

REPOPULATION OF POSTMITOTIC NUCLEOLI BY PREFORMED RNA

II. Ultrastructure

DAVID M. PHILLIPS and STEPHANIE GORDON PHILLIPS

From the Department of Biology, Washington University, St. Louis, Missouri 63130 and the Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

The reconstruction of the nucleolus after mitosis was analyzed by electron microscopy in cultured mammalian (L929) cells in which nucleolar RNA synthesis was inhibited for a 3 h period either after or before mitosis. When synchronized mitotic cells were plated into a concentration of actinomycin D sufficient to block nucleolar RNA synthesis preferentially, nucleoli were formed at telophase as usual. 3 h after mitosis, these nucleoli had fibrillar and particulate components and possessed the segregated appearance characteristic of nucleoli of actinomycin D-treated cells. Cells in which actinomycin D was present for the last 3 h preceding mitosis did not form nucleoli by 3 h after mitosis though small fibrillar prenucleolar bodies were detectable at this time. These bodies subsequently grew in size and eventually acquired a particulate component. It took about a full cell cycle before nucleoli of these cells were completely normal in appearance. Thus, nucleolar RNA synthesis after mitosis is not necessary for organization of nucleoli after mitosis. However, inhibition of nucleolar RNA synthesis before mitosis renders the cell incapable of forming nucleoli immediately after mitosis. If cells are permitted to resume RNA synthesis after mitosis, they eventually regain nucleoli of normal morphology.

INTRODUCTION

Several lines of evidence suggest that ribosomal RNA precursors synthesized in the nucleolus just before mitosis are maintained in the cell in a stable form during mitosis and repopulate nucleoli after mitosis. There is no detectable RNA synthesis during mitosis, from late prophase until telophase (Taylor, 1960; Feinendegen and Bond, 1963). Ribosomal RNA precursor molecules have been isolated from mitotic cells and shown to be stable for the duration of mitosis (Fan and Penman, 1971). It has been demonstrated in amoeba and in cultured mammalian cells that if one prevents the

resumption of ribosomal RNA synthesis after mitosis, nucleoli form nevertheless (Stevens and Prescott, 1971; Phillips, 1972). These nucleoli contain RNA which was presumably formed before mitosis and was stable during mitosis. In addition, it was found that inhibition of the nucleolar RNA synthesis which normally occurs just before mitosis results in the absence of any large, RNA-containing bodies in nuclei which form after mitosis (Phillips, 1972).

In the present study we have made electron microscope observations to complement previous

light microscope cytochemical studies on the postmitotic formation of nucleoli in cells which were inhibited from synthesizing ribosomal RNA before or after mitosis. We have also followed the fate of cells premitotically depleted of nucleolar RNA which have reentered interphase without the concomitant formation of RNA-containing nucleoli.

MATERIALS AND METHODS

L929 cells (originally obtained from the American Type Culture Collection Rockville, Md.) were maintained in monolayer culture in Joklik's modified minimum essential medium (MEM) supplemented with 10% fetal calf serum. Mitotic populations of cells were harvested from roller bottles or Blake bottles by mechanical selection (Terasima and Tolmach, 1963; Robbins and Marcus, 1964). At the time of harvest, the percent mitotic cells was determined in a small aliquot of cells fixed in 3 parts glacial acetic acid: 1 part ethanol, air dried on a slide, and stained with Giemsa's. Mitotic indexes of cells collected for these experiments ranged from 79 to 91%. The population of mitotic cells was pelleted by centrifugation, resuspended in medium, and plated into Falcon plastic 35 × 2 mm Petri dishes. If cells were to be examined by light microscopy, they were plated onto a 22 × 22 mm cover slip which had been inserted into the Petri dish. In cases where cultures were treated with actinomycin D for 3 h before mitotic cells were harvested, removal of the actinomycin D was accomplished by pelleting the mitotic cells, resuspending them in 10 ml Hanks' balanced salt solution, and repelleting them before resuspending them to plate out in medium without actinomycin D. In all experiments, actinomycin D (a gift of Merck, Sharp and Dohme of Canada Ltd., Montreal, Quebec, Canada) was used at 0.08 µg/ml, a concentration found to inhibit nucleolar RNA synthesis preferentially in earlier autoradiographic experiments done with L929 cells in our laboratory.

For electron microscopy, cultures were fixed for 0.5–12 h in 0.5 × Hanks' balanced salt solution containing 2% glutaraldehyde (TAAB Laboratories, Reading, England) and a final collidine concentration of about 0.02 M at pH 7.2. Cultures were then rinsed several times in 0.2 M collidine (total time 1–5 min) and postfixed in 1% OsO₄ in 0.1 M collidine (pH 7.2) for 1–4 h. Cultures were subsequently dehydrated to 100% alcohol. The alcohol was decanted and replaced with a few drops of Epon which was spread over the surface by rocking the Petri dish. After polymerization, the Epon was separated from the Falcon plastic and pieces of Epon containing the cells were mounted with epoxy glue on blank Epon capsules. Sections were cut parallel to the culture

surface, stained overnight in 3% aqueous uranyl acetate and subsequently in lead citrate (Venable and Coggeshall, 1965), and were examined in a Siemens Elmiskop IA microscope. Micrographs of more than 50 pairs of postmitotic cells were taken for each experimental treatment. The morphological appearance of nucleoli was very uniform for each treatment.

