THE FINE STRUCTURE OF THE NUCLEOLUS IN MITOTIC DIVISIONS OF CHINESE HAMSTER CELLS *IN VITRO*

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ABSTRACT

The nucleolus of Chinese hamster tissue culture cells (strain Dede) was studied in each stage of mitosis with the electron microscope. Mitotic cells were selectively removed from the cultures with 0.2 per cent trypsin and fixed in either osmium tetroxide or glutaraldehyde followed by osmium tetroxide. The cells were embedded in both prepolymerized methacrylate and Epon 812. Thin sections of interphase nucleoli revealed two consistent components; dense 150-A granules and fine fibrils which measured 50 A or less in diameter. During prophase, distinct zones which were observed in some interphase nucleoli (*i.e.* nucleolonema and pars amorpha) were lost and the nucleoli were observed to disperse into smaller masses. By late prophase or prometaphase, the nucleoli appeared as loosely wound, predominantly fibrous structures with widely dispersed granules. Such structures persisted throughout mitosis either free in the cytoplasm or associated with the chromosomes. At telophase, those nucleolar bodies associated with the chromosomes became included in the daughter nuclei, resumed their compact granular appearance, and reorganized into an interphase-type structure.

INTRODUCTION

Although a number of studies have been directed toward the ultrastructure and function of the nucleolus in interphase, comparatively little is known of its behavior during cell division. It is generally concluded that the nucleolus disappears in late prophase and remains undetected throughout the remaining mitotic stages until the daughter nuclei are formed. The appearance of the mitotic spindle following the dissolution of the nucleolus led earlier investigators to speculate that the nucleolus was involved in spindle formation (*cf.* 20). Others have reported that the ribonucleoprotein released from the nucleolus during prophase formed a coat around the mitotic chromosomes and later gave rise to new nucleolar material at telophase (11).

A few electron microscope studies on nucleolar behavior in mitosis are available during recent years. Lafontaine (14) and later Lafontaine and Chouinard (15) reported that in *Vicia faba* complete nucleolar dissolution occurred during prophase, after which the elements of the nucleolus could no longer be distinguished from other nuclear and cytoplasmic structures. Nucleolar material in the form of fibrils and granules was observed to reappear along the arms of anaphase chromosomes. Somewhat similar observations were made recently by Stevens in grasshopper neuroblasts (24). In these cells, nucleolar dissolution was reported to occur in two steps during prophase. At anaphase, dense material presumed to

be prenucleolar structures aggregated along the chromosome arms.

The appearance of nucleolar structures at specific sites along anaphase or telophase chromosomes seems to agree with the concept that specific nucleolar organizers exist on certain chromosomes (1, 8, 18, 27). The question which remains to be answered, however, is whether or not these structures represent newly synthesized material, reorganization of previous structures carried over from the parent nucleus, or a combination of both. In a recent study, Hsu *et al.* (9) presented evidence that nucleolar structures persisted throughout mitosis in a number of mammalian cell lines. By means of autoradiography and cytochemical staining, these investigators demonstrated the association of several nucleolar bodies both with the chromosomes and free in the cytoplasm in all stages of mitosis. In addition, a preliminary account of the fine structure of mitotic nucleoli was presented. These authors demonstrated that, at least in mammalian cells, the persistence of nucleoli through mitosis is not an atypical behavior.

The present study is a continuation of our investigations on persistent nucleoli and presents a more detailed account of the ultrastructure and the behavior of the nucleoli in each stage of mitosis in Chinese hamster cells *in vitro*.

MATERIAL AND METHODS

Female diploid Chinese hamster cells (strain Dede) were maintained as monolayer cultures in McCoy's 5a medium supplemented with 20 per cent fetal calf serum. Mitotic cells were selectively harvested by washing monolayer cultures in 0.2 per cent trypsin solution. As many as 80 per cent of the cells collected in this manner were in various stages of mitosis. The cell suspension was immediately centrifuged and the pellet resuspended in one of the following fixatives:

(*a*) 1 per cent osmium tetroxide buffered with veronal-acetate at pH 7.6 containing 0.004 M magnesium and calcium. This solution was made isotonic with sucrose (6).

(*b*) 3 per cent glutaraldehyde buffered with Millonig's phosphate buffer (19) at pH 7.6. After 1 hour in this solution, the cells were washed in buffer and postfixed for 30 minutes in 1 per cent osmium tetroxide buffered in a similar manner. Fixation in each of the solutions was carried out at either 4°C or room temperature.

Subsequent to dehydration in a graded series of ethanol, the pellets were broken into smaller pieces and embedded in either prepolymerized methacrylate (2 parts ethyl:3 parts n-butyl dried in anhydrous powdered Na_2SO_4 and containing 1 per cent benzoyl peroxide) or Epon 812 (16). Those cells fixed at 4°C were dehydrated in cold ethanol; all subsequent procedures were conducted at room temperature.

Thin sections were cut with a glass knife on a Porter-Blum ultramicrotome and picked up on Formvar-coated, stainless-steel grids. Staining was accomplished by inverting the grids in either saturated uranyl acetate for 1 hour or 2 per cent uranyl acetate for 15 minutes (pH 5) followed by Karnovsky's lead hydroxide (13) for 10 minutes. Sections were examined on a Hitachi HU-11A electron microscope operated at 75 and 100 kv.

Light Microscopy

In order to supplement the study of thin sections and for purposes of orientation, thick (0.5- to 1-μ) sections were cut adjacent to thin ones and placed on glass slides. These were either examined directly with a phase contrast microscope or stained with Azure II–methylene blue (22) for brightfield microscopy.

Hypotonic Solution Treatment

In an effort to study the effects of tonicity on nucleolar ultrastructure, some of the cells were subjected to a brief treatment in growth medium rendered hypotonic by adding distilled water at ratios of 4:1 (medium:H_2O), 3:1, 2:1, 1:1, and 1:2. After 5 minutes in these solutions, the cells were centrifuged and fixed in the manner previously described.

