay(T + S)/Ax(1 - S)$, where R_l and R_u, respectively, are the lower and upper confidence limits, $T = X/Y$, $S = t_x(T/X + Y)^{\frac{1}{2}}$, and where t_x is the normal deviate. 95 per cent confidence limits ($t_{0.05} = 1.96$) in this case.

Stage	Embryonic region	Incubation time	No. of nuclei examined	Grain count per unit area (μ^2) over nucleus	Grain count ratio nucleolus/Other part of nucleus		
						95% confidence limit	
					Mean	Lower	Upper
		hrs.					
	Ectodermal tissues						
11	Presumptive	3	9	0.07	8.34	2.91	13.98
	ectoderm	21	14	0.14	12.40	7.95	16.97
26	Optic vesicle	3	16	0.16	10.08	8.18	12.10
		21	10	0.14	8.51	6.35	10.79
35	Optic cup	1	13	0.26	5.95	4.77	7.19
		3	17	0.47	6.75	5.72	7.81
	Mesodermal tissues						
11	Dorsal lip	3	9	0.06	22.69	13.31	32.96
		21	12	0.08	17.75	11.24	34.54
26	Somite	3	9	0.13	10.97	8.08	14.13
		21	17	0.16	9.08	6.98	11.29
35	Trunk muscle	3	9	0.47	9.74	7.64	11.99

TABLE II

Labeling over Nucleolus at Different Incubation Times after 3 Hours' H^3-Uridine Treatment

Stage	Embryonic region	Incuba-tion time	No. of nucleoli examined	Av. size of nucleolus examined	Av. grain count per nucleolus	Mean % grain count for fibrous region of that for total nucleolus
		hrs.		μ^2		
	Ectodermal tissues					
11	Presumptive ectoderm	3	19	1.08	0.63	100.0
		21	23	2.41	2.15	70.0
26	Optic vesicle	3	26	5.21	7.5	80.8
		21	21	3.85	6.3	50.3
35	Optic cup	1	19	4.35	9.0	77.8
		3	17	4.14	11.5	62.4
	Mesodermal tissues					
11	Dorsal lip	3	23	1.59	2.55	100.0
		21	28	1.75	1.81	82.8
26	Somite	3	26	4.09	6.0	80.0
		21	23	3.41	4.9	42.9
35	Trunk muscle	3	10	4.67	10.1	91.2

ured fibrils, have been shown in electron micrographs of OsO_4-fixed materials from several sources (1, 12, 15, 18, 26, 30, 35, 39, 46-49). The presence of RNA in the two components has been demonstrated with electron microscopic cyto-chemistry (1, 17, 27, 34, 46, 49). These facts as well as the sequential labeling of RNA of fibrous and granular regions in the nucleolus suggest that the fibrous regions are involved in formation of ribosome-like particles.

As to the sites of the synthesis of nucleolar RNA present in the nucleolus during amphibian em-bryogenesis, the available evidence in general suggests two possibilities: (a) The nucleolus simply serves as an assembly point for the different RNA's synthesized at various levels of the chromosomes. (b) Nucleolar RNA is synthesized at nucleolus-specific loci of chromosomes which are close to or within the nucleolus. These two alternatives con-cerning the sites of nucleolar RNA synthesis have been repeatedly discussed by a number of workers (see for review Sirlin (42, 43)). In the present studies, uridine incorporation into RNA starts in the primary nucleoli from the early gastrula stage when ribosomal RNA synthesis is just beginning in amphibian embryos (5-8). The present data are in conformity with the ideas that the primary nucleoli synthesize RNA independently of other parts of the nucleus, and that the organized

nucleolus synthesizes RNA in the fibrous region, which later is transferred to the granular region. Throughout the developmental stages studied, the ratio of uridine incorporation per unit area into nucleoli is higher than that for other parts of the nucleus. This ratio remains roughly unchanged after extended incubation with the non-radioactive medium. This finding seems to support the second alternative. However, because of failure to locate the incorporation site in a pulse experiment in the present study, the possibility of a quick transfer of RNA synthesized on the chromosomes to the nucleolus, as suggested by Goldstein and Micou (16) in mammalian tissue cells, cannot be com-pletely ruled out.