For light microscopy, cells were fixed by the addition of a mixture of 3 parts ethanol:1 part acetic acid in a volume equal to the volume of the medium. Adding the fixative to the medium (rather than replacing the medium with fixative) was found helpful in preventing the shrinkage of the nucleus away from the cytoplasm which often occurs as an artifact of acid fixation. After 1.5–3 h of fixation, cells were stained for RNA with Azure B (Flax and Himes, 1952).

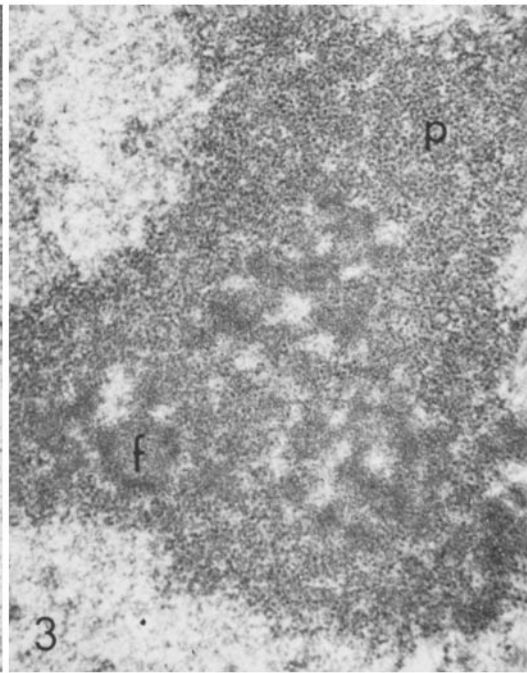
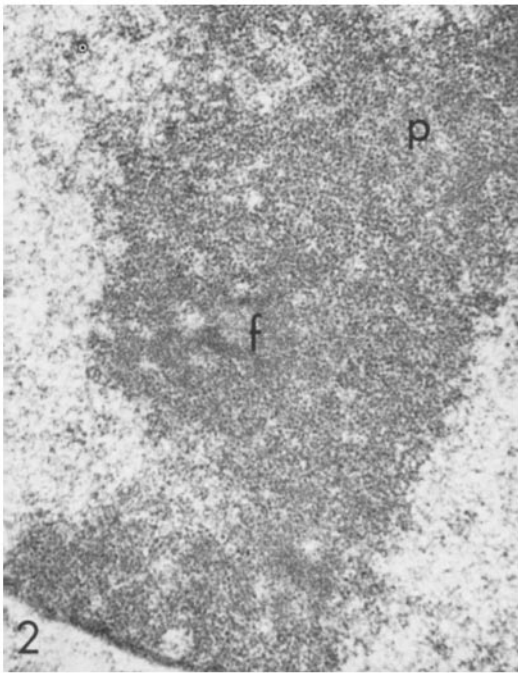
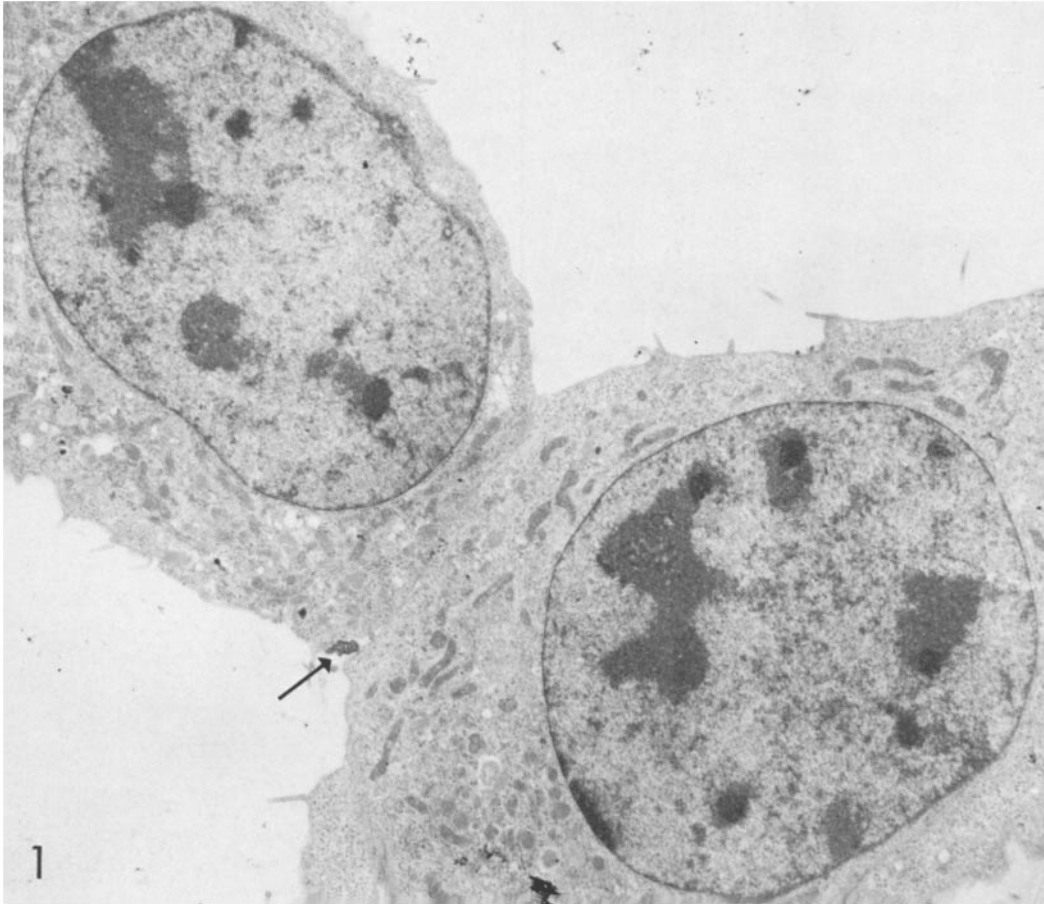
RESULTS