RESULTS

Interphase

The interphase nucleolus of Chinese hamster cells is not grossly different from that described in other mammalian cell types (17, 26). It consisted of at least two distinct structural components: densely packed granules which measured 100 to 150 A in diameter and fine fibrils 50 A or less in diameter (Fig. 1). In addition, a less dense amorphous background material associated with both fibrils and granules was sometimes observed in cells which had been fixed in glutaraldehyde. This material may correspond to the protein matrix described in other mammalian cells by Marinozzi (17). The organization of the above components in the nucleolus was found to be variable from one cell to another. In Fig. 1, the dense granules are seen to occupy the outer surface while the fibrillar elements appear to be concentrated in the central zone. In other nucleoli, however, both granules and fibrils formed large

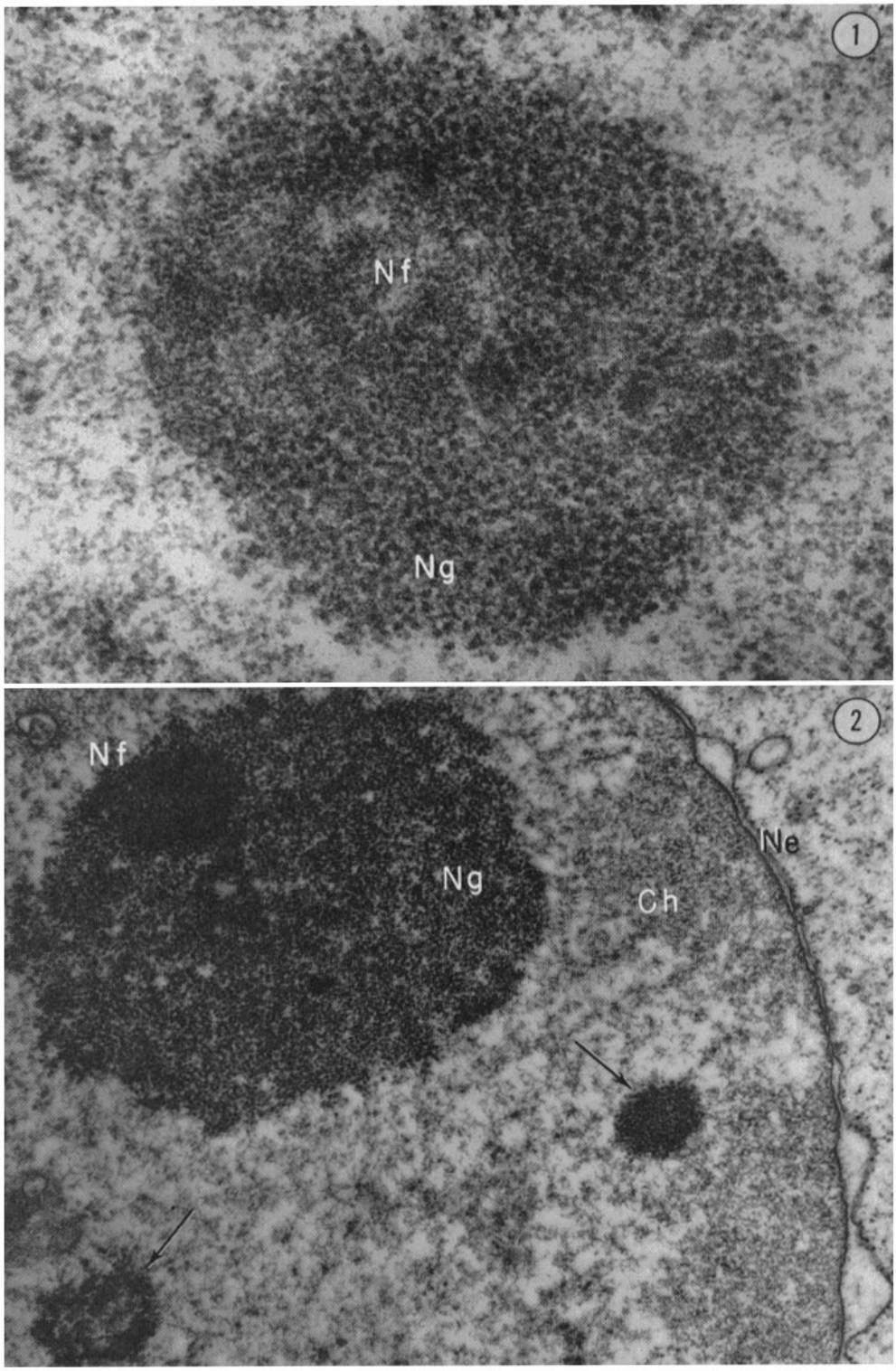
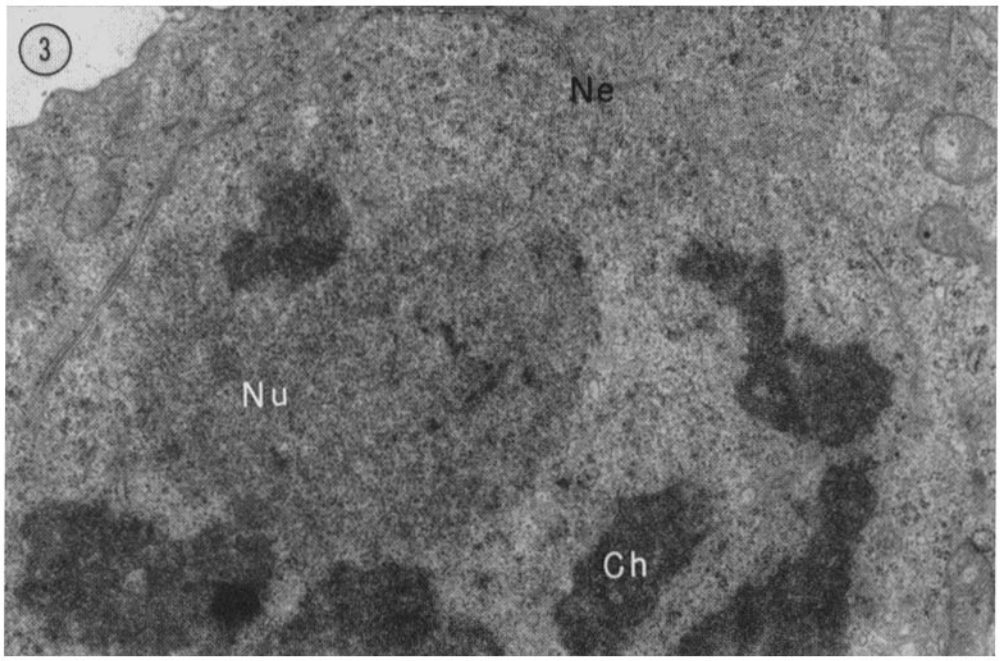


