SITES OF NUCLEOLUS PRODUCTION IN CULTURED CHINESE HAMSTER CELLS

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ABSTRACT

Chinese hamster cell strains in the early passages in culture display wide variation in number of nucleolus-like bodies per cell, though such strains are characteristically euploid. A variety of criteria indicate that the nucleolus-like bodies are true nucleoli. Their Azure B– and fast green–staining properties indicate the presence of RNA and protein; they have typical nucleolar fine structure, including both fibrous and granular components; radio-autography reveals that their patterns of uptake of uridine-^{3}H into RNA are similar to those reported for nucleoli of other cell types; actinomycin D, at a level which selectively inhibits ribosomal RNA synthesis, greatly reduces their RNA synthesis and also causes segregation of fibrous and granular nucleolar components. Colchicine was used to experimentally fragment the nuclei of these cells into a number of separate karyomeres, each presumably containing some, or only one, of the chromosomes of the complement. Almost all the karyomeres contain nucleolus-like bodies which, by the same criteria applied to the multiple nucleolus-like bodies of uninuclear cells, appear to be true nucleoli. The nucleoli of individual karyomeres of the same cell often differ from each other in fine structure while the multiple nucleoli of a uninuclear cell generally resemble each other. The evidence presented in this study indicates that Chinese hamster cells contain many nucleolus-producing sites scattered through the genome.

Diploid, nontransformed Chinese hamster cells in tissue culture were found to possess widely varying numbers of nuclear bodies which appeared cytologically to be nucleoli. This finding seemed inconsistent with numerous studies demonstrating that somatic cells are normally characterized by a set number of nucleoli which consistently arise in association with specific chromosomal loci. The number of nucleoli produced is found to be one per haploid chromosome complement, i.e., two per diploid cell, in a great many organisms, for instance, *Hyacinthus* (de Mol, 1926, 1928), *Crepis* (Nawaschin, 1927), *Vicia* (Heitz, 1931 *a*), *Zea* (McClintock, 1934), *Allium* (Heitz, 1931 *b*), *Drosophila* (Kaufman, 1934), certain species of *Chironomus* (Beermann, 1960), *Xenopus* (Fischberg and Wallace, 1960), frog (Parmenter, 1940), axolotl (Dearing, 1934) and other amphibians (Fankhauser and Humphrey, 1943). However, two nucleoli per genome are characteristic of a few species of grasshopper (Carothers, 1913), ginkgo (Lee, 1954) and *Chironomus tentans* (Balbiani, 1881). These data are consistent with biochemical evidence from *Xenopus* and *Drosophila* that cistrons coding for ribosomal RNA, which is synthesized in the nucleolus (Perry, 1962; Brown

and Gurdon, 1964), are confined to a single "nucleolar organizer" region of the chromosome complement (Wallace and Birnstiel, 1966; Ritossa and Spiegelman, 1965).

The harmony between these biochemical and cytological findings is seductive; nevertheless, it should not beguile the biologist into discounting the large body of discordant data which exists. In some species of animals and plants, nucleoli are reported to arise at telophase from several chromosomal sites (Hsu, Arrighi, and Klevecz, 1965) or from a collection of "droplets" or a matrix coating the chromosomes (Heitz, 1931 b; Derman, 1933: McClintock, 1934; Gates, 1942; Rattenbury and Serra, 1952; Lafontaine, 1958; Lafontaine and Chouinard, 1963; Das, 1963; Stevens, 1965), which suggests that nucleolus-producing sites may be scattered. It is not established, however, that the sites at which material arises at telophase are the sites where this material is actually synthesized. In salivary glands of some dipterans, the polytene chromosomes are associated with numerous "micronucleoli" at multiple, scattered sites (Swift, 1962; Jacob and Sirlin, 1963), and there is evidence that these scattered nucleoli are active in RNA synthesis (Garcia and Kleinfeld, 1966). In some organisms which normally produce two nucleoli per cell, nucleoli may be produced at sites other than the normal "nucleolar organizer" if a "nucleolar organizer" is absent from the nucleus (Heitz, 1931 a; McClintock, 1934; Swift, 1959), and functional "nucleolar organizer" loci can be present in a nucleus in a "repressed" state (Nawaschin, 1927, Crosby, 1957). This observation may be related to the finding that nucleoli isolated from some species contain only a fraction of the ribosomal RNA cistrons present in the genome (Chipchase and Birnstiel, 1963; McConkey and Hopkins, 1964).

The research reported in this paper was directed towards determining the nature and sites of origin of the multiple, nucleolus-like bodies observed in Chinese hamster tissue culture cells. The results indicate that these cells contain many sites at which normal, functional nucleoli may be produced.

MATERIALS AND METHODS

Tissue Culture and Cytochemistry

Fibroblast-type cells derived from macerated female Chinese hamster (Cricetelus griseus) fetuses were propagated on glass in Puck's N-16 medium (Puck, Cieciura, and Robinson, 1957) supplemented with 5% horse serum, 10% fetal calf serum, and 12.5 µg/ml aureomycin. Cells were subcultured by trypsinization every 4-7 days. Experiments were performed on cells in the second to tenth passage grown on coverslips in Yerganian tubes. For light microscopic cytochemistry, cells were fixed in situ with 10% buffered neutral formalin for 1 hr at 4°C and washed in tap water for 3 hr. Control slides for each experiment were treated with RNase (Worthington), 2 mg/ml, pH 6.5, at 37°C for 2 hr. All slides used for radioautography were treated with 5% trichloroacetic acid at 4°C for 15 min, rinsed briefly in one change of 5% cold trichloroacetic acid, rinsed in water, and air dried prior to dipping. Azure B staining for RNA was carried out according to Flax and Himes (Flax and Himes, 1952), and fast green staining for total protein and Feulgen staining were done according to Swift (Swift, 1955). Radioautographs were stained through the emulsion after development with Azure B at 4°C for 8 min. Periodic acid treatment, 0.5% at room temperature for 15 min, was used for removal of silver grains. After grain removal, slides were restained with 0.2% carmine in 45% propionic acid at room temperature for 10 min.