Fine Structure of Nucleoli after Mitosis in Untreated Cells

3 h after mitosis untreated L cells have large, well-organized nucleoli (Figs. 1–3). These nucleoli are irregular in outline and consist principally of a loose meshwork of particulate material. Fibrillar material is distributed in small, often somewhat globular areas embedded within the granular material. These areas, termed fibrillar cores (Recher et al., 1969), are composed of light fibrillar material in the center surrounded by denser fibrillar material. Nucleoli of cells fixed 3 h after mitosis were found to contain a high concentration of RNA as revealed by staining with Azure B (Phillips, 1972).

Postmitotic Nucleoli Formed in the Presence of Actinomycin D

When actinomycin D, 0.08 µg/ml, was added to cells at the time of mitosis, formation of nucleoli accompanied the return to interphase. These nucleoli appeared dense and rounded in cells fixed 3 h after mitosis. In electron micrographs they uniformly had a segregated morphology, often appearing as a ball of fibrillar material with a granular cap (Figs. 4–6). This segregated appearance is characteristic of nucleoli of cells in which RNA synthesis is inhibited (Schoeffl, 1964; Reynolds et al., 1964; Jézéquel and Bernhard, 1964; Phillips and Phillips, 1971). Though the level of actinomycin D used was sufficient to block nucleolar RNA synthesis, the segregated nucleoli