FIGURE 1 Interphase nucleolus with dense 150-A granules (*Ng*) distributed around the periphery, and fine fibrils (*Nf*) 50 A or less in the central zone. Double fixation, glutaraldehyde-OsO_4, embedded in Epon. × 67,000.

FIGURE 2 Section through an early prophase nucleus. The granular elements (*Ng*) of the nucleolus are more uniformly dispersed and the fibrillar zone (*Nf*) becomes smaller and is eventually lost. Small nucleolus-like bodies (arrows) appear in the nucleoplasm and along the condensing chromatin (*Ch*). The outer membrane of the nuclear envelope (*Ne*) exhibits a wavy appearance. OsO_4-fixed, embedded in methacrylate. × 30,000.



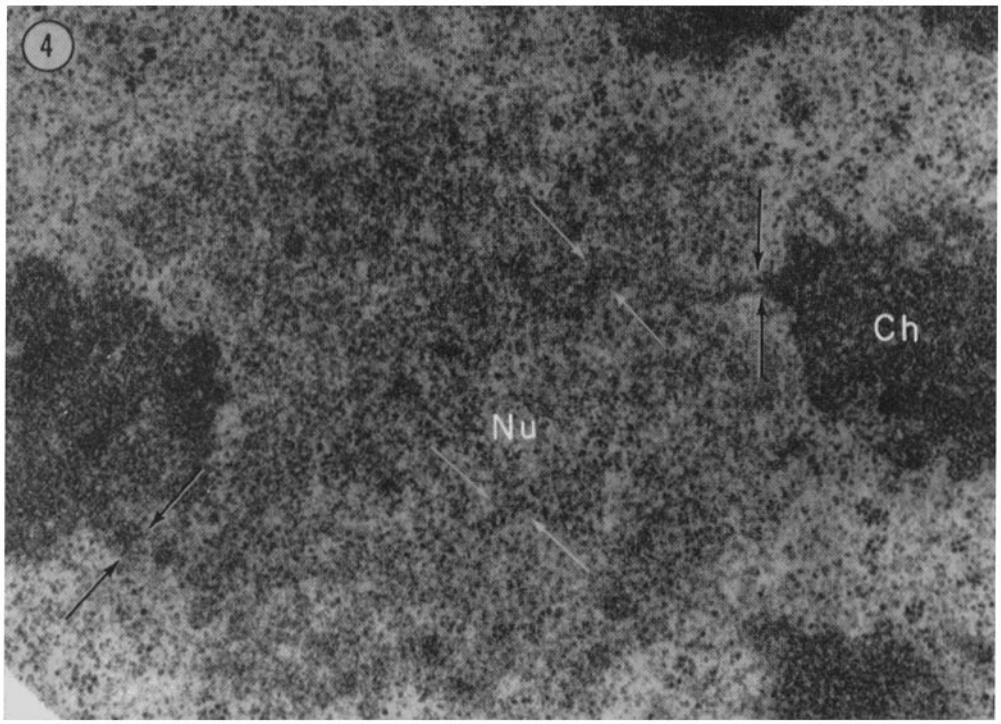


FIGURE 3 Late prophase showing nuclear envelope (*Ne*) dissolution. The large nucleolus (*Nu*) has a density intermediate between that of the chromosomes (*Ch*) and the cytoplasm. Nucleolar organization typical of some interphase cells (Fig. 1) is not evident at this stage. Double fixation, glutaraldehyde-OsO_4, embedded in Epon. × 15,000.

FIGURE 4 Higher magnification of another section through cell shown in Fig. 3. The nucleolus (*Nu*) is composed of granules and numerous fine fibrils which exist in bundles or "chords" (white arrows). An apparent association between nucleolus and chromosome (*Ch*) is indicated by black arrows. × 34,000.