Micronucleus Induction and Radioautography

Treatment of rapidly proliferating cell cultures with 5×10^{-5} M colchicine for 16-36 hr induced the formation of a variable proportion of multi-nucleated cells. Cultures treated with colchicine were washed with three rinses of Hanks' balanced salt solution before being incubated with uridine-^{3}H. Colchicine was returned to the medium for samples chased in a 500-fold excess of cold uridine.

For demonstrating preferential inhibition of nucleolar RNA synthesis in response to low levels of actinomycin D, cells were treated with 0.04 µg/ml of actinomycin D for 20 min before, as well as during, incubation with uridine-^{3}H. Control cultures were treated with higher levels of actinomycin D, 4 µg/ml, in the same manner so as to inhibit almost all RNA synthesis.

Radioautography was carried out on cells incubated in conditioned medium containing 0.1 mc/ml uridine-^{3}H, specific activity 21.0 c/mmole, at 37°C for 5, 15, or 30 min. Cells were then either fixed or rinsed in three changes of Hanks' salt solution and chased in a 500-fold excess of cold uridine in conditioned medium for varying lengths of time. After fixation and air drying, slides were dipped in Kodak NTB-2 emulsion, stored in the dark 1-3 wk, and developed for 2 min at 20°C in D-19 developer.

Electron Microscopy

The fixative for electron microscopy was prepared by adding 20 cc of 25% glutaraldehyde (Eastman) to 80 cc of Puck's N-16 medium and adjusting the pH to 7.4 with several drops of 2,4,6-trimethyl-pyridine (Eastman). Cultures growing on glass coverslips in Yerganian tubes were fixed *in situ* at 0–4°C for 1–3 hr, rinsed in cold 0.2 M collidine, and postfixed for 1–3 hr in 1.3% collidine-buffered OsO_4. The cultures were subsequently dehydrated in alcohol and transferred through propylene oxide to Epon (Luft 7:3; Luft, 1961). After the Epon had been allowed to infiltrate for several hours, the Epon was wiped from the bottom of the coverslip with lens paper and propylene oxide. The Epon on the top of the coverslip was polymerized and separated from the coverslip by repeated cooling on dry ice and

warming in water at room temperature and by careful separation of the coverslip from the Epon with a watchmaker's forceps. Sections cut parallel to the surface of the Epon were stained in 3% aqueous uranyl acetate for 8–24 hr, poststained in lead citrate (Venable and Coggeshall, 1965), and examined with a Siemens Elmiskop I.

RESULTS

Cytological Characteristics of Multiple Nucleolus-Like Bodies

The number of nucleolus-like bodies per cell is highly variable in fetus-derived Chinese hamster cell cultures. As many as 21 bodies in a single nucleus have been observed, though most cells have fewer than seven (Fig. 1). The distribution
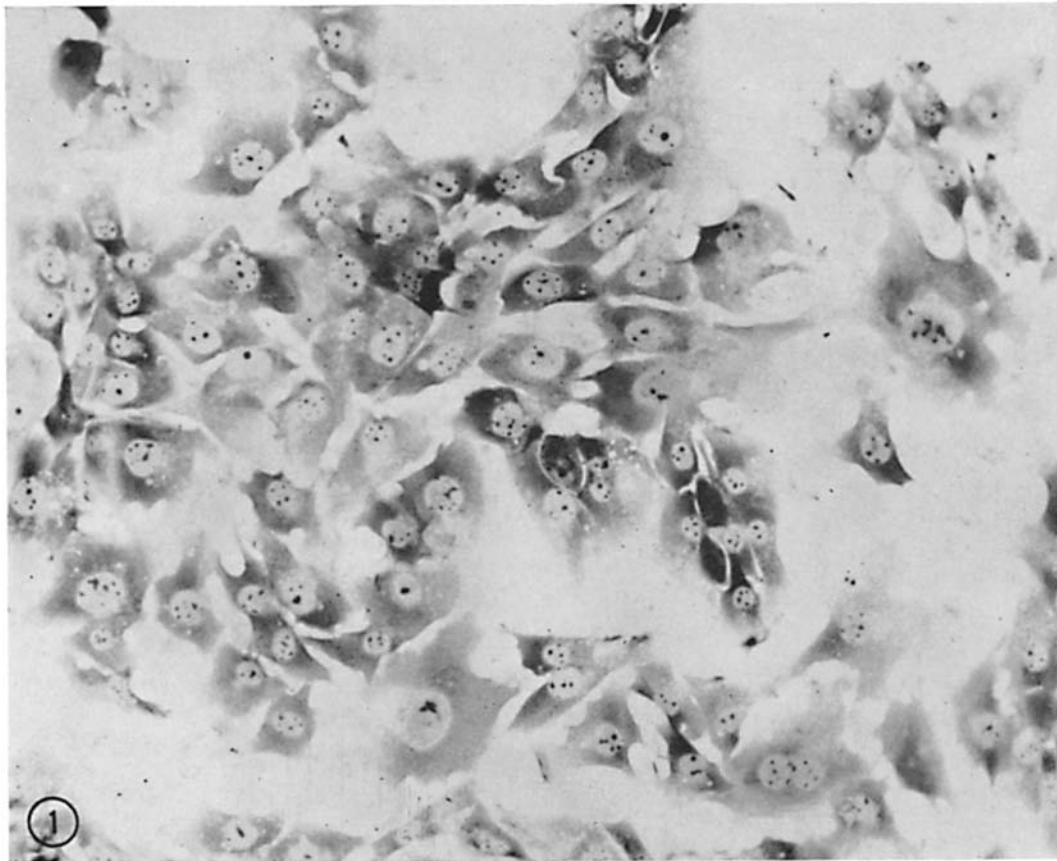