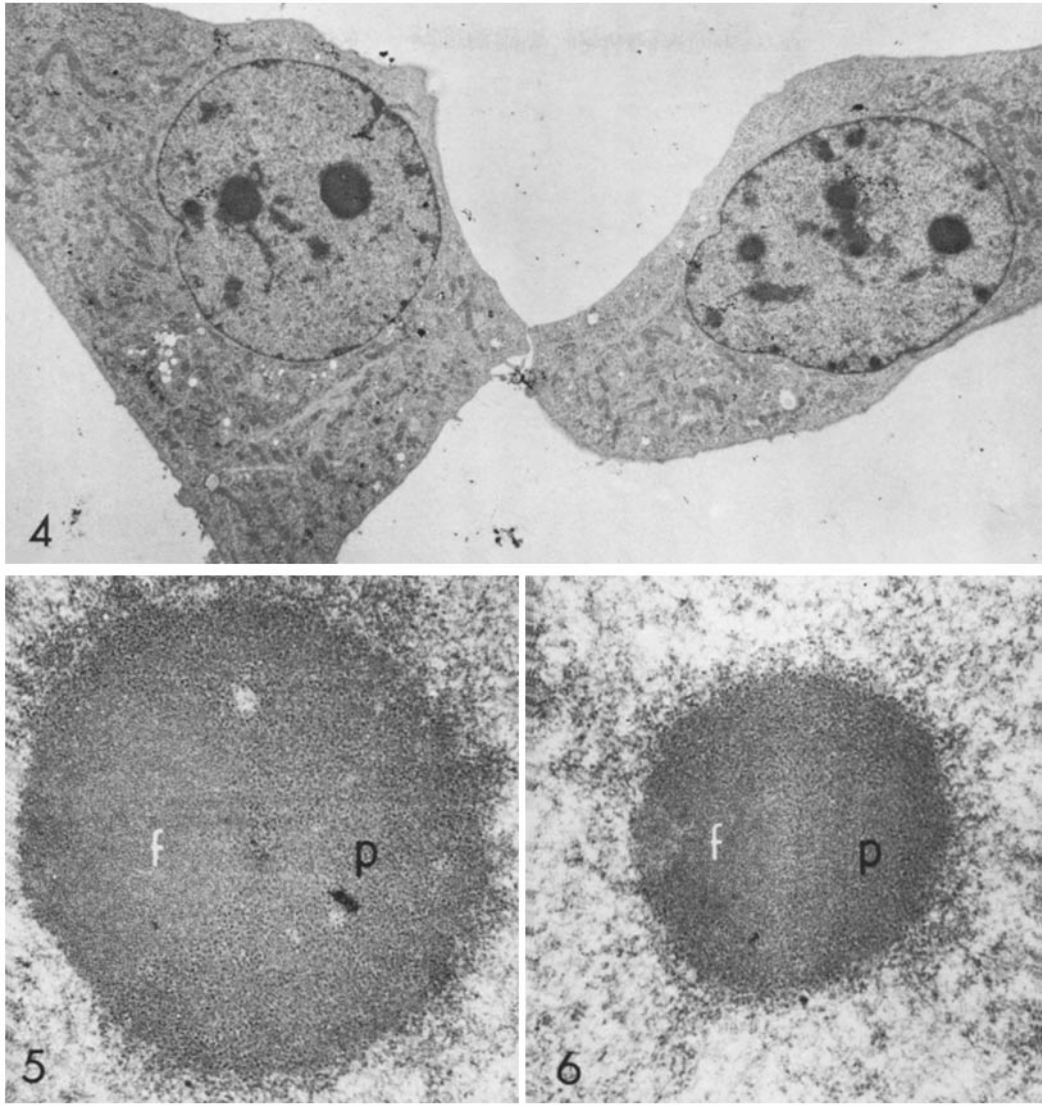


FIGURE 4 Pair of L cells plated into actinomycin D ($0.08 \mu\text{g/ml}$) at the time of mitosis and fixed 3 h later. Nuclei contain a considerable amount of condensed chromatin. Nucleoli have formed, but they have a roundish, compact appearance. $\times 4,500$.

FIGURES 5 and 6 Higher magnification views of two of the nucleoli shown in Fig. 4. Nucleoli appear segregated into particulate (*p*) and fibrillar regions (*f*). ($\times 23,000$).

FIGURE 1 Pair of untreated (control) L cells fixed 3 h after mitotic selection. Large, irregularly shaped nucleoli appear in the nuclei. Arrow indicates remnant of midbody. $\times 6,500$.

FIGURES 2 and 3 Higher magnification views of two of the nucleoli shown in Fig. 1. Most of the nucleoli consist of a loose meshwork of particulate material (*p*) interspersed with occasional fibrillar regions (fibrillar cores) (*f*) containing an outer region denser than the inner region. $\times 23,000$.