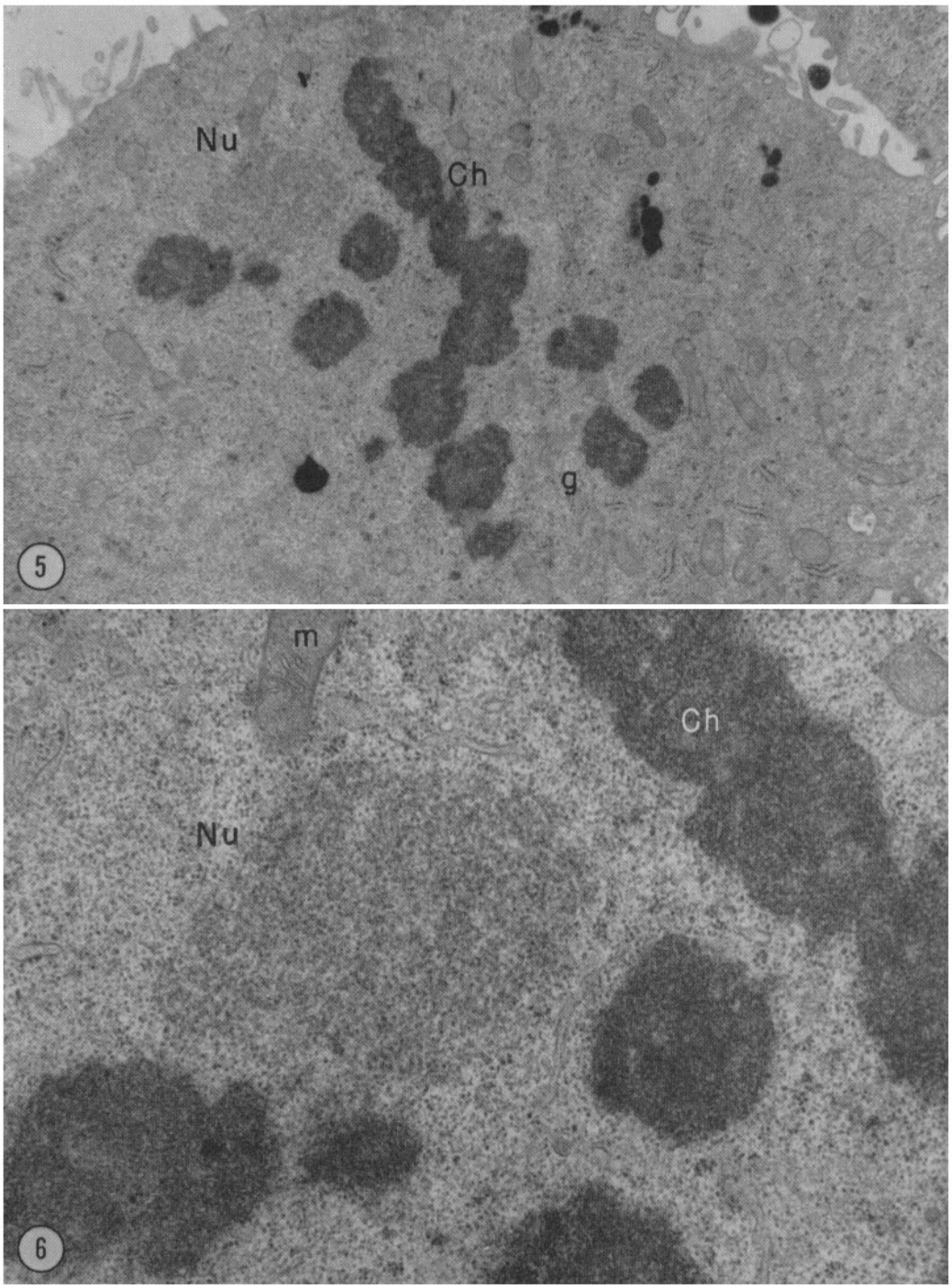


FIGURE 5 Prometaphase. The nucleolus (*Nu*) is seen among several chromosomes (*Ch*). Vesicles of the Golgi apparatus (*g*) are also evident. Double fixation, glutaraldehyde-OsO_4, embedded in Epon. × 7,000.

FIGURE 6 A portion of Fig. 5 under higher magnification. The nucleolus contains a loose mass of fibrils and granules in close association with a chromosomal segment and mitochondria (*m*). × 30,000.

"chords" or bundles which measured 400 to 500 A in diameter. In still a third type, the fibrils and granules appeared uniformly distributed throughout the nucleolus. Occasionally, from one to several "vacuoles" were seen in a nucleolus, and a band of perinucleolar chromatin was sometimes seen around the structure.

The chromatin of interphase nucleoli is also variable but most often confined to a narrow, condensed band in contact with the nuclear envelope. Coarse fibrils and patches of condensed chromatin may be seen scattered throughout the nucleoplasm. In addition, diffuse clusters of dense 100- to 150-A granules similar to those in the nucleolus were often present in the nucleoplasm of interphase cells.

Prophase, Prometaphase, and Metaphase

In early prophase, the nucleoli began to undergo an obvious morphological transition. Distinct zones which were evident in many interphase nucleoli began to disappear and the granular elements became more uniformly distributed and less densely packed (Fig. 2). In addition to the large nucleoli, small masses of dense nucleolar material containing both fibrils and granules were observed to distribute throughout the condensing chromatin. Later, some of the small nucleolar masses became completely dispersed as fibrils and granules, but one to several large nucleoli remained and were usually associated with the condensed chromosomes at the time of nuclear envelope dissolution (Fig. 3). In addition to the nucleolar-like masses associated with the prophase chromosomes, similar but somewhat smaller masses were often seen free in the cytoplasm.

