THE NUCLEOLI IN MITOTIC DIVISIONS OF MAMMALIAN CELLS *IN VITRO*

T. C. HSU, FRANCES E. ARRIGHI, ROBERT R. KLEVECZ, and B. R. BRINKLEY

From the Section of Cytology, Department of Biology, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas

ABSTRACT

In a number of mammalian cell strains nucleoli persisted through mitosis. This phenomenon was especially pronounced in several cell lines derived from Chinese hamster tissues. All the methods employed, including radioautography with tritiated uridine, cytochemical stains (methyl green–pyronin and azure B), fluorescent microscopy (coriphosphine O), ribonuclease digestion, and electron microscopy, demonstrated that the bodies identified as persistent nucleoli in the mitotic stages had the same characteristics as did the nucleoli in the interphase. Persistent nucleoli may attach to the chromosomes or may be free in the cytoplasm. In cells where no persistent nucleoli as such were noted, nucleolar material was observed to attach to the chromosomes in shapeless masses which moved with the chromosomes during anaphase. At least a portion of the nucleolar material was included in the daughter nuclei, presumably for immediate use for protein synthesis after cell division.

In their study on the effect of 5-fluorodeoxyuridine (FUdR) on animal cells *in vitro*, Hsu *et al.* (1) found that mitotic cells recovering from the FUdR block exhibited persistent nucleoli at metaphase and anaphase. In an attempt to analyze the phenomenon further, we used a newly established cell line from a female Chinese hamster and discovered that nucleoli persisted in division stages without any pretreatment. This accidental finding prompted us to investigate in detail the fate of nucleoli during mitotic phases in a number of mammalian cells *in vitro*.

MATERIALS

The following cell strains were employed in this study.

PRIMATES

Human fetal lung: An early subculture of fetal lung fibroblasts.

KB: A human carcinoma line originally established by Eagle (2).

Alouatta villosa: An early subculture of skin fibroblasts from a female howler monkey.

Lagothrix ubericolor: An early subculture of skin fibroblasts from a male woolly monkey.

RODENTIA

LM: A subline of strain L mouse fibroblasts (3).

Don: A diploid fibroblast line from a male Chinese hamster (4).

Dede: A diploid fibroblast line from a female Chinese hamster (5).

Mary L: A diploid fibroblast line from a female Chinese hamster, established in 1963 from a lung culture.

B14FAF28: An aneuploid line from a female Chinese hamster (6).

Peromyscus difficilis: An early subculture of fibroblasts established from a biopsy of an adult male ear.

Aschizomys niigatae: An early subculture of lung fibroblasts established from a male animal collected in Japan by Dr. Murray L. Johnson, University of Puget Sound, Tacoma, Washington.

Aschizomys andersoni: An early subculture of lung fibroblasts established from a male animal collected in Japan by Dr. Murray L. Johnson, University of Puget Sound, Tacoma, Washington.

EDENTATA

Myrmecophaga tridactyla: An early subculture of skin fibroblasts from a male giant anteater.

Tamandua tetradactyla: An early subculture of skin fibroblasts from a female collared anteater.

MARSUPIALIA

Pt-K1: A permanent epithelial cell line from a kidney of a female wallaby, *Potorous tridactylus*, established by the University of California Naval Biological Research Laboratory, Oakland, California.

CARNIVORA

Ferret: An early subculture of skin fibroblasts from a male ferret.

Black bear: An early subculture of skin fibroblasts from a male black bear, *Ursus americanus*.

PERISSODACTYLA

Hinny: An early subculture of skin fibroblasts from a hinny, the hybrid between stallion and donkey.

All cell lines were grown as monolayer cultures in a modified McCoy's 5a medium supplemented with 20 per cent fetal calf serum. The KB line was carried in Eagle's basal medium supplemented with 10 per cent calf serum.

METHODS

Cells in the logarithmic growth period were dislodged from the culture flasks with 0.2 per cent trypsin solution. They were immediately centrifuged without pretreatments such as colchicine and hypotonic solution, and fixed as a pellet for approximately 20 minutes with either Carnoy's solution (3 parts ethyl alcohol to 1 part glacial acetic acid) or 50 per cent acetic acid. The cells fixed in Carnoy's solution were washed in fresh fixative twice and dried in air according to the method of Moorhead *et al.* (7). When cells were fixed with 50 per cent acetic acid, squash preparations were made and either left unstained or stained with dilute acetic orcein. Squash preparations were sealed with Krönig's wax for temporary storage and observation, and the coverslips were removed by the method of Conger and Fairchild (8). The slides were rinsed twice in alcohol and dried in air for furthur processing.

The following techniques were applied.

ACETIC ORCEIN: This stain was used extensively in the present study for convenience. Temporary acetic orcein preparations were examined and photographed with phase contrast optics. The contrast of the nucleoli was generally lower than that of the chromosomes.

AZURE B: The cells were fixed in 50 per cent acetic acid and squashed directly in the fixative. After the coverslips were flipped off and the preparations rinsed with alcohol, the slides were stained with an aqueous solution of azure B (0.1 per cent) for approximately 1 minute. The slides were then rinsed in tap water, air-dried, and mounted in Permount. The chromosomes stained blue, whereas the nucleoli showed a lavender coloring.

METHYL GREEN-PYRONIN (PMG): Cells were fixed with Carnoy's solution and air-dried. The preparations were stained with methyl green and pyronin according to the procedure of Kurnick (9). The chromosomes stained green, and the nucleoli stained red.

ACRIDINE DYE FLUORESCENT MICROSCOPY: Cells were either fixed with Carnoy's solution and air-dried or squashed in 50 per cent acetic acid. The preparations were rinsed in McIlvain's citrate phosphate buffer (pH 5.6), and stained for 2 minutes in coriphosphine O diluted 1:100 from the original 1 per cent stock solution in McIlvain's buffer. The preparations were then rinsed in 5 changes of the buffer for 1 minute each, mounted in the buffer, and sealed with Krönig's wax. They were examined with the aid of a Leitz fluorescence attachment fitted to a Reichert Zetopan microscope. The chromosomes fluoresced yellow, and the nucleoli, red.