FIGURE 1   Azure B–stained preparation of a culture of rapidly proliferating female Chinese hamster cells in the 9th passage. The nucleoli stain metachromatically. In the early passages, cells of fetus-derived Chinese hamster cell strains are 95% diploid, yet the number of nucleoli per cell is highly variable. × 400.
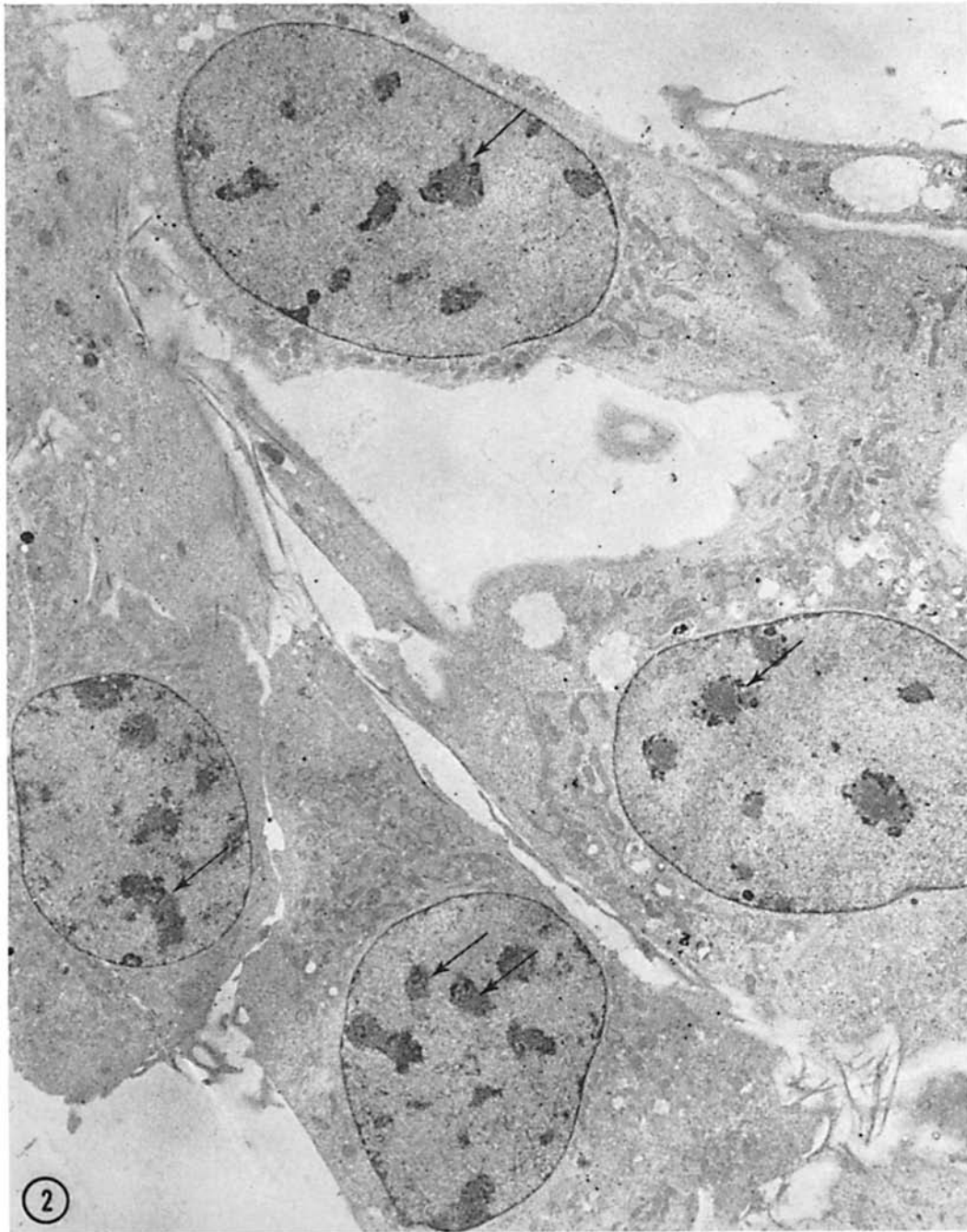
FIGURE 2 Low magnification electron micrograph of four Chinese hamster fibroblasts. Note the multiple nucleoli in each cell which are similar to nucleoli described in other cell types. The nucleoli are somewhat variable in structure from one cell to the next, but those within a single nucleus tend to be similar in appearance. Arrows indicate nucleoli shown in Figs. 3–5. × 6,100.
