NUCLEOLAR NECKLACES IN CHICK EMBRYO MYOBLASTS FORMED BY LACK OF ARGININE

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ABSTRACT

The round nucleoli of chick embryo myoblasts, when grown in a culture medium devoid of arginine, unravel in several days into 5-20 μ long, beaded strands termed nucleolar necklaces (NN). Addition of arginine reverses this change. The NN contain protein, RNA, and traces of DNA as determined cytochemically by enzyme digestion and by acridine-orange fluorescent staining. When a cell containing the beaded strand is treated with agents, such as actinomycin D, that prevent rRNA polymerase action, the strand collapses and condenses into a small dense nucleolus with segregated regions of ribonucleoprotein (RNP) and deoxyribonucleoprotein (DNP). The properties of the NN appear to resemble those of the nucleolar necklaces of amphibian oocytes. Cycloheximide or puromycin inhibition of general protein synthesis does not lead to NN formation. We suggest that NN formation during arginine starvation may be a result of a singular depletion of some rapidly turning over, arginine-rich proteins that normally attach to ribosomal RNA precursor molecules during their synthesis in the processing towards maturation of the ribosomes.

INTRODUCTION

When trypsinized chick-embryo muscle cells are grown in certain media they may form flattened fibroblast-like cells, the nuclei of which contain one or several 5–20 μ long, beaded strands. These strands are derived from the nucleoli, as first shown by Weissenfels (1). The strands consist of granules aligned in a chain which have been termed "nucleolar necklaces" (NN).[1] These necklaces resemble those developed from nucleoli of amphibian eggs (2). Similar necklaces have been found to form in chick fibroblasts in the presence of 2 mM adenosine by Lettré et al. (3).

The interest in the nucleolar necklaces lies in the possibility that studies of their development may provide knowledge of the intermediate steps in the synthesis of ribonucleoprotein (RNP) complexes, as well as of the cause for the compactness of the nucleolar body which is not surrounded by a limiting membrane. In this paper we report that compact nucleoli of chick embryo myoblasts in culture are converted to beaded strands when arginine is limiting. Histochemical properties of the NN and the reactions of the NN-containing cells to various antibiotics are also examined.

MATERIALS

Complete Medium

Eagle's basal medium (4) was supplemented with the antibiotics, streptomycin, penicillin, and mycostatin, together with 10% bovine fetal serum and glutamine.

[1] *Abbreviations:* DNP, deoxyribonucleoprotein; NN, nucleolar necklaces; RNP, ribonucleoprotein; SS, Earle's salt solution devoid of Mg and Ca.

Incomplete Medium

Earle's inorganic salt solution was supplemented with antibiotics, glutamine, and bovine fetal serum in the concentrations used in the complete medium.

Preparation of Cell Inoculum

Two ventricles of 16-day old chick embryos were washed in Earle's salt solution devoid of Mg and Ca (SS), then chopped finely with a razor blade in a small Petri dish containing 7 ml of 0.5% crystalline trypsin (Worthington Biochemical Corp., Freehold, N.J.) in SS. After 15 min at 38°C, 0.5 mg or less of Varidase was added if stringiness due to released deoxynucleoprotein (DNP) was present. Intermittently the suspension was sucked back and forth through a wide-mouth pipette to aid in comminution. When almost all of the tissue particles had disappeared and the suspension was cloudy, it was diluted with SS to 12–15 ml, transferred to a conical tube, the particles were permitted to settle for 10 min, and the supernatant cell suspension was used as inoculum.

Growth of Cells

The cells were grown in vials, 18 × 60 mm, each containing a cover slip. To 1 ml of complete medium in the vial, 0.05 ml of cell suspension was added. The medium was changed on the next day and every few days subsequently. The cells were incubated in 5% CO_2 at 37°C.

Polaroid photographs were made with Polapan Type 52.

Cell fluorescence was examined with a Zeiss ultraphot microscope, with the exciting UV beam passing through the objective onto the cells.