RADIOAUTOGRAPHY: In one experiment, cultures were pulse labeled for 20 minutes with H^3-uridine (specific activity 6.0 c/mM) at a final concentration of 8 μc/ml. Immediately after the labeling period, the cells were fixed with 50 per cent acetic acid. In the second experiment, cultures were labeled for 1 minute with H^3-uridine (specific activity 13.0 c/mM) at a final concentration of 10 μc/ml. The cul-cultures were rinsed with warm medium containing 5 μg/ml of non-radioactive uridine, and allowed to incubate in this chasing medium for an additional 45 minutes before fixation. Acetic orcein squash preparations were made for photography before the application of Kodak AR 10 stripping film. After proper exposure time (14 days for the first experiment and 42 days for the second), the slides were developed and restained with dilute Giemsa's stain. Bright field microscopy was used to study the radioautographs.

RIBONUCLEASE TREATMENT: Air-dried cells stained with azure B were placed in a Petri dish containing a moistened filter paper. Ribonuclease solution (2 mg/ml, Worthington Biochemical Corporation, Freehold, New Jersey) was placed directly over the cells with a Pasteur pipette and covered with a large coverslip. After digestion at 37°C for 2 hours, the preparations were rinsed with tap water and stained again with azure B.

ELECTRON MICROSCOPY: Mitotic cells of monolayer cultures were collected by light trypsiniza-

tion. They were collected by centrifugation and fixed immediately in either Palade's 1 per cent osmium tetroxide buffered with Veronal-acetate at pH 7.6 or 2.5 per cent glutaraldehyde buffered with Veronal-acetate at pH 7.6 (10). The cells were resuspended, centrifuged again, and covered with fresh fixative for 1 hour at 0–4°C and 25°C. The cells fixed in glutaraldehyde were rinsed in Veronal buffer after 1 hour and postfixed in 1 per cent osmium tetroxide for 30 minutes.

After fixation, the cell pellets were dehydrated in a graded series of ethanol and embedded in prepolymerized methacrylate (3 parts *n*-butyl to 2 parts ethyl methacrylate) containing 1 per cent benzoyl peroxide.

Thin sections were cut with glass knives on a Servall MT-2 Porter-Blum ultramicrotome, picked up on Formvar-coated stainless steel grids, and stained for 20 to 30 minutes with a saturated aqueous solution of uranyl acetate. Some sections were poststained in lead citrate for 20 minutes.

Examinations were made on a Hitachi HU-11A electron microscope operated at 75 and 100 kv with a 20-μ objective aperture.

RESULTS

Cytological Verification of Persistent Nucleoli

The cellular components which were presumed to be persistent nucleoli were subjected to a variety of cytochemical tests to ascertain whether they were RNA-rich bodies and had an ultrastructure similar to that of the nucleoli in interphase. All tests suggested that these were true nucleoli. They appeared red in PMG preparations (chromosomes green), lavender-tinted in azure B (chromosomes blue), fluoresced red with acridine dyes (chromosomes yellow), and were labeled with H^3-uridine. Radioautography was especially convincing for the following reasons: (*a*) the objects were photographed before and after film development so that nucleoli were unequivocally identified in the original pictures; and (*b*) the time between labeling and fixation was so short that no cell was expected to be in the DNA synthetic stage. Thus uridine should not be incorporated into the DNA of the mitotic cells which were the objects of inquiry.

The Behavior of Persistent Nucleoli in Chinese Hamster Cells

The nucleoli were very conspicuous during prophase of all Chinese hamster cell strains. Radioautographs with H^3-uridine labeling showed a heavy uptake of this RNA precursor into the nucleoli (Fig. 1). It is of interest to note that the nucleoli in Fig. 1 were actively synthesizing RNA during prophase (the culture was labeled for 20 minutes), apparently more so than the semicondensed chromosomes. Also, the label in the largest nucleolus appears to have a definite pattern.

In most metaphase figures of strains Dede and Mary L, a number of nucleoli were present in each cell. Many appeared to attach to the ends of chromosomes (Fig. 2), while others were free in the cytoplasm (Fig. 3). The number of persistent nucleoli per cell was difficult to estimate because the nucleoli were of various sizes and shapes, some so small as to escape notice. For example, besides the three conspicuous nucleoli in the cytoplasm in Fig. 3, at least two small nucleoli (Nu) can be seen attached to the chromosomes. The end-to-end connections between chromosomes (double-shafted arrows) may also represent tiny pieces of nucleolar material pulled by chromosomal movement.

Nucleoli attached to metaphase chromosomes generally moved with the chromosomes during anaphase (Figs. 4 and 5). The cell shown in Fig. 4 was from an H^3-uridine labeling experiment. The culture was labeled for 1 minute with H^3-uridine, after which it was incubated for 45 minutes in a medium containing an excess quantity of nonradioactive uridine. The silver grains over a persistent nucleolus attached to one group of daughter chromosomes were heavier than in any other area of corresponding size. Since the cells were labeled 45 minutes before fixation, the precursor was probably introduced during the late G2 period. The nucleolus apparently retained its integrity through all its movements in mitosis.

As can be seen from Fig. 4, the distribution of nucleoli in the daughter cells was apparently not equal. Fig. 6 provides another example in which two large nucleoli, free in the cytoplasm, were located near one daughter nucleus while none was at the sister half. In one instance, we observed that one of the two newly formed daughter cells contained a large nucleolus in the cytoplasm outside the nucleus, whereas the sister cell had no visible nucleoli. Of particular interest is the observation that nucleolar material can be pulled and stretched during anaphase when it is attached to the ends of both sister chromosomes (Fig. 7). In

the cell depicted in Fig. 8, two stretched nucleoli simulate the appearance of chromatin bridges.

During metaphase, anaphase, and telophase numerous granules of varying sizes were seen in the cytoplasm (Figs. 5 to 8). To judge from staining reactions, such granules were clearly rich in RNA. It is not known, however, whether they represented partially disintegrated nucleoli.

In metaphase and anaphase figures where conspicuous nucleoli were not present, RNA-rich bodies could still be detected along chromosomes by PMG staining, especially when the preparations were examined with phase contrast optics. Figs. 9 and 10 were photographed through a yellow-green filter to enhance the pyronin staining; therefore the chromosomes, being green, are shown only as negative images. In Fig. 9 a few medium-sized lumps of RNA-rich bodies are located alongside the chromosomes; but in Fig. 10 only numerous pyronin-positive granules are present. Note that the distribution of pyronin-positive bodies is very uneven along the metaphase chromosomes.