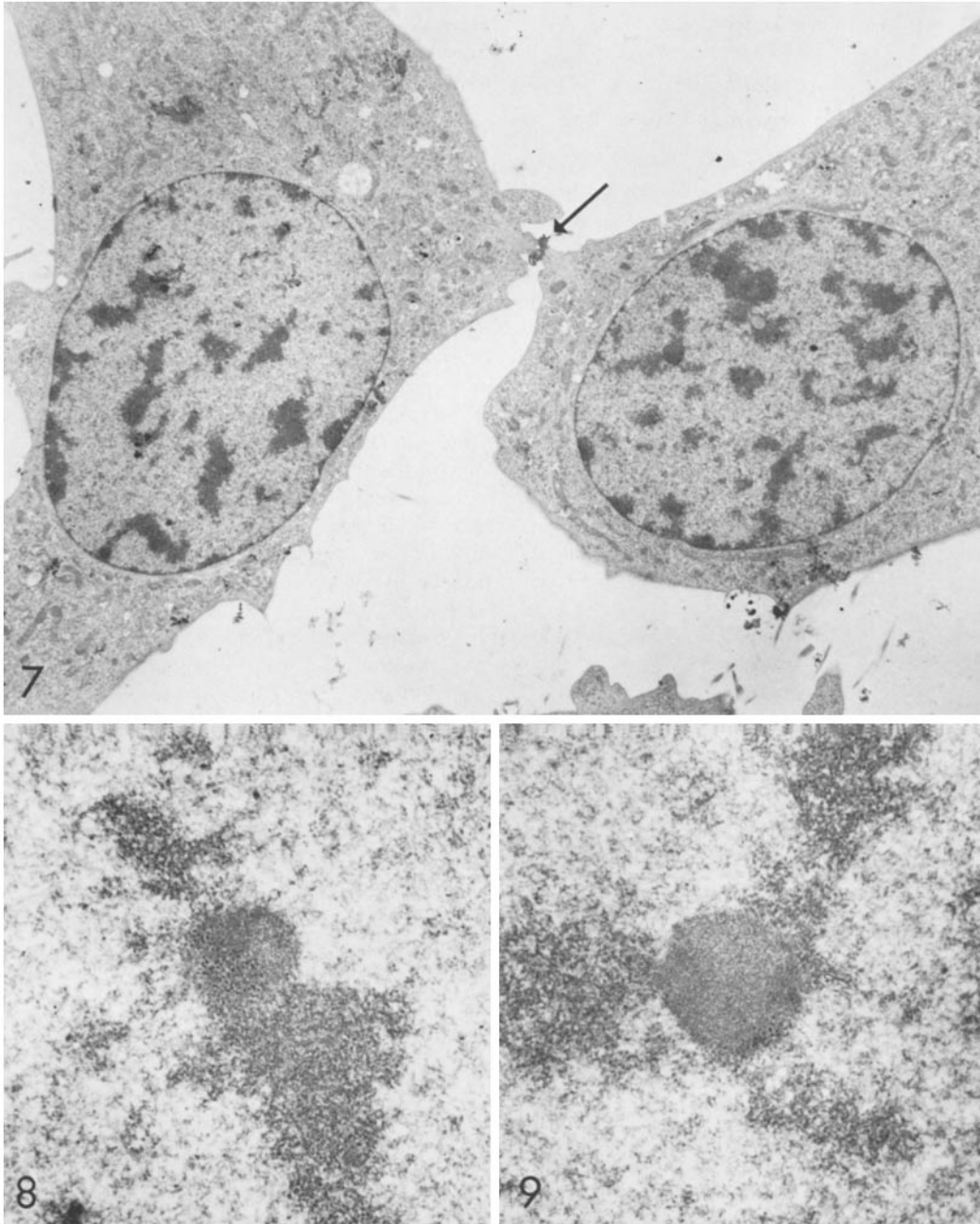


FIGURE 7 Pair of L cells which were incubated for 3 h before mitosis with actinomycin D, washed at the time of mitosis, allowed to return to interphase in the absence of actinomycin D, and fixed 3 h after mitosis. Much clumped chromatin is present in the nucleus, but no obvious nucleoli are visible. Arrow indicates remnant of midbody. $\times 5,000$.

FIGURES 8 and 9 Higher magnification views of areas of the nuclei of the two cells shown above. Small roundish bodies appear within the clumped chromatin. These bodies are composed of segregated dense fibrillar and lighter fibrillar components with a suggestion of a rim of particulate material. Their morphology is thus reminiscent of nucleoli of actinomycin D-treated cells. They are probably the precursor of larger nucleolus-like bodies seen at later times after mitosis. $\times 23,000$.

were shown by Azure B staining to contain RNA concentrated in a caplike region (Phillips, 1972).

Nucleoli of Cells Treated before Mitosis with Actinomycin D

It was previously shown that cells in which nucleolar RNA synthesis was inhibited for 3 h before mitosis failed to form RNA-containing nucleoli in the first 3 h after mitosis. That is, at the light microscope level their nuclei were devoid of large bodies which stained for RNA (Phillips, 1972). The nuclei did contain phase-dense, fast green stainable material which was thought to consist of decondensing chromatin and nucleolar proteins.

In the experiments to be described, cells were treated with actinomycin D for the last 3 h before mitosis and were allowed to return to interphase in the absence of actinomycin D. They were fixed for electron microscopy at intervals after mitosis. At the level of the electron microscope, as was the case at the light microscope level, no bodies resembling normal nucleoli were seen in cells fixed 3 h after mitosis (Figs. 7-9). This was a consistent finding in cells fixed at this time. A considerable amount of condensed chromatin was seen in the nuclei; apparently, the actinomycin D pretreatment caused an abnormal lag in decondensation of the chromatin after mitosis. Very small rounded bodies could be discerned in the nuclei. These were composed mostly of fibrillar material which was segregated into more and less electron-opaque components. Sometimes a rim of particulate material was apparent (Figs. 8 and 9). These tiny bodies (about 0.5 μm in diameter) which occurred at 3 h after mitosis were most likely the precursors of the nucleoli seen at later times during the recovery from premitotic inhibition of RNA synthesis.

By 8 h after mitosis, roundish fibrillar bodies were much more numerous than earlier and they were larger in size (Figs. 10-12). They were still composed principally of segregated light fibrillar and denser fibrillar regions and were often seen to be closely associated with condensed chromatin. Peripheral dense granules occurred in the nucleolar bodies, but the granular region was small in comparison to the fibrillar regions. Nuclei of many, but not all, cells fixed 8 h after mitosis contained small dense spheres in addition to the larger nucleolar bodies. These electron-opaque

spheres differed from chromatin in their appearance; they were frequently seen in configurations suggesting that they were fusing with the nucleolar bodies. Possibly they represented accumulations of nucleolar structural proteins.