Acridine-Orange Staining and Enzyme Digestion

Cells growing on the cover slips in the vials were washed with saline, fixed on the cover slips in ethanol-acetic acid (3:1 by volume) at 4°C for 10 min, washed with 70% alcohol three times, and kept overnight in 70% alcohol at 4°C. The cover slips were then brought down through 40, 20, 10% alcohol into acetate buffer (0.1 M) pH 5.6. At this stage the cells were digested for 1 hr, 37°C, at pH 5.6 or 7.0 with pancreatic RNase (0.1–1 mg/ml), or T_1-RNase (0.03 mg/ml), or DNase (0.5 mg/ml) containing 0.02 M $MgSO_4$, or Varidase (1 mg/ml), or Pronase (Calbiochem, Los Angeles, Calif.) (1 mg/ml). Then the cells were washed in the acetate buffer pH 5.6, stained in 0.05% acridine orange dissolved in the buffer for 15 min at 20°C, rinsed four times for 10 min each in the buffer, mounted in the buffer containing 20% glycerol, and examined with phase and fluorescence optics.

To follow the action of the enzymes under the microscope, cells were fixed and stained with acridine orange; then the cells were sealed under the cover slip in a droplet of enzyme solution and the changes in fluorescence were observed either at room temperature or at 38°C.

METHODS AND RESULTS

Conditions for the Development of Nucleolar Necklaces

To obtain NN in chick myoblasts, Weissenfels (1) used the medium of Morgan, Morton, and Parker (5) supplemented with a 5% chick embryo extract and 3% serum from the human umbilical cord. After some 10 days in unchanged medium, NN began to appear. In our experiments Eagle's basal medium (4) supplemented with 10% bovine fetal serum was used, which we shall designate as the "complete medium." When myoblasts were grown in this medium and the medium was changed every 3 days no NN were formed, although when the medium was unchanged for 10 days some poorly formed NN developed. It was then found that after growth had been established on the cover slip in 1–4 days, NN would develop in the next several days when the medium was changed to one devoid of amino acids, i.e., "incomplete medium."

STEPS IN THE DEVELOPMENT OF THE NN DURING AMINO ACID STARVATION are presented in Fig. 1 *a–c*. The small nuclei are probably diploid and the large are tetraploid (1). As seen in the living cell with phase optics, the nucleoli first enlarged to form a lumpy mass, then extended as a flattened strand which elongated and narrowed and became more dense and granular (Fig. 1 *d–h*). Some 30% or more of the cells developed NN. Accompanying the appearance of the NN was the formation of fat droplets and a diminution in mitochondria. The NN appeared earliest in fibroblasts, but also developed in myoblasts which were already in a syncytium.

REVERSAL OF NN FORMATION BY ARGININE: Cells deprived of amino acids for about 5–7 days and containing NN could recover if a complete medium was provided. In the complete medium the NN rounded up, after 2 days, into usually two nucleoli per cell, as shown in Fig. 1 *j*, and the number of mitochondria increased greatly. By the fourth day (Fig. 1 *j*) the large fat droplets had begun to emulsify into smaller ones (lower left).