The uneven "coat" of nucleolar material along the chromosomes is further shown in Fig. 11 *a*, which was taken from an azure B preparation. In black and white photography, the lavender-colored nucleolar material shows lighter contrast than the blue chromosomes. Most of such substance was digestible by ribonuclease. Fig. 11 *b* shows the same cell after ribonuclease treatment. The nucleolar material as well as the cytoplasmic background no longer took up the stain, indicating the removal of ribonucleic acid. When these two figures are compared, many areas originally showing only RNA staining revealed chromosomes after digestion (arrows in Fig. 11 *a*). However, not all the RNA-rich material was completely removed by ribonuclease; in the areas where chromosomes aggregated, a certain purplish hue was still detectable. It is possible that, after staining, the binding of the dye to RNA was so intimate that the enzyme was no longer able to digest it.

Cells of strain Dede examined with the electron microscope were seen to contain from one to several prominent nucleoli in both the interphase nuclei and the cytoplasm of dividing cells. During interphase, the nucleoli appeared as compact bodies containing numerous electron-opaque granules. The granules resembled cytoplasmic ribonucleoprotein particles in size and staining characteristics. Though nucleoli in some interphase cells appeared to differentiate into "nucleolonemata" and "pars amorpha" (11), most appeared to lack such specific organization.

Although preliminary, our electron microscope studies show that the nucleoli underwent a rather subtle morphological change during cell division. The structures became less compact and thus less electron opaque. The ribosome-like granules were more dispersed and appeared to be associated with a reticulum of fine fibrils with a diameter of 50 A or less. These "loose masses" persisted throughout mitosis and were often seen in close proximity to the denser chromosomes (Fig. 12). In a few sections, the chromosomal material appeared to connect with the nucleoli.

From the electron micrographs examined thus far, it is apparent that some of the nucleolar material which moves with the chromosomes to the poles during anaphase becomes included in the daughter nuclei. Fig. 13 is a section taken at telophase which shows the formation of nuclear envelope around the chromosomes. Note that although nuclear envelope is not yet complete, some nucleolar material is obviously enclosed inside the envelope (Nu_1). In studies of persistent nucleoli in other organisms, it has generally been concluded that such material is excluded from the re-forming daughter nuclei (12, 13). A detailed study of the ultrastructure and behavior of persistent nucleoli in dividing cells will be reported in the future.

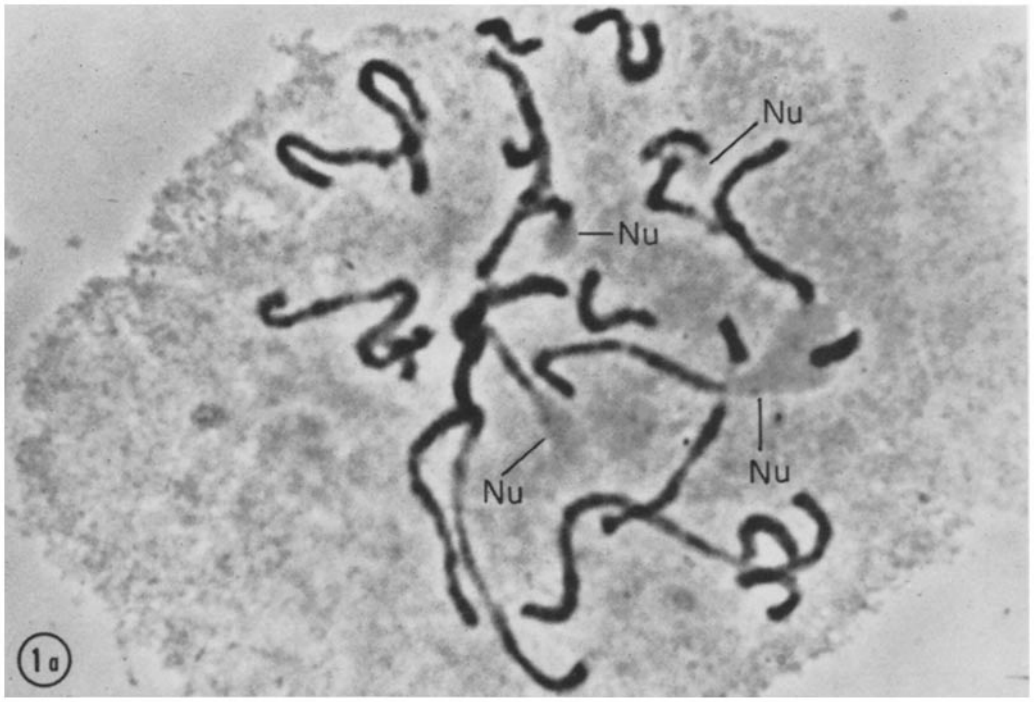
Frequency of Cells with Persistent Nucleoli

A survey of 18 cell strains available in this laboratory was conducted to determine whether the phenomenon of persistent nucleoli in mitotic divisions was limited to the Chinese hamster cells. After it was realized that nucleolar material was

FIGURE 1 A late prophase cell from strain Don, pulse-labeled with H^3-uridine (8 μc/ml) for 20 minutes.

Fig. 1 *a*, acetic orcein squash. Note several nucleoli of different sizes (Nu).

Fig. 1 *b*, autoradiograph of the same. Note heavy label on nucleoli. × 1300.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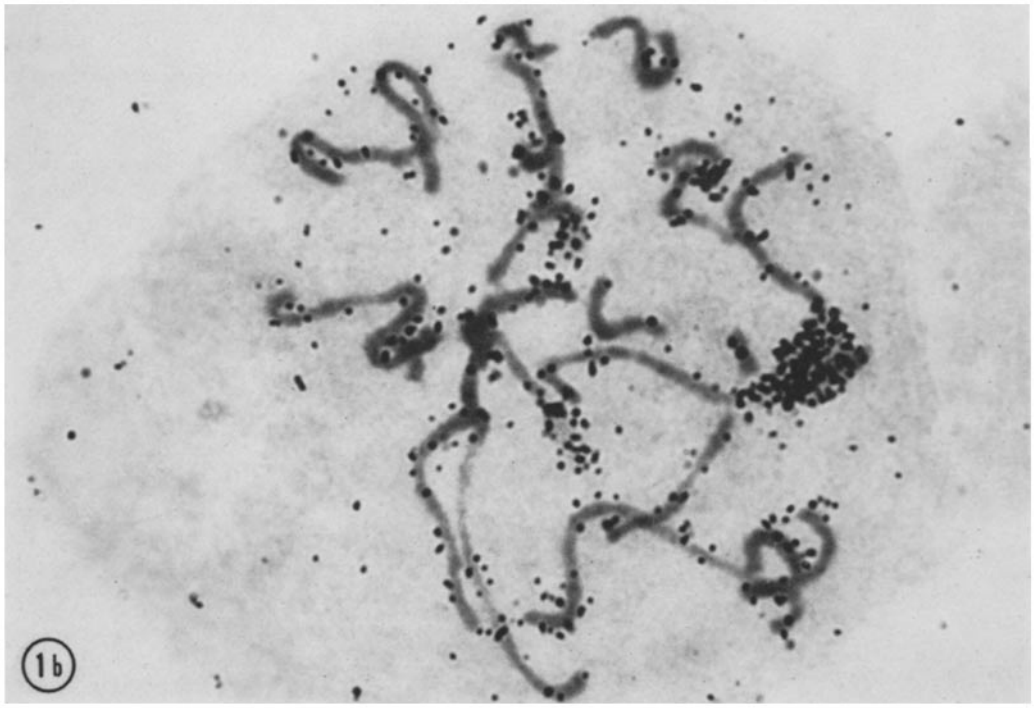