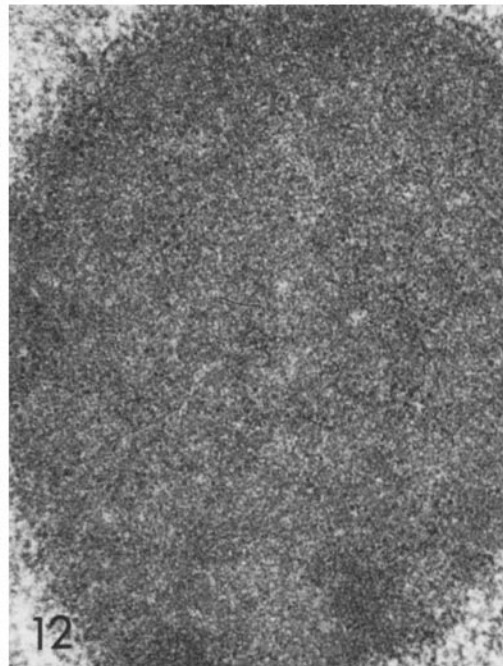
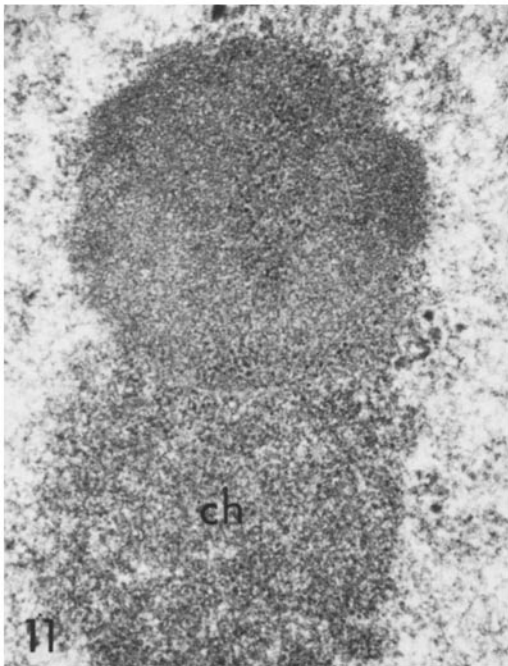
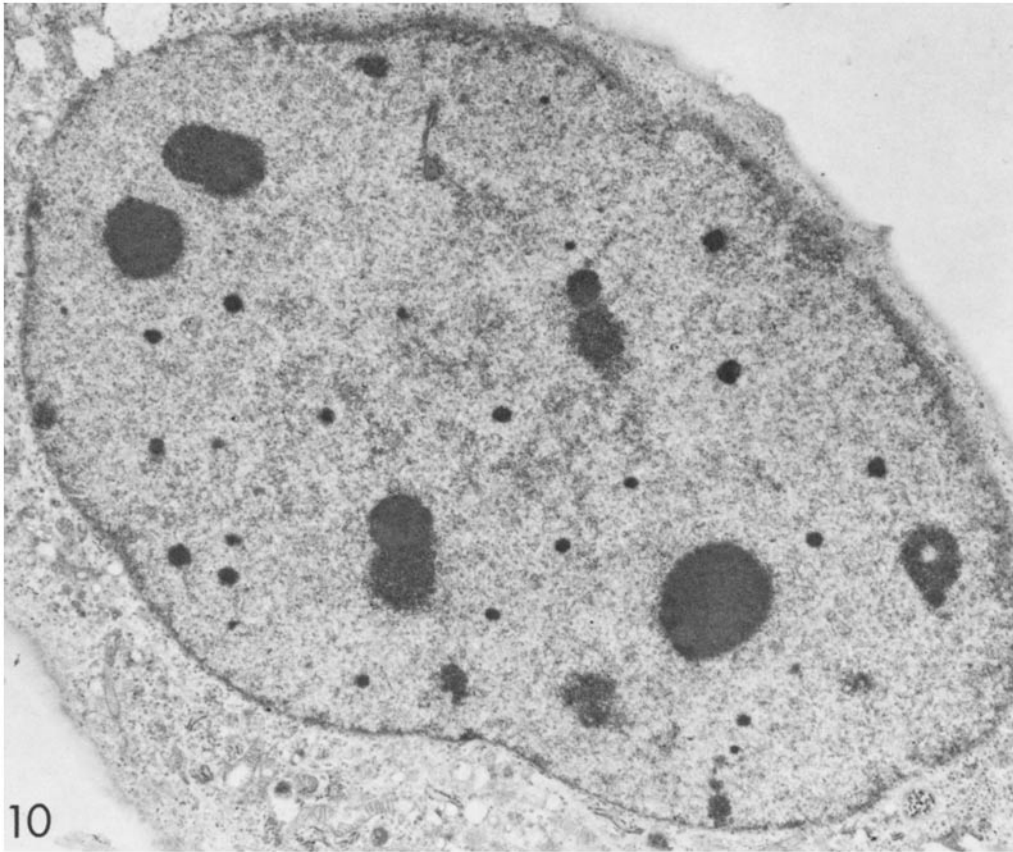
In light microscope preparations, the nucleolar bodies seen 8 h after mitosis were found to stain for RNA. They generally had a visibly segregated appearance, with one portion more intensely stained than the other. These bodies occurred in higher numbers per nucleus than did the nucleoli of normal, untreated control cells. The bodies continued to increase in size with time after mitosis. They became more intensely stainable for RNA and gradually lost their segregated morphology. The cytoplasm of the cells recovering from actinomycin D appeared (on the basis of staining) to contain less RNA than control cells. However, in cultures fixed 18-24 h after mitosis, many division figures were seen. This is about the normal interdivision time in untreated L cells. Thus, the deficiency in nucleolar RNA metabolism did not appear to have a grossly detrimental effect on progress through the cell cycle.

By electron microscopy, cells fixed 23 h after premitotic actinomycin D treatment appeared to have recovered their normal nucleolar morphology. Nucleoli were no longer segregated in appearance; fibrillar and granular regions were interspersed in a reticular-appearing matrix (Figs. 13-15).

DISCUSSION

In this study we have shown that postmitotic nucleolar RNA synthesis is not required for the formation of nucleoli in the G_1 nucleus. In previous work (Phillips, 1972) we found that nucleoli which formed in the presence of actinomycin D contained RNA; in the present study we have shown that these nucleoli possess the fine structure typical of nucleoli in actinomycin D-treated cells (Schoeff, 1964; Jézéquel and Bernhard, 1964; Phillips and Phillips, 1971).