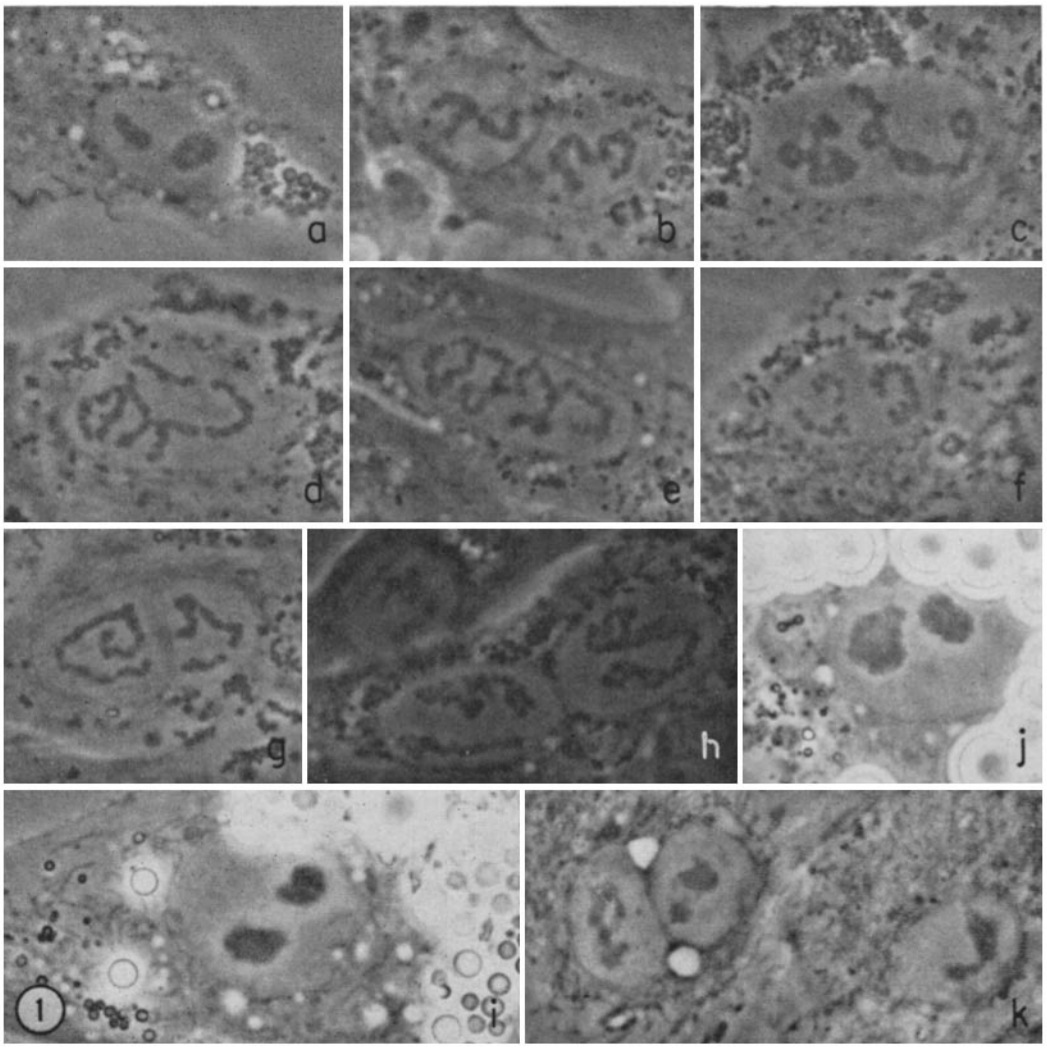

FIGURE 1 Conversion of nucleoli to nucleolar necklaces on medium lacking amino acids (incomplete medium) and reversion to normal on complete medium. (*a–c*) Steps in development of nucleolar necklaces. (*d–h*) Nucleolar necklaces. (*i–j*) Reformation of nucleoli from nucleolar necklaces by change to complete medium. (*k*) Development of nucleolar necklaces greatly delayed by addition of arginine to incomplete medium. × 1560.

Supplementation of the incomplete medium with amino acids both singly and in various combinations indicated that the lack of arginine primarily, and to a lesser extent lysine, resulted in NN formation. When the incomplete medium was supplemented with arginine (50 μg/ml) the formation of NN was considerably delayed (Fig. 1 *k*). Lysine at 100 μg/ml but not at 50 μg/ml delayed the NN formation slightly. Methionine and histidine, each at 50 μg/ml, together did not prevent NN formation, though a mixture of arginine, methionine, and histidine each at 50 μg/ml permitted good growth and prevented NN formation. When maximum NN formation had developed, arginine (100 μg/ml) added to their medium resulted in a conversion of the NN back to normal nucleoli in 2 days.

Fetal bovine serum (10–15% by volume) was required both for growth in a complete medium and for the subsequent development of NN in an

incomplete medium. The cells grew poorly when the fetal bovine serum was only 3%.