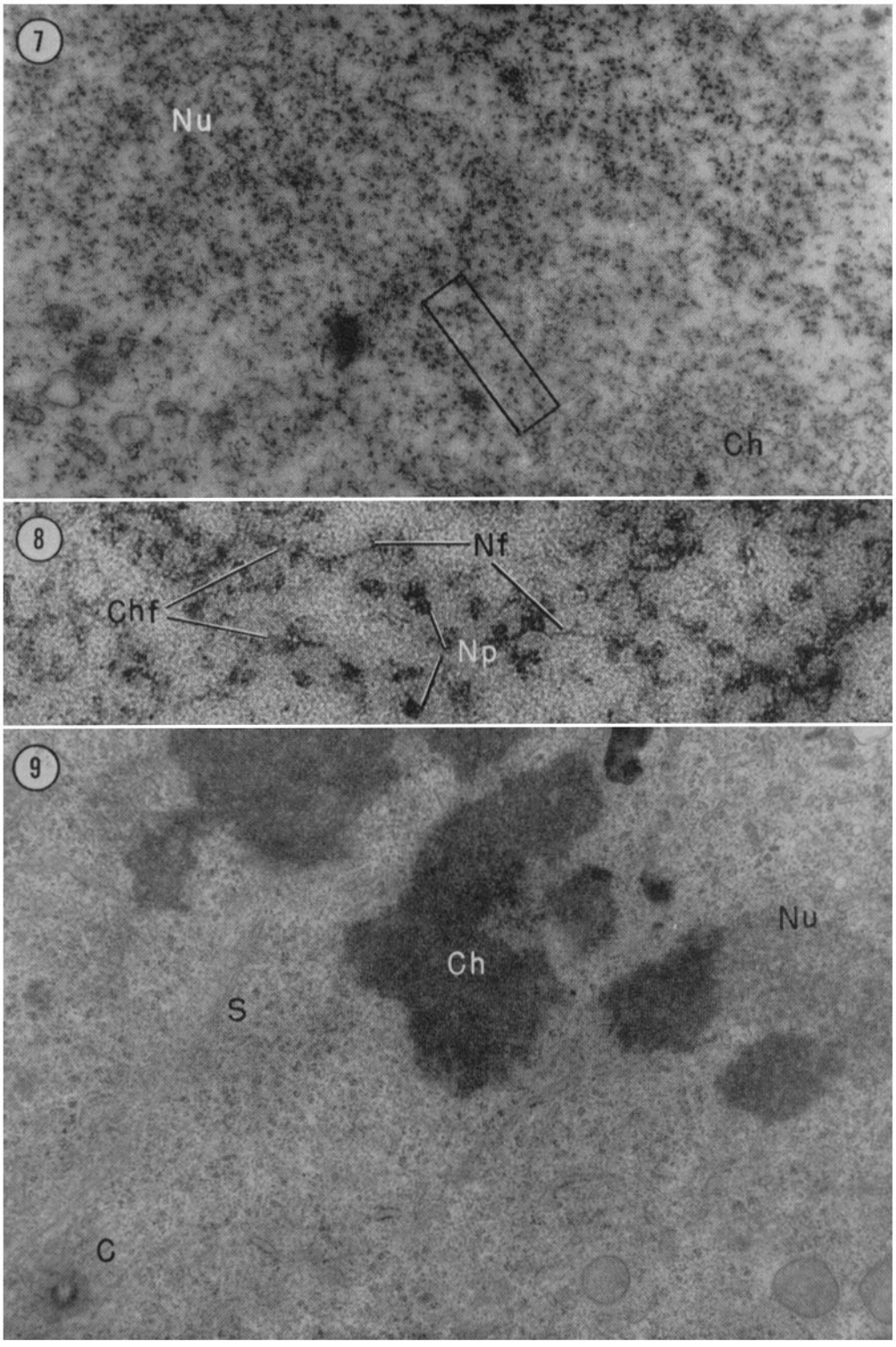
The prometaphase nucleoli showed an electron density intermediate between that of the chromosomes and the cytoplasm. When examined at higher magnifications, they exhibited a fine structure considerably different from that of the interphase nucleoli. The fibrillar elements and amorphous background material contributed to much of the gross structure while the granular component became more widely dispersed (Figs. 3 to 6).

In a number of sections, the prometaphase nucleoli appeared to be associated with more than one chromosome (Figs. 3, 4, and 9). In such cases, it was often possible to detect points of connection between fibrils of the nucleolus and those of the chromosomes (Fig. 4). These attachment points appeared as 400-A "chords" or "bundles" consisting of smaller fibrils. Such chords appeared to continue into the nucleolus where they looped back and forth or anastomosed into a diffuse reticulum (white arrows in Fig. 4). Dense, round profiles which were similar in size and structure to the nuclear chords and probably represented cross-sections of chords were also evident. Evidence on the association of the individual nucleolar fibrils with those of the chromosomes (Fig. 7) was also obtained from nucleoli which had been expanded by mild hypotonic treatment (two parts of growth medium to one part of distilled water). While the usual organization was lost in such preparations, the 50-A fibrils were greatly extended and possible points of connection could be seen between the 50-A fibrils and the larger fibrils of the chromosome (Fig. 8). The possibility that the points of connection between persistent nucleoli and the chromosomes are merely chromosomal strands superficially extending into the

FIGURE 7 A prometaphase nucleolus (*Nu*) expanded by hypotonic solution treatment prior to fixation. Individual fibrils and granules can be clearly seen. The 100-A fibrils of the chromosomes (*Ch*) are more clearly visible after hypotonic swelling. The area in the rectangle indicates the attachment between the nucleolus and the chromosome. OsO_4 fixed, embedded in methacrylate. × 44,000.

FIGURE 8 Higher magnification of the structures within the rectangle of Fig. 7. Note the attachment of 50-A nucleolar fibrils (*Nf*) to the larger (100-A) chromosomal fibrils (*CHf*). Also note the fine structure and association of the nucleolar granules (*Np*) with the nucleolar fibrils. × 213,000.