1b

always present in mitosis, either in the form of distinct, roundish bodies (Figs. 2 and 3) or broken up into shapeless masses surrounding the chromosomes (Fig. 11), it was difficult to become objective in determining the persistent nucleoli as such. However, every effort was made to record metaphases with morphologically distinct nucleoli as positive and all others as negative. The percentage of metaphase figures with nucleoli for each strain is as follows:

Strain	Per cent
Human fetal lung	54.00
KB	18.00
Alouatta villosa	9.00
Lagothrix ubericolor	5.00
LM	4.00
Don	90.00
Dede	98.00
Mary L	92.00
B14FAF28	63.00
Peromyscus difficilis	74.00
Aschizomys niigatae	87.00
A. andersoni	90.00
Giant anteater	27.00
Collared anteater	16.00
PT-K1	22.00
Ferret	20.00
Black bear	6.00
Hinny	70.00

It is clear from the list above that most Chinese hamster cells showed persistent nucleoli, whereas the L cells were almost completely negative. However, in most metaphases and anaphases of L cells, especially those following FUdR inhibition and thymidine reversal (unpublished data), heavy, diffuse material suggesting disintegrated nucleoli covered the chromosomes. We conclude that nucleolar material is always present in mitotic phases, but not always in the form of nucleoli which can be recognized as such by conventional cytological criteria.

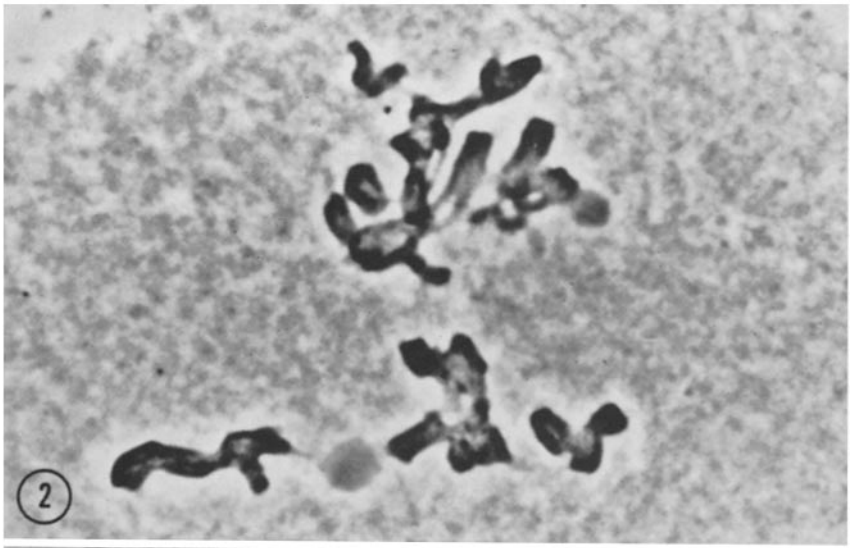
DISCUSSION

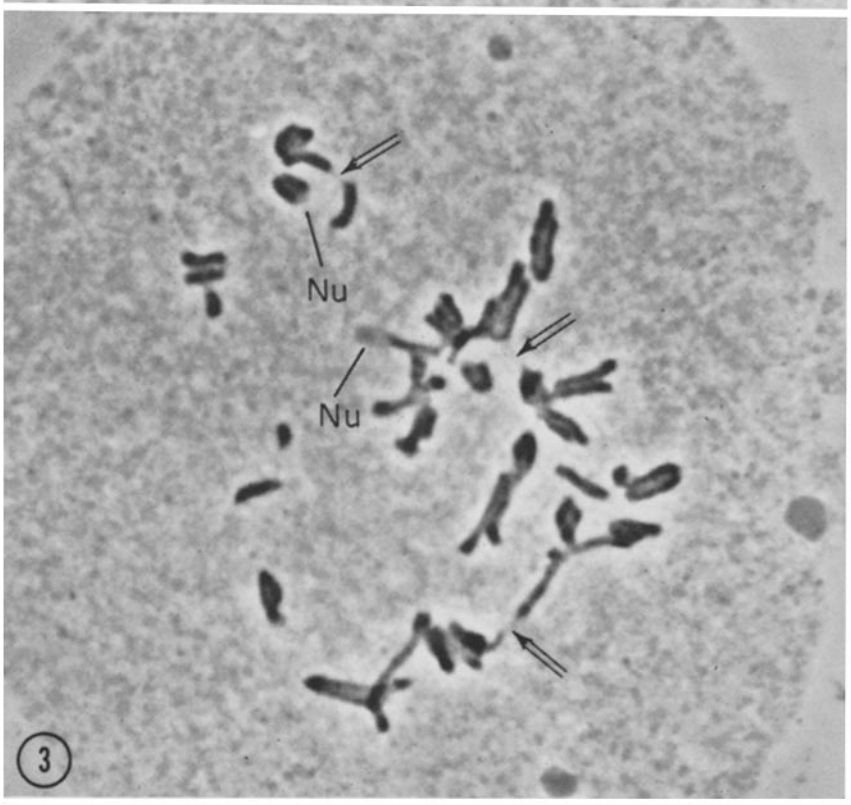
The data presented here merely confirmed the classical concept of the behavior of the nucleolus in mitosis; *i.e.*, that nucleoli disintegrate at late prophase and the nucleolar material forms a mass which coats the chromosomes. The persistent nucleolus is merely a variation of this behavior; *i.e.*, the nucleolar material does not break up completely during metaphase and anaphase. In plant materials, persistent nucleoli have been noted in some species as a normal feature in mitosis (14), but in animal cells such bodies have not been frequently described except under certain experimental conditions. Heath (12) was able to induce persistent nucleoli in chick cells *in vitro* by adding cobalt salts to the growth medium, and Hsu *et al.* (1) thought that they had induced persistent nucleoli in mammalian cells by treatments with FUdR and thymidine. Our data demonstrate that persistent nucleoli are not a result of FUdR treatment, although FUdR inhibition of DNA synthesis might increase the frequency and exaggerate the size. In fact, persistent nucleoli were noted by Love and Suskind (15) in a number of mammalian cells, using the toluidine blue–molybdate procedure; however, without flattening the cells by either squashing or air drying, the photomicrographs of Love and Suskind were not convincing until we studied our own material.