Concerning the second possibility, Pelling (37) has reported an autoradiographic experiment suggesting the nucleolus-associated chromatin as the ultimate site of nucleolar RNA synthesis in Chironomus salivary gland chromosomes. This is contrary to our tentative conclusion, indicated above, that in the nucleus of amphibian embry-onic cells the initial site of uridine incorporation may be in the central fibrous region of the nucleo-lus but not in the chromatin surrounding the nucleolus, which may be called the "nucleolus-associated chromatin" (41, 44), as observed at the light microscopic level. By autoradiographic experiments, Sirlin et al. (45) have shown that in

the larval salivary gland cells of *Smitta* nucleolar RNA synthesis begins in the nucleolus proper and that the "nucleolus-associated chromatin" does not contribute, to any observable extent, to the synthesis of nucleolar RNA. However, these authors could not demonstrate the presence of intra-nucleolar chromatin by autoradiographs of thymidine incorporation (45) and by electron micrographs (21). Recently Perry (38) has proposed that the nucleolar RNA and the RNA of extranucleolar parts of the nucleus in tissue culture cells are synthesized independently of each other, and that the former is preferentially suppressed by a low concentration of actinomycin D. In most of the interphase nuclei of animal cells, as well as in *Smitta* polytene chromosomes, intranucleolar chromatin fibrils cannot be distinguished as separate entities under light microscopy, especially if they happen to be diffused throughout the body of the nucleolus. Recently, electron microscopic cytochemistry (1, 17, 32, 34, 49) and autoradiography (17–19) have clearly demonstrated the existence of DNA or chromatin within the nucleolus at the ultrastructural level. In embryonic cells of *Triturus pyrrhogaster*, some attempts were made to study the cytochemical nature of the nucleolus with electron staining and specific enzyme digestion (27, 28). The presence of chromatin material was also found both in the primary nucleoli and in the fibrous region of organized nucleoli. With similar technique, Stevens (46) has demonstrated the close association between the nucleolar organizer chromatin and the fibrous component of the nucleolus in the salivary gland cell of *Chironomus*. This makes it probable that in amphibian embryos the submicroscopic chromatin fibrils in the fibrous region of the nucleolus participate in nucleolar RNA synthesis and correspond to the "nucleolar organizer." Recently, Eakin (12) has found that the nucleolus of differentiating amphibian cells becomes remarkably reduced in size by actinomycin D and has assumed that this reduction is due to interference with DNA-dependent RNA synthesis.

From the numerous studies on RNA synthesis in the cell, it is often assumed that the compound is synthesized in the nucleus, on chromatin and the

nucleolus, and is transferred to the cytoplasm. As demonstrated by the present autoradiographic experiments, the appearance of labeling over the cytoplasm after 21-hour incubation in non-radioactive solution suggests the transfer of RNA from the nucleus to the cytoplasm in amphibian embryos. According to Brown and his associates (5, 6, 8), few ribosomes appear in the cytoplasm before the tail bud stage in *Rana* and *Xenopus*. From this stage on, the amount of cytoplasmic ribosomes begins to increase (5, 6, 8, 23). The distribution pattern of labeled RNA at the different stages is consistent with the hypothesis that cytoplasmic ribosomes are derived from the nucleolus during embryogenesis. This is also supported by the biochemical studies of Brown and Gurdon (7), which indicate that the anucleolate mutant of *Xenopus* is incapable of synthesizing ribosomes and ribosomal RNA.

Transfer of nuclear materials into cytoplasm may be possible by direct passage through nuclear pores at interphase and also by their discharge at the time of mitosis, when the nuclear envelope breaks down. A previous study (24) on the nucleus of developing cells has shown some morphological evidence suggestive of nucleo-cytoplasmic interaction, including a remarkable abundance of nuclear pores at the invagination of the nuclear envelope and characteristic protrusion of the outer nuclear membrane into the cytoplasm. It should be pointed out that some silver grains are detected over the nuclear pores and the protruded nuclear membrane at the time when labeled RNA may be transferred from the nucleus into the cytoplasm.

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