A second observation of this study is that inhibition of nucleolar RNA synthesis for a period before mitosis results in the absence of nucleoli from the cells in the ensuing G_1 . Though G_1 cells treated before mitosis with actinomycin D do not contain bodies resembling typical nucleoli by either light or electron microscopic criteria, they do contain small fibrillar spheres visible in the electron micro-



scope which, with time, may develop into nucleoli. This observation suggests that the ribosomal RNA precursors normally found in the nucleolus play a role in maintaining the morphological appearance of the nucleolus. This may be related to the finding that ribosomal proteins which are synthesized in the cytoplasm (Craig and Perry, 1971) during suppression of ribosomal RNA synthesis do not appear in the nucleolus (Wu et al., 1971) and cannot be utilized later to make mature ribosomes (Craig and Perry, 1971). Apparently, coordinate synthesis of the ribosomal precursor RNA and ribosomal proteins is required for their successful assembly into a ribosomal particle. It seems likely that this assembly normally occurs in the nucleolus soon after synthesis of the ribosomal precursor RNA (Warner and Soeiro, 1967; Liau and Perry, 1969). The RNP particles thus formed may be the major constituents determining the morphological as well as the biochemical (Kumar and Warner, 1972) features of the nucleolus.

A third observation of our study is that cells depleted of their nucleolar RNA by actinomycin D treatment before mitosis eventually form nucleoli if allowed to recover from mitosis in the absence of actinomycin D. At early times (3 h) after mitosis small fibrillar prenucleolar bodies are seen. These, with time, increase in size and stainability for RNA. However, it takes nearly a whole cell cycle before nucleoli in these cells have recovered a size and morphology which are indistinguishable from the size and fine structure of nucleoli of untreated control cells. This would suggest that cells are capable of organizing nucleoli at times other than telophase though the normal pattern is to organize nucleoli at telophase at the nucleolar organizer sites (Heitz, 1931; McClintock, 1934) from pre-existing nucleolar RNA and protein.

We would like to thank David Desper and Miriam

Lifisics for their competent and cheerful assistance during the course of these experiments.

Supported by National Science Foundation Research Grant GB-29214.

Received for publication 27 December 1972.

REFERENCES

- CRAIG, N., and R. P. PERRY. 1971. Persistent cytoplasmic synthesis of ribosomal proteins during the selective inhibition of ribosomal RNA synthesis. *Nat. New Biol.* 229:75.
- FAN, H., and S. PENMAN. 1971. Regulation of synthesis and processing of nucleolar components in metaphase-arrested cells. *J. Mol. Biol.* 59:27.
- FEINENDEGEN, L. E., and V. P. BOND. 1963. Observations on nuclear RNA during mitosis in human cancer cells in culture (HeLa-S₃), studied with tritiated cytidine. *Exp. Cell Res.* 30:393.
- FLAX, M. H., and M. H. HIMES. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.* 25:297.
- HEITZ, E. 1931. Die Ursache der gesetzmässigen Zahl, Lage, Form, und Grösse pflanzlicher Nucleolen. *Planta (Berl.)* 12:775.
- JÉZÉQUEL, A. M., and W. BERNHARD. 1964. Modifications ultrastructurales du pancréas exocrine de rat sous l'effet de l'actinomycine D. *J. Microsc. (Paris)* 3:279.
- KUMAR, A., and J. R. WARNER. 1972. Characterization of ribosomal precursor particles from HeLa cell nucleoli. *J. Mol. Biol.* 63:233.
- LIAU, M. C., and R. P. PERRY. 1969. Ribosome precursor particles in nucleoli. *J. Cell Biol.* 42:272.
- MCCCLINTOCK, B. 1934. The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z. Zellforsch. Mikrosk. Anat.* 21:294.
- PHILLIPS, D. M., and S. G. PHILLIPS. 1971. Distinctive characteristics of nucleoli of two established cell lines. *J. Cell Biol.* 49:803.
- PHILLIPS, S. G. 1972. Repopulation of the postmitotic nucleolus by preformed RNA. *J. Cell Biol.* 53:611.

FIGURE 10 Cell treated for 3 h before mitosis with actinomycin D and allowed to return to interphase in the absence of actinomycin D. Fixed 8 h after mitosis. Large roundish nucleolar bodies occur in the nucleus. At the light microscope level they are found to stain for RNA. Small, roundish dense fibrillar blobs are probably proteinaceous. They often appear to be in association with the nucleolar bodies. $\times 8,500$.

FIGURES 11 and 12 Higher magnification view of two nucleolar bodies shown in Fig. 10. They are composed principally of fibrillar components and are closely associated with clumped chromatin (*ch*). $\times 23,000$.