INHIBITORS OF PROTEIN SYNTHESIS added to complete medium slowed growth but did not cause NN development. When cells, after being transferred to incomplete medium, were given the same inhibitors, acetoxycycloheximide (0.025 μg/ml) or puromycin (5 μg/ml) or emetine (0.04 μg/ml), and observed for several days, all these inhibitors produced similar results, i.e., only intermediate stages towards NN development. At high concentrations of puromycin (10 μg/ml) the nucleoli were broken up into large granules that were loosely connected together. Thus, mere inhibition of total protein synthesis does not result in complete development of NN, that is, does not reproduce the effect of arginine deficiency.

INHIBITORS OF METHYLATION OF 45S RIBOSOMAL-PRECURSOR RNA were tested and found not to affect NN formation. Although 45S RNA is methylated (at the 2'OH of ribose) mainly before further processing, undermethylated 45S RNA does not accumulate but continues to be cleaved to the 32S stage (Vaughan et al., 6). When amethopterin (25 μg/ml) and trimethoprim (5 μg/ml), which are folic acid inhibitors, were added to cells that were developing NN, they slightly delayed growth and NN formation. Ethionine (100–200 μg/ml), an inhibitor of *S*-adenosyl methionine formation, caused a slight condensation of the NN. Thus, NN formation may be caused by factors before the methylation step.

Collapse of NN by Inhibitors of DNA and RNA Synthesis

A number of inhibitors which attach to DNA and block RNA synthesis caused the NN to condense and form usually two small dense nucleoli per cell. Some of these nucleoli contained several pale vesicles or caps. The collapse of the NN was time- and temperature dependent. The collapse was not enhanced or prevented by dinitrophenol (50–100 μg/ml). The appearance of these collapsed nucleoli was similar to that of condensed nucleoli derived from normal cells when treated with these reagents. According to Simard (7), the condensed nucleoli are seen by electron microscopy to have segregated regions of fibrillar and granular RNP materials.

The following inhibitors of DNA-dependent RNA synthesis, when incubated with the NN-containing cells, caused the NN to collapse and condense to rounded small nucleoli in 2–5 hr at 36°C: actinomycin D (0.25 μg/ml), mithromycin (1 μg/ml), and mitomycin C (2 μg/ml). Actinomycin D (0.25 μg/ml) for 6 hr at 20°C produced only an incipient condensation.

Other compounds had less effect on the NN. Ethidium bromide (2 μg/ml) for 2 hr at 36°C caused an incipient condensation which was complete in 20 hr; it also caused the formation of spatulate mitochondria which possibly reflected the action of this dye on the inhibition of mitochondrial RNA synthesis (8). Desamino–actinomycin D (10 μg/ml) caused the collapse of the NN only after 20 hr at 36°C. Proflavine (5 μg/ml) for 2 hr at 36°C caused only a start in condensation; at 10 μg/ml the nucleoplasm which was normally transparent under phase became distinctly granular.

Components of the NN as Determined by Enzyme Digestion and Acridine-Orange Fluorescence

After alcohol-acetic acid fixation the cells were digested with pancreatic RNase. Although no change in the NN was observed with phase optics (Fig. 2 *a*), the fluorescence staining with acridine orange differed. The orange fluorescence had disappeared, indicating a loss of RNA; the nucleoplasm fluoresced greenish indicative of DNA, and the NN had a very pale greenish-white fluorescence suggestive of the presence of DNA (Fig. 2 *b*). After DNase digestion and acridine-orange staining the NN fluoresced orange but the nucleoplasm was devoid of greenish fluorescence (Fig. 2 *c*). These experiments indicate that the NN contain some RNA and a small amount of DNA. Estimates of the amount of DNA associated with a normal cell nucleolus are between 2.5 and 10% of the DNA of the nucleus, depending on the cell type (9).