Lafontaine and Chouinard (16), in their detailed ultrastructural studies on mitosis in *Vicia faba*, observed that in prophase the nucleolar material was dispersed throughout the intact nucleus in the form of ribosome-like particles and fine fibrils. The continuity of the nucleolar material was lost during late prophase and metaphase, but such material reappeared in anaphase in the form of ribosomal particles. These authors therefore concluded that the nucleolus is lost in

FIGURE 2 A metaphase cell from strain Dede. Three persistent nucleoli are attached to the chromosomes. Acetic orcein squash. × 1300.

FIGURE 3 A metaphase cell from strain Dede. Three persistent nucleoli free in the cytoplasm. Note two small nucleoli still attached to chromosomes (*Nu*). Double-shafted arrows point to places where ends of chromosomes show signs of association, probably relics of tiny nucleoli. Acetic orcein squash. × 1300.

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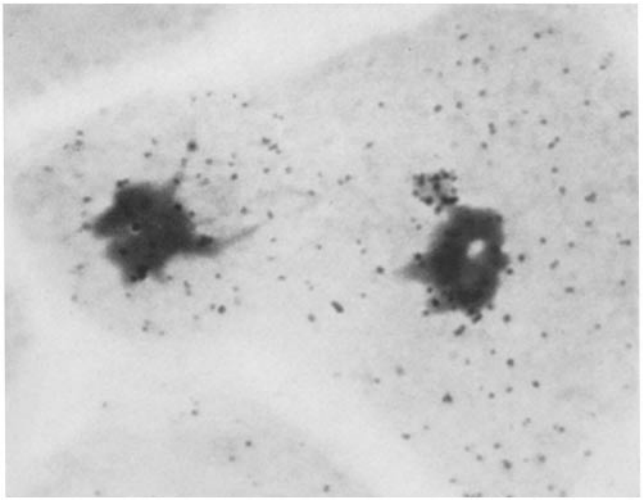

FIGURE 4 Radioautograph of a telophase cell of strain Dede showing a persistent nucleolus with heavy label. The culture was labeled with H^3-uridine (10 μc/ml) for 1 minute and was allowed to incubate for 45 minutes in medium containing non-radioactive uridine. Note also cytoplasmic label. × 1300.

late prophase and is resynthesized in anaphase and telophase. Jones (17) found, in mitotic division of erythroblasts, packages of ribosomal particles which he interpreted as partially disintegrated nucleoli. Our own electron micrographs, although preliminary, suggest that such packages of granules in division may indeed be the remnants of nucleoli. Whereas at low magnification the mitotic nucleoli appeared somewhat homogeneous (Fig. 12), high resolution micrographs of these structures revealed many ribosome-like particles associated with numerous fine fibrils.

Apparently the persistent nucleoli are very susceptible to hypotonicity. No nucleolar material could be demonstrated in any preparation pretreated with a hypotonic solution, such as is commonly employed in preparing slides for chromosome analysis. No diffused material connecting the chromosome tips (Fig. 3) can be detected in any preparation for karyological studies. The relic of such connection may occasionally be seen as an end-to-end orientation, not connection, of two chromosomes in metaphase. The terminal association between acrocentric chromosomes in human metaphases belongs to this category.

In materials with pronounced sticky chromosomes (cells irradiated with ultraviolet light, tumors, etc.), the chromosomes can be completely dispersed by hypotonic solution treatment, except where interchanges occur. More than likely, the sticky chromosomes represent an overproduction or an abnormal distribution of nucleolar material in mitosis which literally glues the chromosomes together and may cause division difficulties.

The stretching of nucleoli between chromosomes in anaphase (Figs. 7 and 8) is another evidence for nucleolar involvement in sticking chromosomes. It should be added, in this connection, that to study induced chromosome anomaly (by means of radiation, chemicals, viruses, etc.) by enumerating anaphase bridges, one must use DNA specific stains such as the Feulgen reaction, for stretched nucleoli with non-specific nucleic acid stains such as orcein and carmine may be mistaken for chromatin bridges. Studies on the role of nucleoli in induced sticky chromosomes are in progress and will be reported at a later date.

It is not unreasonable to consider that the daughter nuclei, after the completion of mitosis, conduct active protein synthesis. If the newly formed daughter nuclei are devoid of a considerable quantity of ribosomes, they must first synthesize ribosomes. The nucleolar material which moves with the chromosomes during anaphase (Fig. 11), and is included in the daughter nuclei during telophase (Fig. 13), is probably used as ready-made synthetic sites inside the nuclei following cleavage. After the early stages of the G1 phase, new synthesis of nucleolar material would ensue to meet the growing needs.