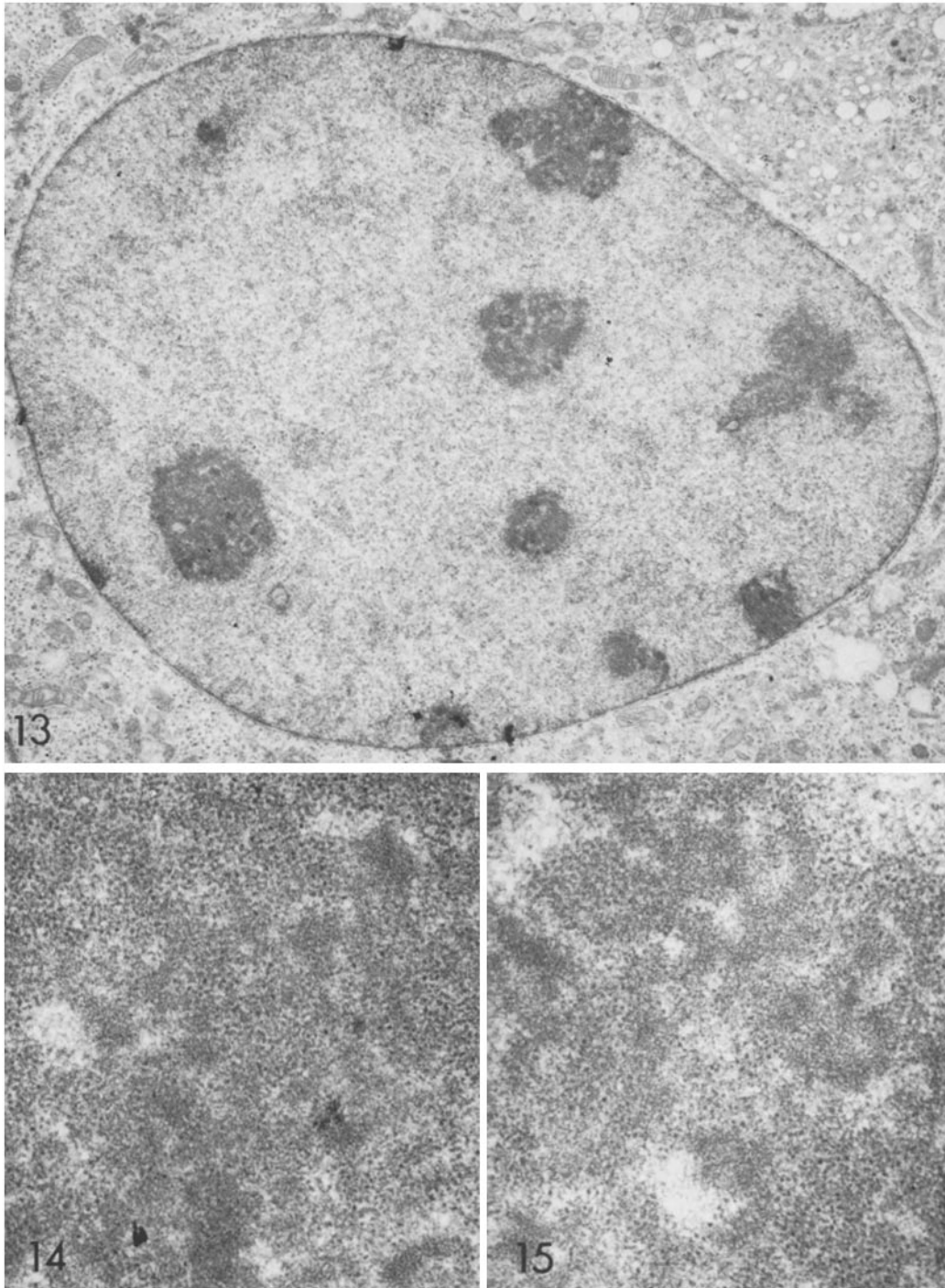


FIGURE 13 Cell from culture treated 3 h before mitosis with actinomycin D, washed at the time of mitotic selection, and plated out in the absence of actinomycin D. Fixed 23 h after mitotic selection. Large nucleoli occur in nucleus. They are somewhat roundish in outline but are otherwise indistinguishable from nucleoli of untreated control cells as seen in Fig. 1. $\times 8,500$.

FIGURES 14 and 15 Higher magnification view of two of the nucleoli shown in Fig. 13. Interspersed particulate and fibrillar components are apparent. $\times 26,000$.

- RECHER, L. J., J. WHITESCARVER, and L. BRIGGS. 1969. The fine structure of a nucleolar constituent. *J. Ultrastruct. Res.* **29**:1.
- REYNOLDS, R. C., P. O'B. MONTGOMERY, and B. HUGHES. 1964. Nucleolar "caps" produced by actinomycin D. *Cancer Res.* **24**:1269.
- ROBBINS, E., and P. I. MARCUS. 1964. Mitotically synchronized mammalian cells: a simple method for obtaining large populations. *Science (Wash. D. C.)*. **144**:1152.
- SCHOEFL, G. I. 1964. The effect of actinomycin D on the fine structure of the nucleolus. *J. Ultrastruct. Res.* **10**:224.
- STEVENS, A. R., and D. M. PRESCOTT. 1971. Reformation of nucleolus-like bodies in the absence of post-mitotic RNA synthesis. *J. Cell Biol.* **48**:443.
- TAYLOR, J. H. 1960. Nucleic acid synthesis in relation to the cell division cycle. *Ann. N. Y. Acad. Sci.* **90**:409.
- TERASIMA, T., and L. S. TOLMACH. 1963. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp. Cell Res.* **30**:344.
- VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.
- WARNER, J. R., and R. SOEIRO. 1967. Nascent ribosomes from HeLa cells. *Proc. Natl. Acad. Sci. U. S. A.* **58**:1984.
- WU, R. S., A. KUMAR, and J. WARNER. 1971. Ribosome formation is blocked by camptothecin, a reversible inhibitor of RNA synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **68**:3009.