FIGURE 9 At metaphase, the nucleolus (*Nu*) is shown associated with two chromosomal fragments at the equatorial plate. The centriole (*C*) and spindle tubules (*S*) are also shown. *Ch*, chromosome. Double fixation, glutaraldehyde-OsO_4, embedded in Epon. × 15,000.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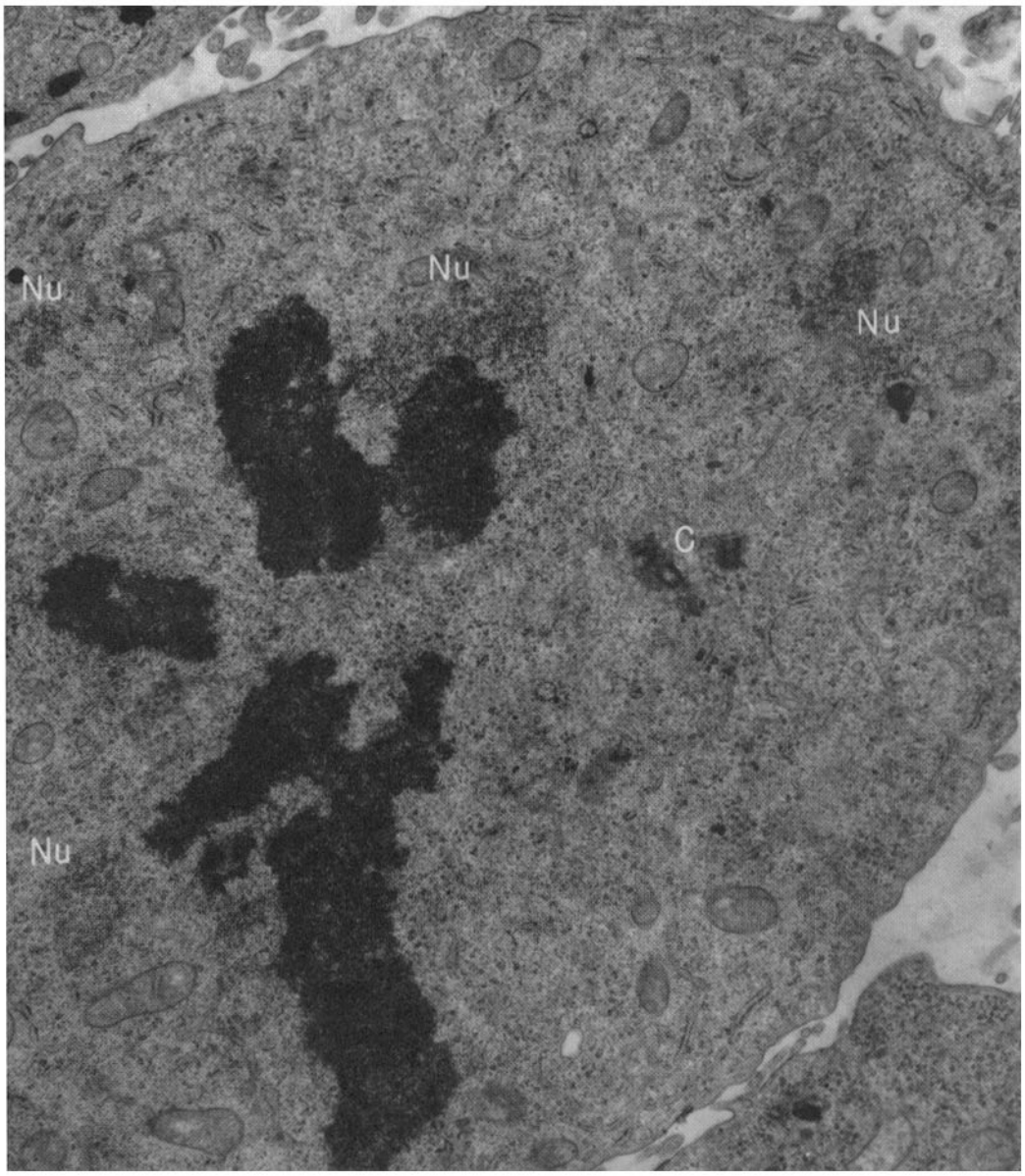


FIGURE 10 A metaphase cell showing a nucleolus associated with a chromosome at the equatorial plate. Note also the nucleolus-like bodies (*Nu*) free in the cytoplasm. This cell had three centrioles (*C*) at one pole. Double fixation, glutaraldehyde-OsO_4, embedded in Epon. × 13,000.

nucleolar masses must also be considered. Their precise nature can not be unequivocally established until cytochemical studies are completed at the ultrastructural level.

The granules of prometaphase nucleoli, although more widely dispersed, appeared similar in structure and staining behavior to those of the interphase nucleoli. However, they usually appeared more diffuse and less densely stained than the cytoplasmic ribosomes. After hypotonic swelling, many of the nucleolar granules appeared hollow while others seemed to contain even smaller substructures (Fig. 8).

At metaphase, the nucleolar bodies which were

attached to the chromosomes could be found at the equatorial plate (Figs. 9 and 10). Again, a single nucleolus appeared to be associated with more than one chromosome, similar to what has been observed by Hsu *et al.* (9). The nucleoli which remained free in the cytoplasm were usually seen throughout the cell (Fig. 10), but at times they may migrate to the polar regions along with numerous vesicles and membrane-bounded structures.

Anaphase and Telophase

Our previous light microscope studies showed that the nucleolar material moved passively with the chromosomes during anaphase (9). When examined with the electron microscope, the anaphasic nucleoli appeared somewhat more electron opaque than, but otherwise similar in structure to those of previous stages. The dense fibrils and granules were distributed along the chromosome arms and often formed cap-like masses over the ends of the chromosomes (Fig. 11). At early telophase, the chromosome regions nearest the centriole fused into a single mass; but the distal portions of the chromosomes were still free and were often covered with nucleolus-like material (Figs. 12 and 13). As telophase progressed, these bodies were characterized by further aggregation of the dense granules at the free ends of the chromosomes. Also at this stage, smaller masses of similar structure appeared at various sites along the chromosomes.

Nuclear envelope reformation began in late anaphase. Numerous smooth-surfaced vesicles which preceded the chromosomes to the poles (Figs. 10 and 11) aligned themselves along the arms of the chromosomes and eventually enclosed all the chromatin and its associated nucleolar material by late telophase (Fig. 14) The nucleolar masses which remained free in the cytoplasm throughout mitosis were excluded from the new daughter nuclei.