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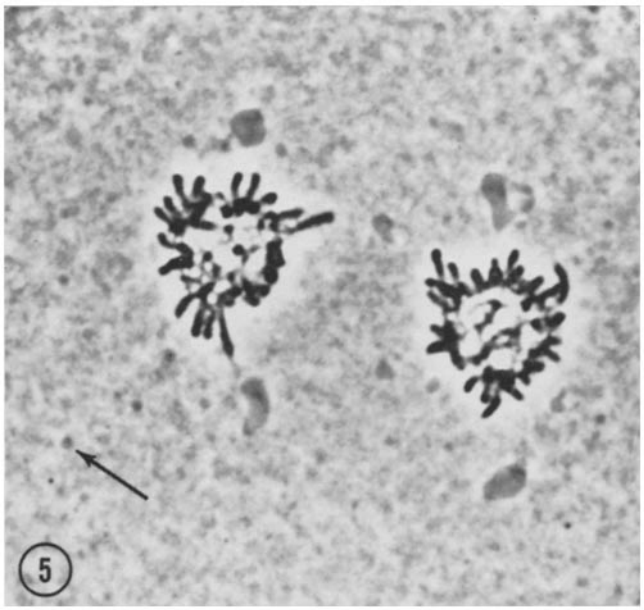

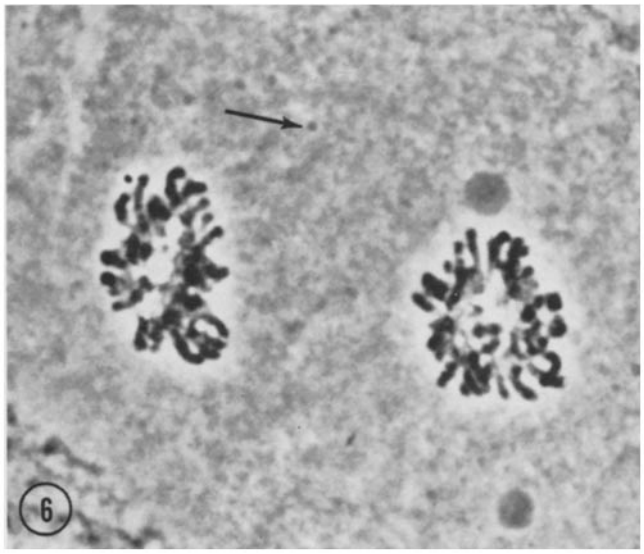

FIGURE 5 A late anaphase cell from strain Dede showing persistent nucleoli. Arrow points to one of the numerous granules in the cytoplasm, possibly parts of broken nucleoli. Acetic orcein squash. × 1300.

FIGURE 6 An early telophase cell from strain Dede showing two large nucleoli at one side. Arrow points to one of the numerous granules in the cytoplasm, possibly parts of broken nucleoli. Acetic orcein squash. × 1300.

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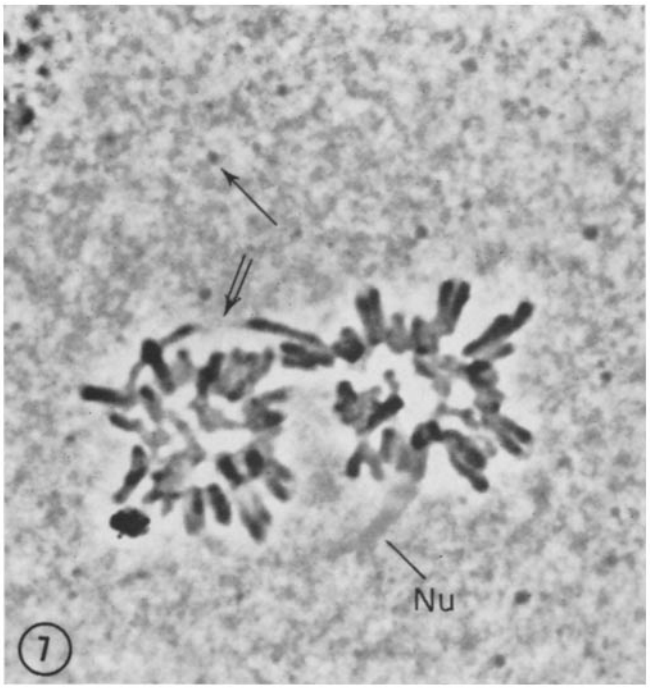
For References, see p. 553



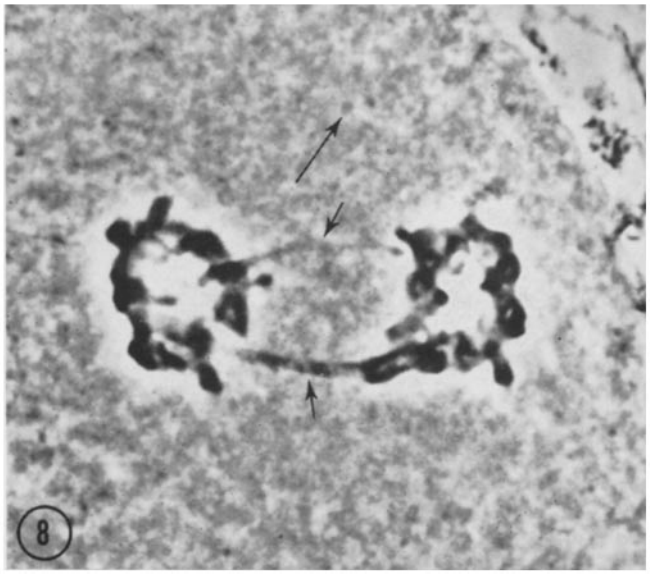
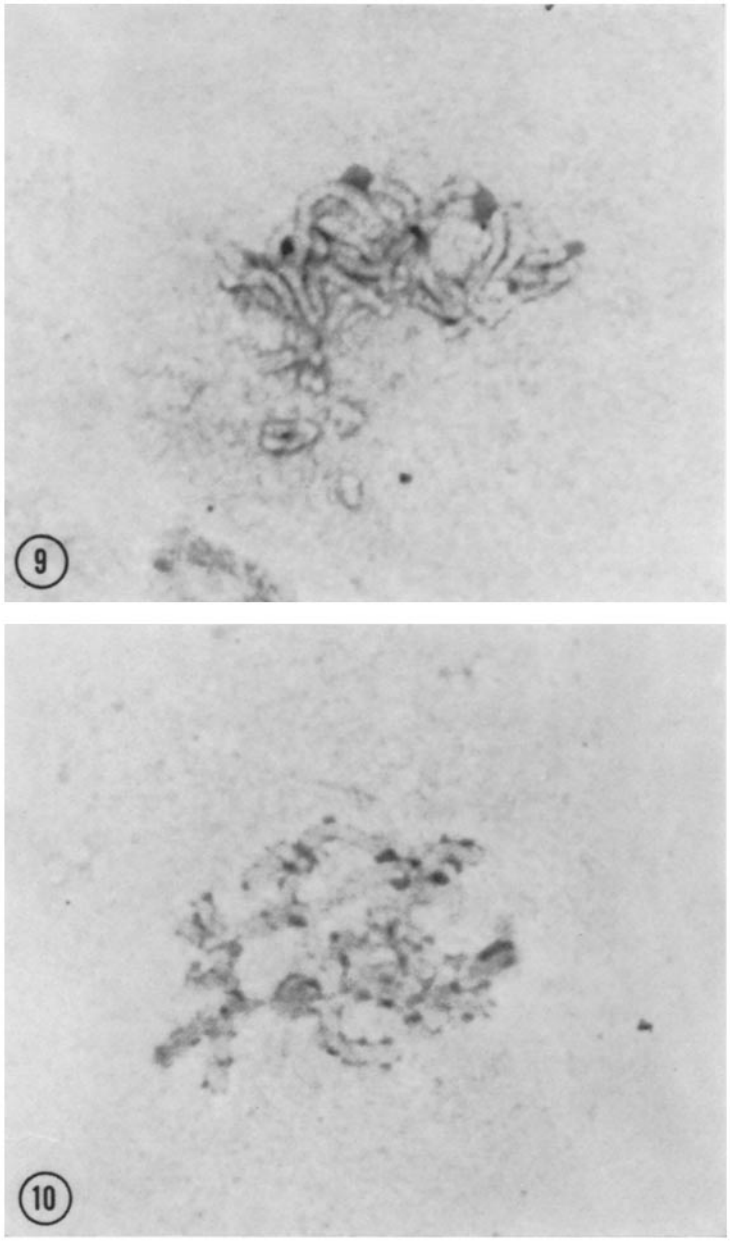