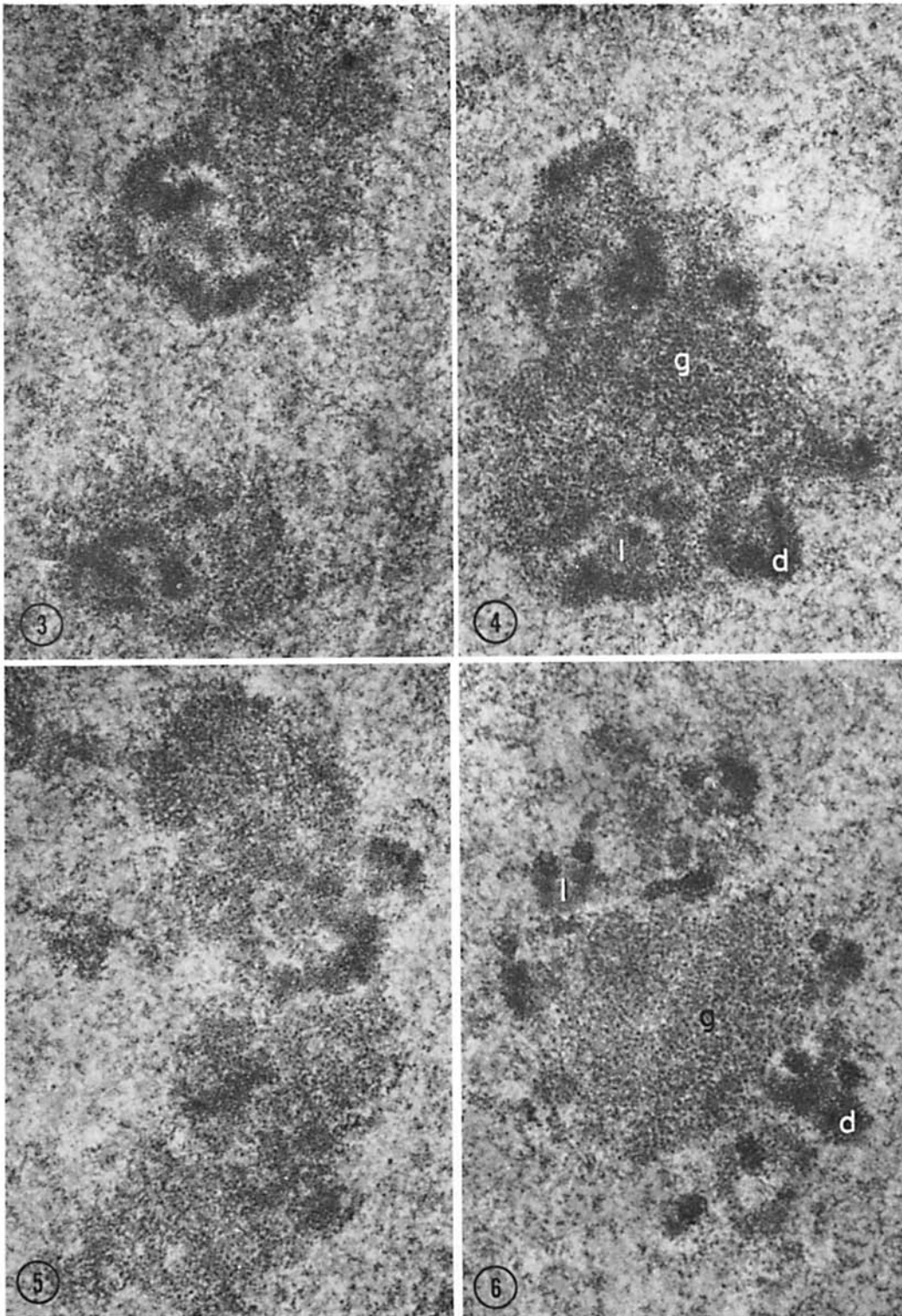
FIGURES 3–6   Higher magnifications of nucleoli indicated by arrows in Fig. 2. Three distinct nucleolar zones may be discerned in each nucleus: a granular component (g), an electron-opaque fibrous component (d), and a less dense fibrous component (l). × 51,000.
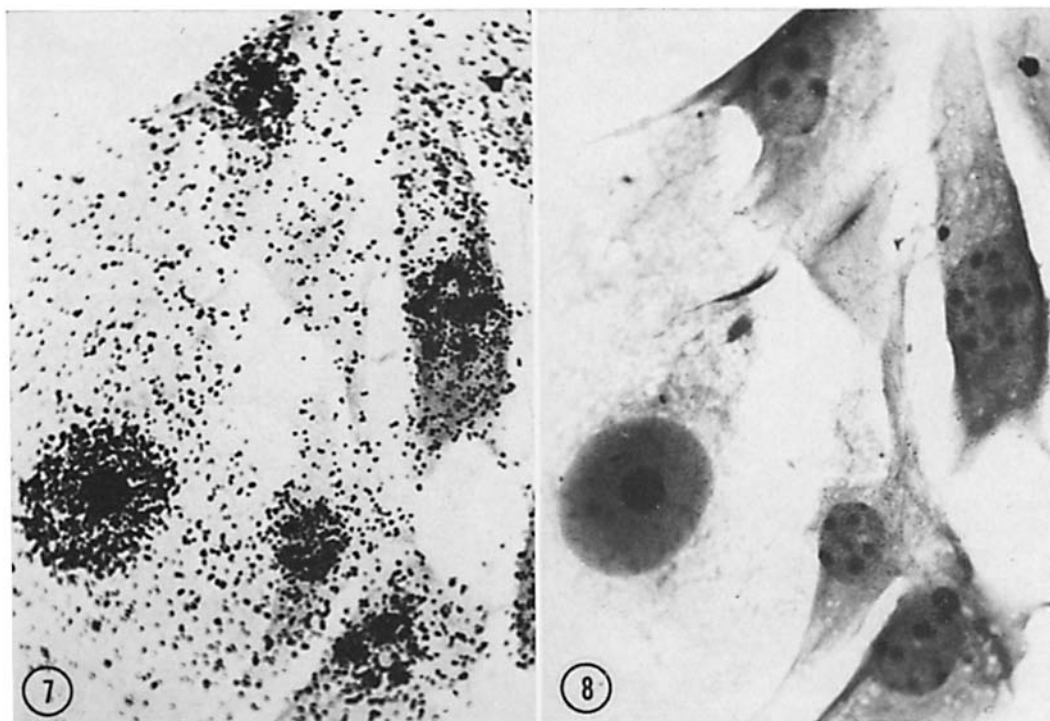
Figures 7 and 8   Cells incubated for 15 min with uridine-^{3}H, sp. act. 21.0 c/mmole, 0.1 mc/ml, and "chased" for 1 hr in a 500-fold excess of cold uridine. Extremely dense accumulations of silver grains in the radioautograph in Fig. 7 (2-wk exposure) correspond to nucleoli which are visible when the silver grains are removed (Fig. 8) regardless of number of nucleoli per cell. $\times$ 1,200.