DISCUSSION

Structural Features of Interphase Nucleoli

The fine structure of the interphase nucleolus of the Chinese hamster cell is similar to that of nucleoli in many plant and animal cells. Its two major components, the 100- to 150-A granules and the fine 50-A fibrils, probably correspond to similar structures described in other mammalian cells (17, 26), dipteran salivary gland cells (10, 25), and plant cells (15). However, the precise segregation of the nucleolar components into distinct zones, nucleolonema, and pars amorpha (8), was not consistently observed in Chinese hamster cells. Instead, at least three distinct morphological types of interphase nucleoli have been noted. Although nucleolar structures have been shown to be labile to fixation conditions (20), it is unlikely that the nucleolar pleomorphism in Chinese hamster cells was the result of fixation or polymerization damage. All types were seen in cells fixed in each of the fixatives and embedded in either methacrylate or Epon. Furthermore, when more than one nucleolus was present in a single nucleus, they were invariably of a similar morphology.

It is possible that such structural variation reflects functional changes in the nucleolus as the cell goes through a variety of metabolic stages in interphase. Whether different phases of the division cycle such as G_1, S, and G_2 possess different nucleolar structures remains to be investigated. Our attempts to use synchronized cell systems may help to elucidate this point.

A number of studies have been made to determine the chemical composition of the structural components of the nucleolus. The existence of RNA in the 150-A granules and the 50-A fibrils has been reported (17). Also, a number of authors have proposed that the granular elements of the nucleolus represent precursors of cytoplasmic RNA (3, 5, 21). Thus, the particles would be continuously synthesized in the interphase nucleolus and passed out into the cytoplasm.

The role of the fibrillar elements, on the other hand, has not been clearly established. After actinomycin D treatment, which inhibits the synthesis of both nucleolar and chromosomal RNA, one sees a partial segregation of the dense granules and fibrillar elements (4, 12, 24, 26). The persistence of RNA in the form of fibrils after the disappearance of granules from the nucleoli of *Chironomus* and pancreas cells when nucleolar synthesis has been arrested (12) suggested to Stevens (23) that the fibrils may act as structural and permanent forms of nucleolar RNA. The predominance of fibrillar components in persistent nucleoli of Chinese hamster cells after RNA synthesis has ceased provides further support that such elements might be structural entities of the nucleolus. This does not exclude, however, the possibility that the

RNA fibrils are in some way responsible for the formation of nucleolar granules (23).

Transitions in Nucleolar Structure during Mitosis

The dispersion of nucleolar particles and the disappearance of fibrillar zones in the nucleolus are concomitant with a reduction in RNA synthetic activity in late prophase (9). The disruption of dense bodies from the main nucleolus and the dispersion of nucleolar material into fine fibrils and granules suggest that portions of the nucleolus of Chinese hamster cells undergo dissolution similar to that described in *Vicia* root tips (15) and grasshopper neuroblasts (24). The bulk of the nucleolus persists throughout mitosis, however, although structural changes occur. In late telophase, when RNA synthesis is resumed and the nuclear envelope reappears, the nucleolus again becomes highly granular and begins to reorganize into a structure similar to that seen in interphase cells.

In our previous report (9), we presented observational evidence from light microscopy to show that nucleoli were found in all mitotic stages. Other investigators using different types of cells (15, 24) postulated that the nucleolar material detected in telophase may represent new synthesis or regrouping of prenucleolar material, since they failed to observe nucleoli in metaphase and in anaphase. Although the possibility of regrouping of prenucleolar material during telophase of mammalian cells cannot be ruled out, it has been found by Arrighi (2) that no RNA synthesis occurs in metaphase and in anaphase of the Chinese hamster cells. Following a 1-minute H^3-uridine labeling and immediate fixation for autoradiography, the interphase, prophase, and late telophase cells showed uptake of this radioactive precursor, but metaphase and anaphase elements were free from label. Her experiment emphatically suggests that RNA synthesis stops during these stages, a conclusion supporting that of Das (7) using plant root-tip cells with a 3-minute labeling period. However, mammalian cells labeled for 1 minute but chased for 10 and 20 minutes with non-radioactive uridine revealed label in metaphase and anaphase figures. The cells were apparently in the late G_2 or prophase stages when H^3-uridine was applied. Thus, it is highly unlikely that synthesis of new nucleoli material takes place during mid-mitotic stages.

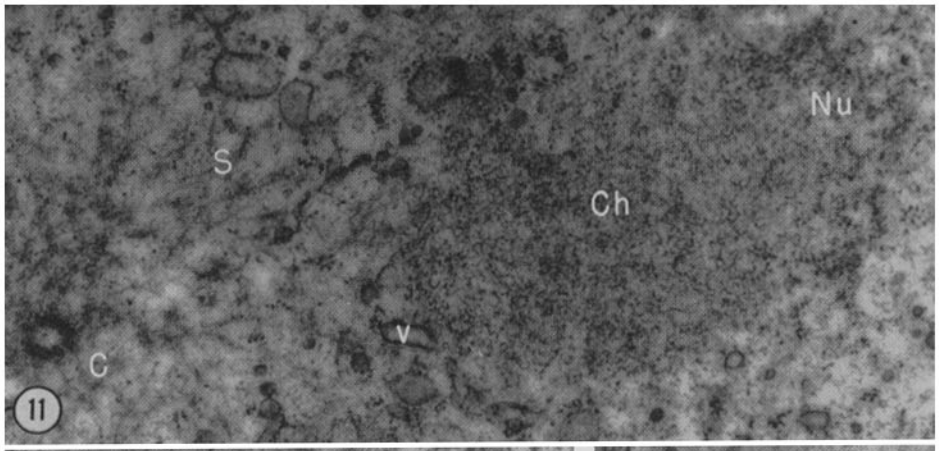
It is not possible at the present time to elaborate on the functional significance of persistent nucleoli in mammalian cells. Two hypotheses may be proposed: (*a*) they are active structures which remain associated with the chromosomes and are carried into the daughter nuclei to serve either as a primer for new nucleolar material or as a source of nucleolar RNA to the young daughter cell while a new nucleolus is being organized; or (*b*) they are passive structures which because of rapid mitosis in tissue culture cells fail to undergo complete dissolution. The facts that the persistent nucleoli directly attach to the chromosomes, move with them, become incorporated into the daughter nuclei, and resume RNA synthesis argue against the latter possibility. At any rate, our data provide morphological evidence for the continuity of nucleolar material from one cell generation to the next in mammalian cells *in vitro*.