FIGURE 7 An anaphase cell from strain Dede showing a nucleolus stretched between the two daughter chromosome groups (*Nu*) and a relic of association of sister chromatids (double arrow). Long arrow points to one of the numerous granules in the cytoplasm. Acetic orcein squash. × 1300.

FIGURE 8 An early telophase cell from strain Dede showing two chromatin bridge–like structures (short arrows) between daughter chromosome groups. These are apparently nucleolar material being pulled by the departing sister chromosomes. Long arrow points to one of the numerous granules in the cytoplasm. Acetic orcein squash. × 1300.



FIGURES 9 and 10 Metaphase cells from strain Dede, fixed in acetic alcohol, air-dried, and stained with pyronin–methyl green. Phase-contrast. Photographed with a yellow-green filter. Methyl green–positive material (chromosomes) shows no contrast, whereas pyronin-positive material (nucleolar bodies) shows high contrast. Note no large nucleoli but numerous medium-sized and small lumps in both cells. × 1300.

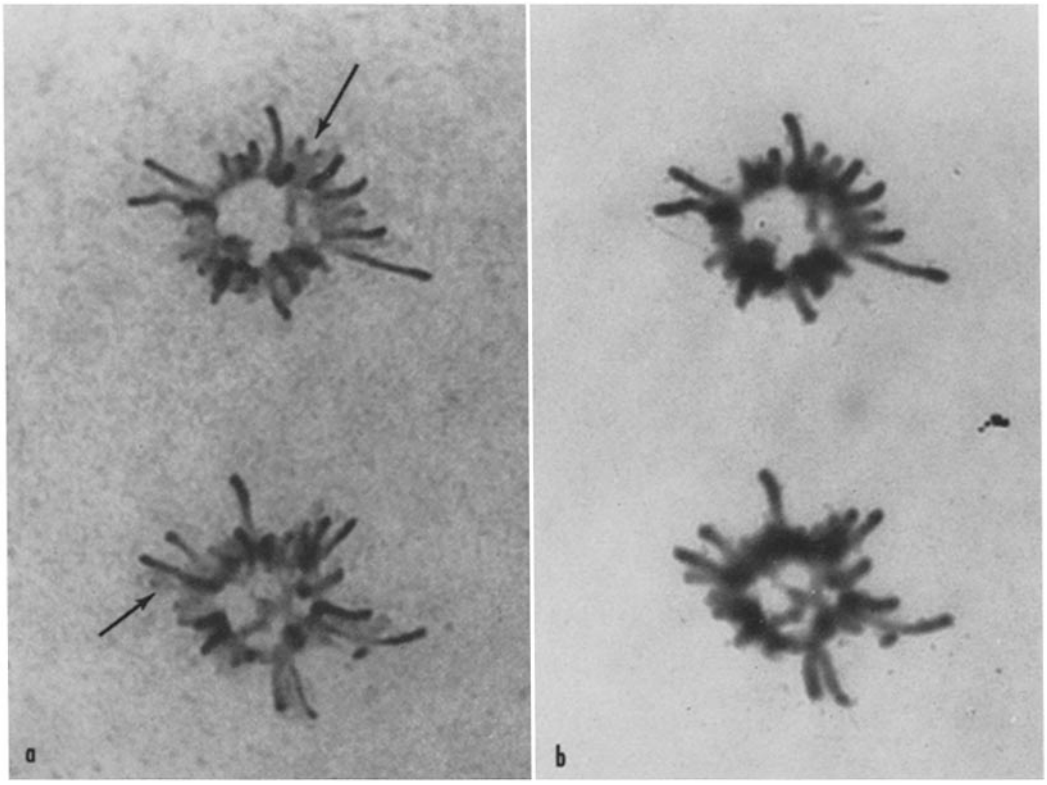


FIGURE 11 An anaphase cell from strain Dede, fixed in acetic acid and stained with azure B. Bright field photography with a blue and a green filter.

Fig. 11 *a*, the cell before ribonuclease digestion. Note dense cytoplasmic staining, and amorphous nucleolar material (gray) mingled with the chromosomes (black).

Fig. 11 *b*, the same cell after ribonuclease digestion. Note nearly complete removal of nucleolar material around the chromosomes and complete removal of cytoplasmic background. × 1300. Note also that in several places (arrows in Fig. 11 *a*) chromosomes are evident where they were originally covered by nucleolar material.