of the number of nucleolus-like bodies per cell varies from one culture to the next and is related to generation time (Phillips, S. G., in preparation). The cultures used in this study were checked periodically for possible alterations in karyotype by making propionic carmine-stained squash preparations of cultures treated for 4 hr with colchicine. Cells with one or two chromosomes more or less than the diploid number (22) were occasionally seen, but cells with more than 24 chromosomes occurred with a frequency of less than 5%. This degree of variation is not nearly enough to account for the observed variation in nucleolar number on an assumption of one or two nucleolus-producing sites per genome.

The nucleolus-like bodies stain metachromatically with Azure B (Fig. 1) and are unstained by this dye after RNase digestion. The bodies are Feulgen negative and stain with 0.01% fast green at pH 2. Thus, these nucleolus-like bodies contain RNA and protein.

Varying numbers of nucleoli per nucleus are

seen in electron micrographs of Chinese hamster tissue culture cells. This finding suggests that the nuclear bodies seen in the light microscope correspond to the multiple nucleoli seen in electron micrographs. The nucleoli of Chinese hamster tissue culture cells possess both granular and fibrous components and display typical nucleolar morphology. The nucleolar appearance varies somewhat from cell to cell. For instance, some nucleolar profiles include a larger relative amount of granular component, a more distinct nucleolonema, or a more irregular shape than others. In micrographs in which a number of nucleoli are observed in the same cell, however, all the nucleoli almost always appear morphologically similar to one another. Figure 2 and the enlargements (Figs. 3–6) show more or less typical multinucleolate cells. Nucleoli are regular shaped with interspersed fibrous and granular components. Two morphologically distinct fibrous components are observed, one considerably more electron opaque than the other.
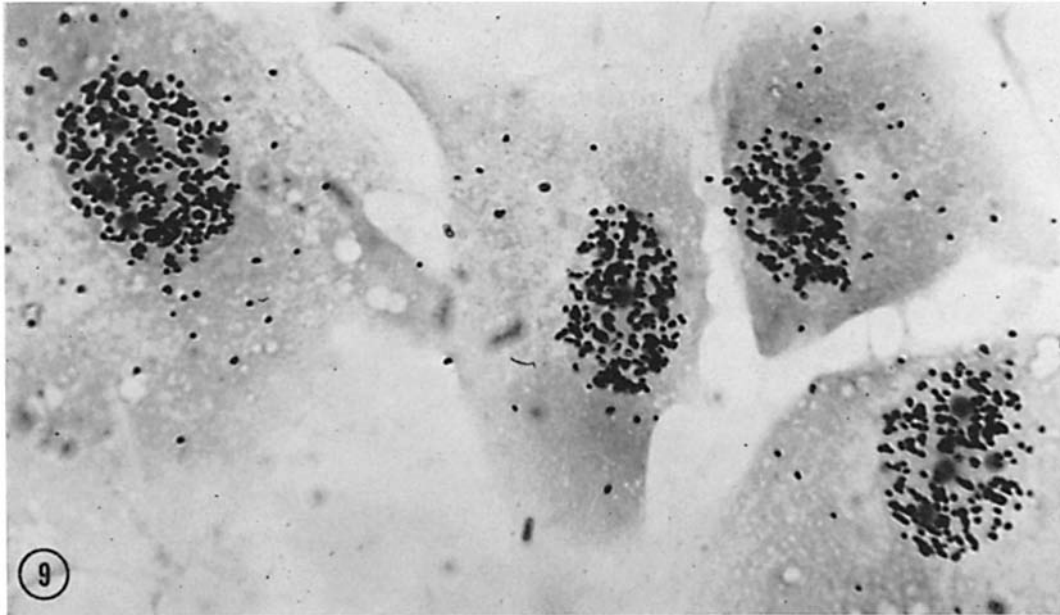
Figure 9 Radioautograph (3-wk exposure) of cells treated with 0.04 μg/ml actinomycin D for 20 min prior to and during a half-hour incubation with uridine-^{3}H, sp. act 21.0 c/mmole, 0.1 mc/ml. Nucleoplasm is clearly labeled; nucleoli are not. × 2,100.

The uridine-^{3}H incorporation patterns of the multiple nucleolus-like bodies conform to the patterns described for nucleoli of other cell types. In radioautographs of cells fixed after a 5-min incubation period with uridine-^{3}H, the nuclei are labeled uniformly over nucleoplasm and nuclear bodies; silver grains generally appear over all the bodies of a nucleus. Label over the nucleolus-like bodies increases relative to the grains over the nucleoplasm with increasing incubation times in uridine-^{3}H. In 500-fold excess cold uridine "chase," the incorporation of uridine-^{3}H into RNA continues at a high rate, presumably because of the presence of intracellular precursor pools (Figs. 7 and 8). Thus, the density of silver grains over nucleolus-like bodies also increases

relative to the nucleoplasmic grain density with increasingly longer chases up to 4 hr. Longer chases were not useful for radioautographic characterization of nuclear bodies because of heavy cytoplasmic label. The radioactivity is removable with RNase. Treatment of cultures with 4 μg/ml of actinomycin D, a fairly heavy dose, inhibits almost all incorporation of uridine-^{3}H.