Comparison with normal cell nucleoli is shown in Fig. 2 *d* and *e*. Digestion with RNase did not change their phase image recognizably (Fig. 2 *d*). This may be because they contain only 8–15% RNA on a dry weight basis (9). That RNA had been digested was indicated by absence of orange fluoresence in both the nucleolus and cytoplasm after acridine-orange staining. A pale greenish-white fluorescence was localized only in the nucleus; the nucleolus appeared as a darker area

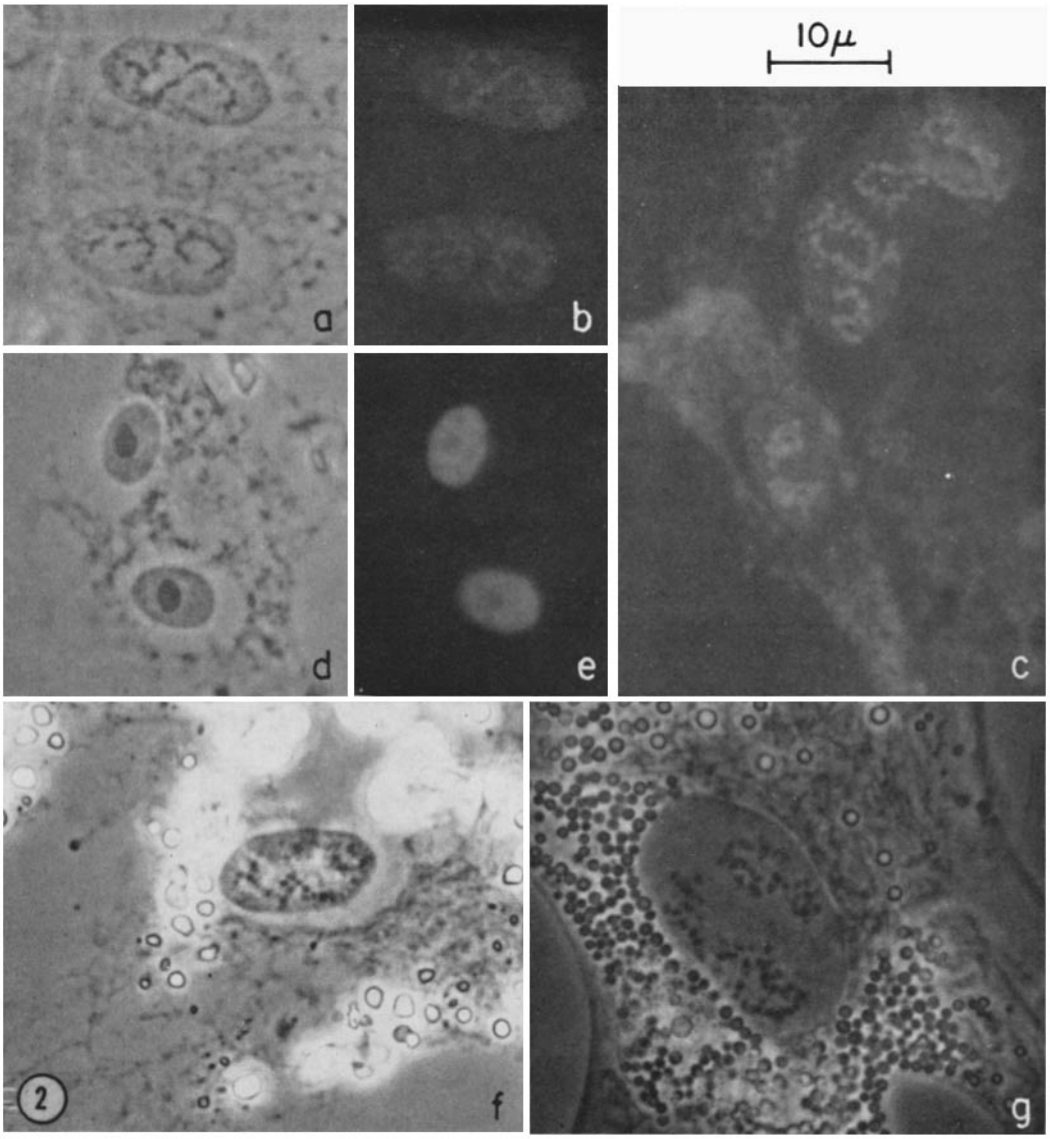

FIGURE 2 Composition of nucleolar necklaces by enzyme digestion and acridine-orange fluorescence. (*a*) RNase digestion of NN, and phase. After acetic-alcohol fixation the cover slips were treated for 1 hr at 37°C, pH 7 with 0.01% pancreatic RNase, subsequently stained with acridine orange, and photographed with phase optics. The nucleolar necklaces appeared unchanged. (*b*) RNase digestion and fluorescence. The cells in *a* were photographed by fluorescent light, showing the faint greenish-white fluorescence of the nucleolar necklaces and the nucleoplasm indicative of DNA. The orange RNP fluorescence is lacking. (*c*) DNase digestion of NN and fluorescence. After acetic-alcohol fixation the cover slips were treated for 1 hr at 37°C, pH 7 with 0.5% DNase containing 0.02 M $MgSO_4$ and subsequently stained with acridine orange and photographed with fluorescent light. The nucleolar necklaces as well as the cytoplasm were orange fluorescent indicative of RNP; the nucleoplasm lacked greenish DNA fluorescence. Exposure time 2 min as in *b*. Similar results were obtained with Varidase digestion. (*d*) RNase digestion of normal cells, and phase. The cells were treated as in *a*. (*e*) RNase digestion of normal cells and fluorescence. The cells of *d* were photographed by fluorescent light, exposure time 3 min. (*f*) Incipient pronase digestion, acridine orange stain, and phase. The cells were treated with pronase (1 mg/ml) at 20°C, pH 7 for a few minutes and stained with acridine orange, pH 5.6. Rapid protein digestion occurred with loss of orange fluorescence indicative of the loss of RNP, but green fluorescence of the nucleus remained for a longer time. (*g*) Glutaraldehyde fixation, enzyme digestion, and phase. Cells which were fixed with 2% glutaraldehyde for 10 min could not be digested by RNase, DNase, Varidase, or trypsin and only very slowly by pronase. × 1560.