FIGURE 11 Anaphase chromosome (*Ch*) with nucleolar material (*Nu*) arranged along one arm. Numerous small vesicles (*v*) precede the chromosomes to the centrioles (*C*) and later give rise to the nuclear envelope. *S*, Spindle Filaments. OsO_4 fixed, embedded in methacrylate. × 25,200.

FIGURE 12 Telophase. The centromeric ends of the chromosomes anastomose into a single mass which is surrounded by nuclear envelope (*Ne*) membranes. The arms of the chromosomes (*Ch*) may be coated with nucleolar material (*Nu*). Also at this stage, small masses of nucleolus-like structures appear throughout the chromatin (areas indicated by broken lines). OsO_4 fixed, embedded in methacrylate. × 11,700.

FIGURE 13 Higher magnification of a chromosome (*Ch*) shown in Fig. 12. Note the appearance of numerous dense granules in the nucleolus (*Nu*). × 27,400.

FIGURE 14 Telophase. The forming nuclear envelope (*Ne*) encloses the nucleolus (*Nu*). Note the appearance of large "chords" (arrows) which are composed of numerous dense granules. *Ch*, chromosome. OsO_4-fixed, methacrylate-embedded. × 41,500.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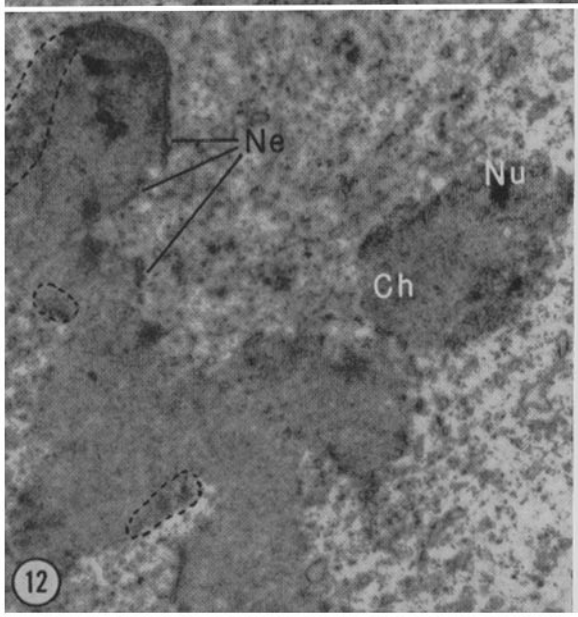
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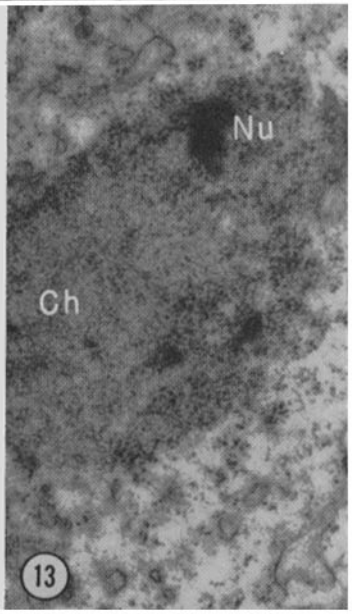
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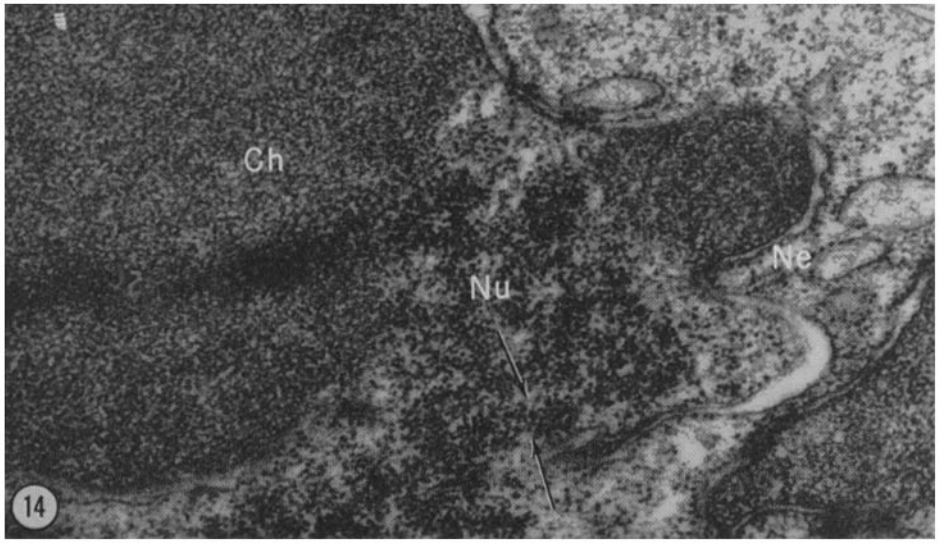
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