Low levels of actinomycin D selectively inhibit the synthesis of ribosomal RNA (Franklin and Baltimore, 1962; Perry, 1963). If the multiple nucleolus-like bodies of Chinese hamster cells are active in ribosomal RNA synthesis, one would predict that low levels of actinomycin D would preferentially inhibit the incorporation of

Figure 10 Chinese hamster cells treated for 75 min with 0.04 μg/ml actinomycin D. Compare circular profiles of transected nucleoli with irregular profiles seen in untreated cells in Figs. 2–6. Arrows indicate nucleoli shown in Figs. 11 and 12. × 8,000.

Figures 11 and 12 Enlargements of the four nucleoli indicated by arrows in Fig. 10. Actinomycin D causes segregation of the three nucleolar components. The light fibrous (*l*) and the granular (*g*) components are displaced to opposite sides of the spherical nucleolus, and the dense fibrous component (*d*) separates them. × 65,000.
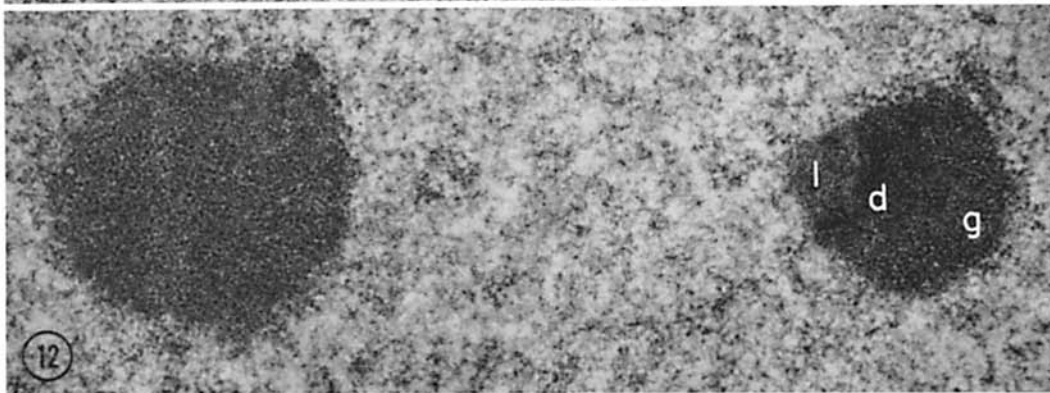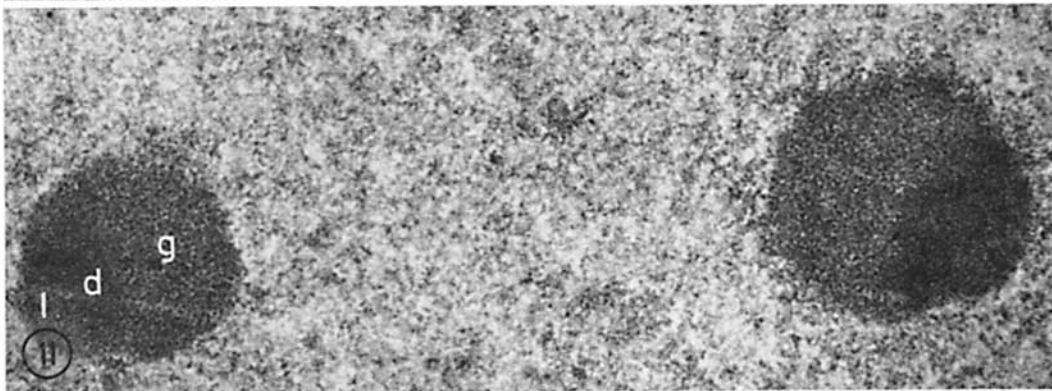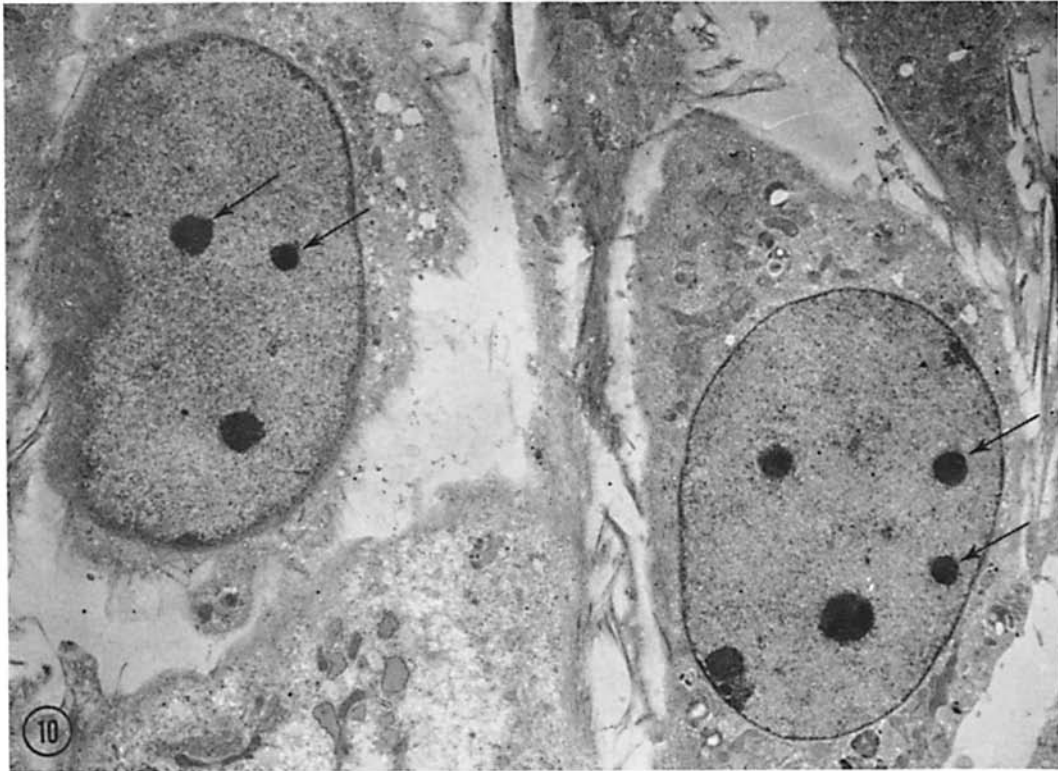
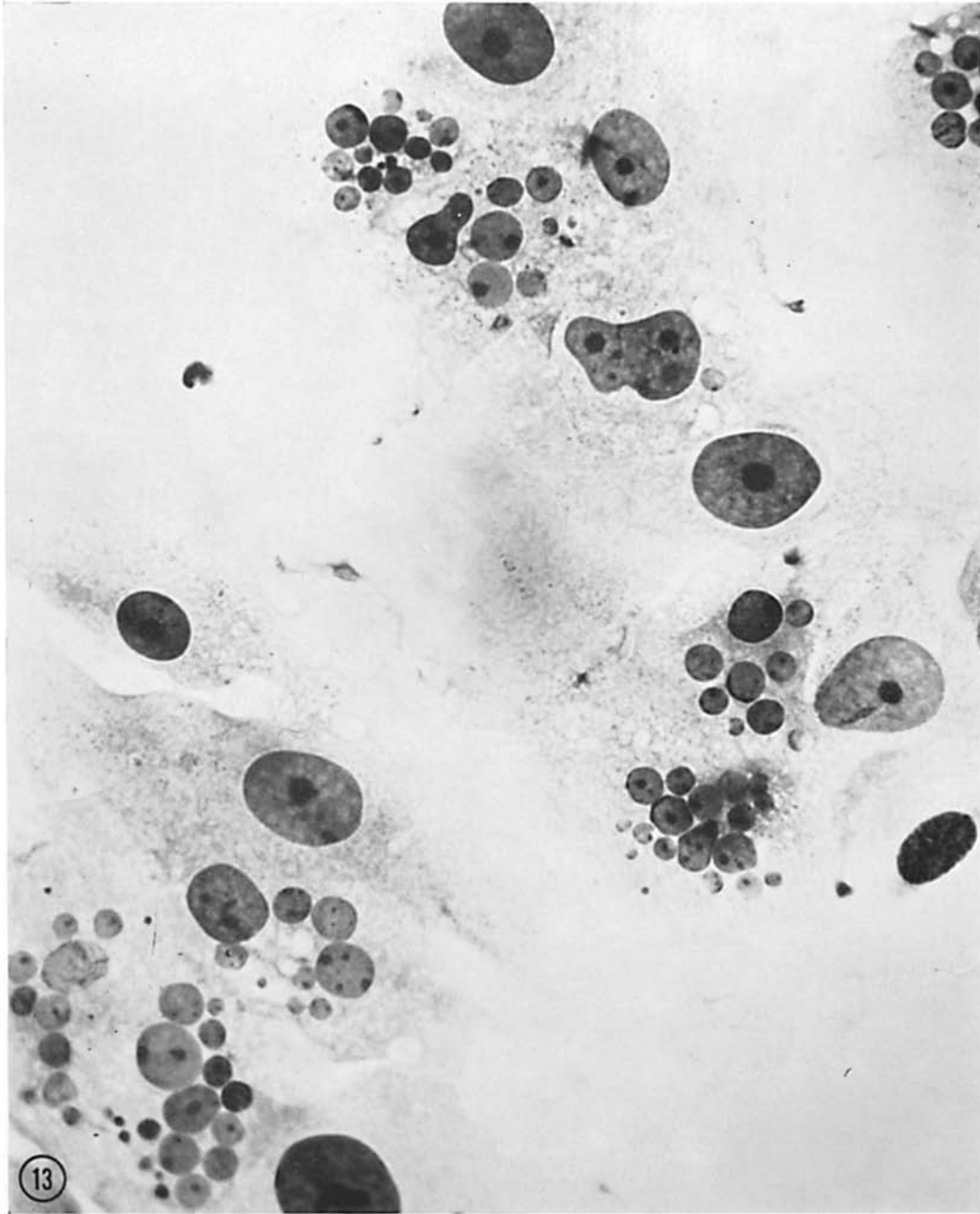FIGURE 13   Culture treated for 19 hr with 5×10^{-5} M colchicine. Several cells with numerous micronuclei as well as some uninuclear cells appear in this field. Nucleoli are visible in almost all nuclei. $\times$ 900.

uridine-^{3}H into RNA of the bodies. In cultures treated with 0.04 μg/ml of actinomycin D for 20 min before and during a half-hour incubation with uridine-^{3}H, label over the nuclear bodies is greatly reduced (Fig. 9). The inhibition appears to affect equally well all the nucleolus-like bodies of a cell.