because of its lower DNA concentration compared to the surrounding nucleoplasm (Fig. 2 *e*).

Digestion with trypsin or pronase after acetic-alcohol fixation caused the dissolution of NN as well as of the remainder of the cell. The NN remained after RNase and DNase digestion but not after proteolytic digestion. This result indicated that the NN are composed primarily of protein. Because the NN were fixed before digestion, the proteins may have held the NN together even after DNase digestion. Incipient proteolytic digestion (Fig. 2 *f*) and acridine-orange staining indicated that the orange fluorescence due to RNP in both the NN and cytoplasm rapidly became dispersed, but the green fluorescence due to DNA dispersed more slowly.

After fixation with 2% glutaraldehyde for 10 min, no visible digestion could be observed by RNase, DNase, or trypsin, and pronase acted very slowly (Fig. 2 *g*). Richards and Knowles (10) suggested that glutaraldehyde forms stable Michael-type adducts with the ϵ amino groups of lysine, and Brutlag et al. (11) noted that, with formaldehyde as fixative, lysine-rich histones became insoluble in acid and that about 4% of the DNA became cross-linked to the histones.

DISCUSSION

Nucleoli of chick embryo fibroblasts and myoblasts form granular filaments, i.e. nucleolar necklaces (NN), after about 5–10 days if the complete medium in which they have grown is not renewed. The formation of the NN results primarily from a lack of arginine and, less so, of lysine. Arginine is as essential amino acid in the chick; the urea cycle is absent (Tamir and Ratner [12]). A concentration of at least $2\text{–}4 \times 10^{-4}$ M arginine is required for growth. Trace amounts of arginine may be furnished by the normal degradation of the cultured cells' own protein (Eagle et al. [13]). Arginine may be broken down in muscle to furnish creatine. The small amount of free arginine contained in the bovine fetal serum that is added to the incomplete medium is insufficient for normal growth; nor can the proteins of the serum themselves supply the necessary arginine. However, some serum components are necessary for normal growth as indicated by the fact that 10% fetal bovine serum is adequate but 3% is inadequate.