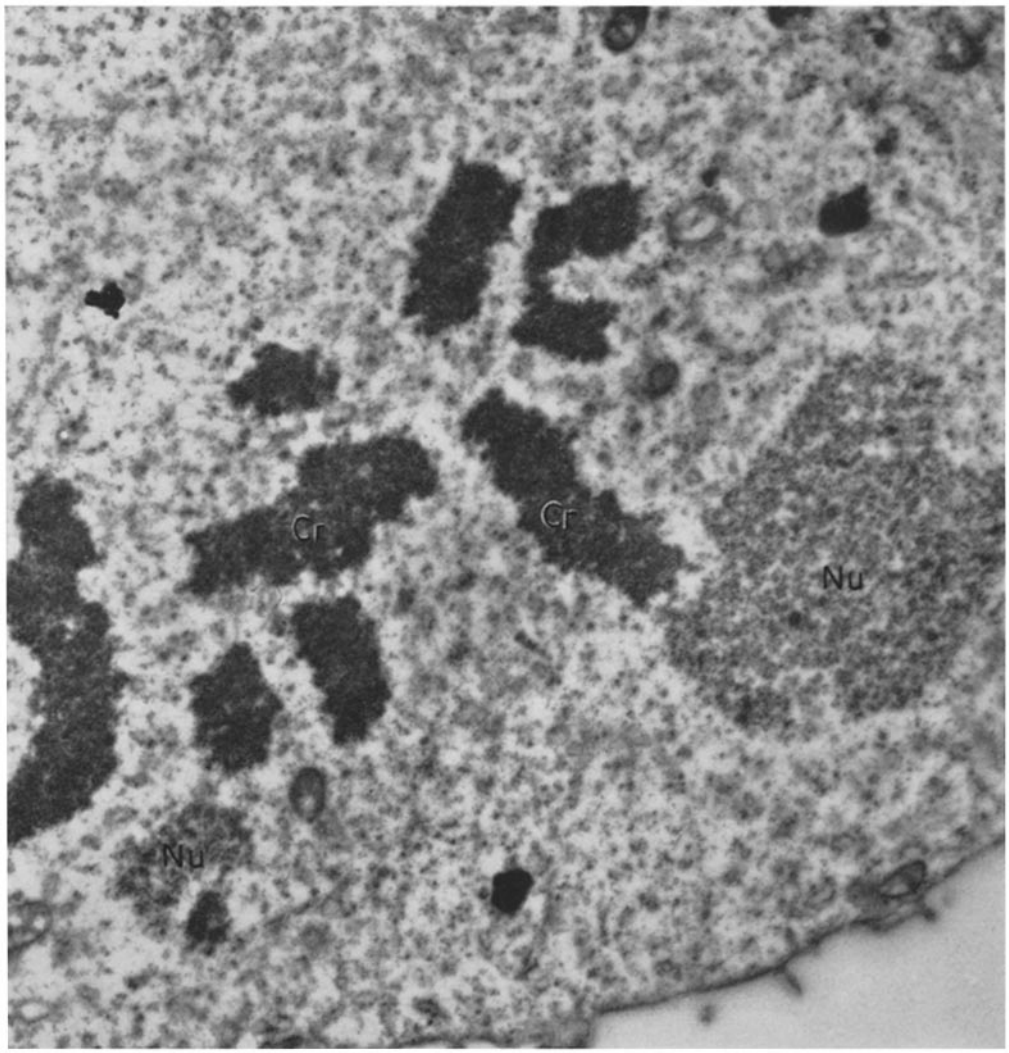


FIGURE 12 Survey electron micrograph of a dividing cell, showing a large and a small nucleolus (*Nu*) associated with the chromosomes (*Cr*). Fixed in osmium tetroxide, embedded in methacrylate, and stained with uranyl acetate. × 15,000.

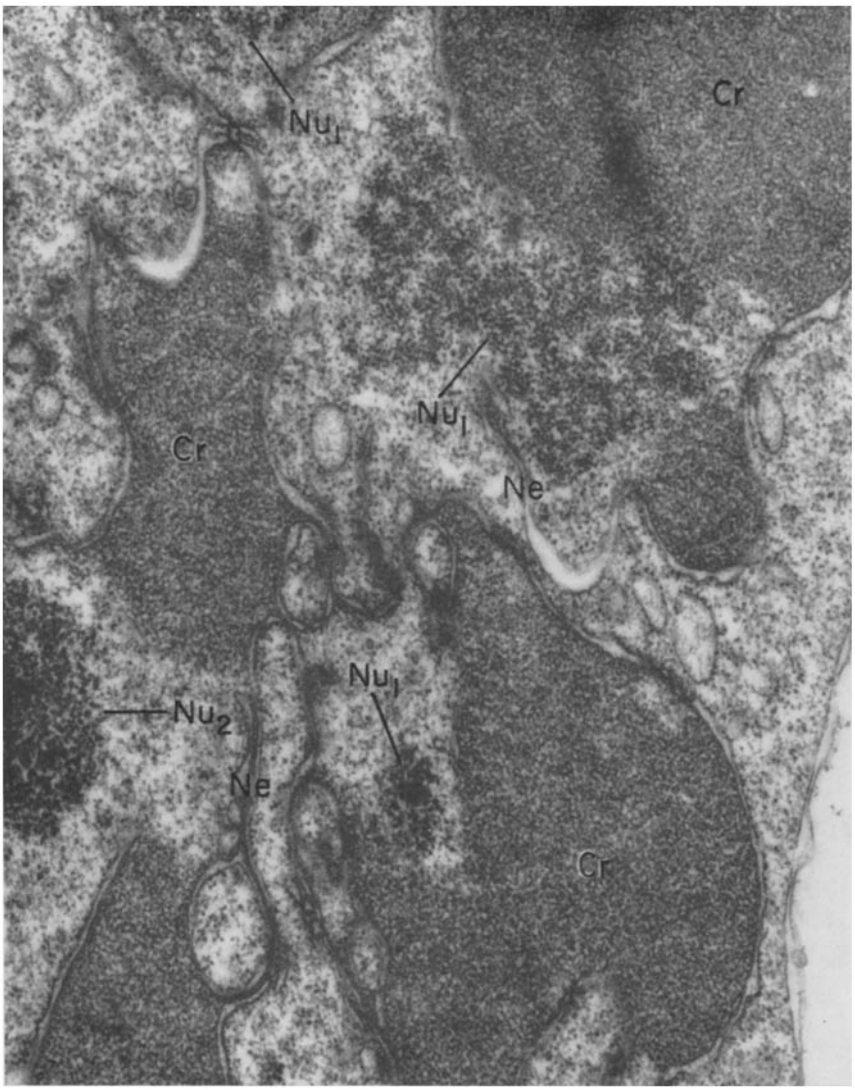


FIGURE 13 Section through telophase chromosomes (*Cr*), showing re-formation of the nuclear envelope (*Ne*). Nucleoli which will be included in the new nucleus (Nu_1) have been indicated. Note also the nucleolus which may or may not be enclosed in the nuclear envelope (Nu_2). × 32,000.

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