In an incomplete medium devoid of amino acids, NN develop in a few days; their development can be slowed down or even reversed merely by the addition of arginine. During arginine starvation the nucleolus swells and becomes less dense; a coiled rope-like structure then becomes apparent, and this structure unwinds to form a beaded or granular filament. The morphological changes in the formation of the NN are similar to those that have been described for the transformation of the spherical nucleoli of amphibian oocytes into NN (2), except that in the oocyte the NN may be of greater length.

As determined by enzyme digestion and by acridine-orange fluorescent staining, the NN contain protein, RNA, and traces of DNA. On digestion with DNase after acetic-alcohol fixation, the NN do not fall apart, perhaps because they are embedded in a gel of precipitated protein; only proteolytic digestion results in the solubilization of the NN. However, amphibian oocyte NN, without previous fixation, were observed by Miller and Beatty (2) to fall apart in the presence of DNase, leaving only granules of the necklaces as residue, thus providing evidence that a DNA thread was the backbone of the NN in that cell type.

In the normal nucleus there is some material or association of materials that renders it internally "sticky," holding it together in a compact spherical mass even without a bounding membrane. This characteristic may also explain the tendency in older cells for the fusion of two nucleoli to form one large nucleolus.

Our observations with phase optics on cells grown in arginine-deficient media indicate that, in hypotonic media, materials leach out more readily from swollen nucleoli in starving cells (Fig. 1 *a*, 1 *k*) than from normal nucleoli. This loss of stickiness could permit the unraveling of the nucleolus and the extension of the NN strand by Brownian movement. Evidence that basic proteins may be involved in this stickiness is suggested by the finding of Allfrey and Mirsky (14) that, when arginine-rich histones were added to lampbrush chromosomes isolated from amphibian oocytes, collapse of the loops occurred in 3 min. The arginine-rich histones were more effective in this action than were the lysine-rich histones or polylysine. In the nucleoli two kinds of arginine-rich proteins that turn over rapidly are the preribosomal subunits (9), and the arginine-rich histones that, according to Sadgopal and Bonner (15), continue to be synthesized in nondividing cells.

Various antibiotics have been reported to cause

the condensation of a normal nucleous into a small, dense spherical unit which contains segregated regions of fibrillar RNP, granular RNP, DNA, and amorphous protein (7, 9). In our study, the antibiotics actinomycin D, mithromycin, and mitomycin C were found to cause the collapse of NN and their condensation into small dense nucleoli with segregated regions.

We have noted three nucleolar states: the normal compact one, the unwound NN state, and the condensed one with segregated components. Studies by Miller and Beatty (2) suggest that the chromatin loop coding for rRNA consists of a double strand of DNA along which RNA polymerase molecules move sequentially and simultaneously to spin out feathery arrays of 45S fibrils. Based on this model, the following tentative hypotheses are suggested to explain the nucleolar states. The NN state would be formed by the lack of certain arginine-rich proteins; the coating with protein of the 45S RNA feathers would be slowed down, as well as the formation of the sticky arginine-rich precursor RNP particles; as a consequence of the charge repulsion of the RNA feathers and the loss of stickiness, the chromatin would unwind to form the NN state. The condensed state of the nucleolus, as caused by the action of actinomycin D on the NN, might represent a depletion of the feathery arrays; perhaps the actinomycin might act to dislodge RNA polymerase molecules with their feathers from the DNA, thus permitting the DNA to pull itself along with some other nucleolar material into a ball.

Whether or not the lack of arginine, which is primarily responsible for the formation of the NN of chick embryo fibroblast and myoblast cells, is also responsible for the formation of the NN of the amphibian oocytes, as well as the formation of lateral loops of lampbrush chromosomes, remains to be determined.

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