In electron micrographs, the multiple nucleoli of cells treated with low levels (0.04 μg/ml) of actinomycin D appear much rounder than nucleoli of control cells (Fig. 10). After a 1-hr incubation in actinomycin D, the fibrous and granular components are segregated to opposite sides of the spherical nucleoli; the denser fibrous component is generally located centrally between the less dense fibrous component and the granular component (Figs. 11 and 12). The changes in nucleolar morphology observed in these cells are basically similar to actinomycin D-induced alterations which have been observed in nucleoli of other cell types (Schoefl, 1964; Reynolds, Montgomery, and Hughes, 1964).

Nuclear Bodies of Multimicronucleated Cells

Extended colchicine treatment of rapidly proliferating cell cultures produces some cells with many small nuclei. Most of the cells of a culture treated for an extended period with colchicine have only one nucleus; these are presumably cells which have not entered mitosis during the period of colchicine exposure, as it appears that the cells arrested in metaphase by colchicine give rise to cells with micronuclei. [This observation was demonstrated by the treatment of a culture with colchicine (5 × 10^{-5} M) for 5 hr, harvesting the cells blocked in metaphase, and replating them in fresh medium. 12 hr later, when the replated metaphase cells returned to interphase, 98% of the replated cells collected from one culture and 94% from a second culture possessed more than one nucleus.] Among the multimicronucleated cells of a colchicine-treated culture, it is not unusual to find cells with as many as 22 (the diploid number) micronuclei, each presumably containing a single chromosome; however, cells with lower numbers of micronuclei are more frequently observed. Cells with 44, or slightly fewer, micronuclei are occasionally seen. These cells may arise from cells which were in anaphase when colchicine was added to the medium or from tetraploid cells which occur spontaneously in these cultures at a low frequency (less than 5%).

Most of the micronuclei of multinucleated cells clearly contain nucleolus-like bodies (Fig. 13) which stain metachromatically with Azure B. However, in smaller and more compacted micronuclei it is sometimes difficult to ascertain whether or not a nuclear body is present. In some cells, all the micronuclei are small and compact and lack nucleolus-like bodies, which suggests that these are cells which have not completely emerged from telophase or are necrotic.

In electron micrographs, colchicine-induced micronuclei are bounded by a nuclear envelope which is similar in appearance to the nuclear membrane of control cells (Figs. 14–17). In most sections of multimicronucleated cells, nucleoli are observed in a number of the nuclei. Micronuclei sometimes contain more than one nucleolus. Nucleoli in micronuclei appear structurally normal, containing both granular and light and dark fibrous components. Nucleoli of different micronuclei of the same cell often vary in morphology, although nuclei of the same micronucleus do not (Figs. 15 and 17).

The RNA synthetic capacity of nuclear bodies of micronuclei was tested radioautographically. The concentrations of silver grains over nuclear bodies in radioautographs of multimicronucleated cells incubated with uridine-^{3}H indicate that the individual nucleolus-like bodies in many, and possibly all, nuclei actively incorporate uridine into RNA (Figs. 18–21). Cells with small, compacted nuclei lacking nuclear bodies are labeled very lightly compared with cells in which nuclear bodies are visible. Low levels of actinomycin D (0.04 μg/ml) inhibit the incorporation of uridine-^{3}H into the nuclear bodies of colchicine-induced multimicronucleated cells (Figs. 22–27), whereas the nucleoplasm is clearly labeled in radioautographs of these cells. This finding suggests that the RNA synthesis of nucleolus-like bodies of individual micronuclei represents ribosomal RNA synthesis, which is more sensitive to actinomycin D than is general nuclear RNA synthesis.

In electron micrographs, nucleoli of actinomycin D-treated, multimicronucleated cells show morphological changes similar to those observed in uninuclear cells, i.e., spherical shape with segregated appearance. These changes are, however, less obvious in the smaller nucleoli (Figs. 28 and 29).

DISCUSSION

A variety of criteria applied in this study indicate that nuclear bodies, which commonly range up to 10 or more per cell in Chinese hamster cultures known to be more than 95% diploid, are true nucleoli. Their Azure B– and fast green–staining properties indicate that they contain RNA and protein. Electron microscopy reveals them as typical nucleoli, possessing characteristic fibrous and granular nucleolar components. They are found to incorporate uridine-^{3}H into RNA after 5-min exposure to the isotope. Longer periods of incubation, or incubation followed by cold uridine chase, result in the enrichment of nucleolar label relative to general nucleoplasmic ("chromatin") label, which would be expected as a result of greater stability of nucleolar RNA species. Actinomycin D, at a level known to inhibit ribosomal RNA synthesis selectively (Franklin and Baltimore, 1962; Perry, 1963), greatly reduces the incorporation of labeled RNA precursor into these bodies. Actinomycin D also causes segregation of fibrous and granular components like the segregation which occurs in response to this drug in nucleoli of other cell types (Reynolds, Montgomery, and Hughes, 1964; Schoefl, 1964; Geuskens and Bernhard, 1966). Since these results suggest that each nuclear body is an autonomous site of ribosomal RNA synthesis, they not only establish the identity of these bodies as true nucleoli, but seem to exclude the possibility that the multiple nucleoli arise by fragmentation from a smaller number of primary nucleoli produced at organizer loci.

Multiple nucleolus-like "blobs" have been described in nuclei which completely lack a nucleolar organizer locus in embryos of *Xenopus* (Elsdale, Fischberg, and Smith, 1958; Esper and Barr, 1964) and *Chironomous* (Beermann, 1960) and in corn microspores (McClintock, 1934). Though the "blobs" formed in these anucleolate nuclei contain RNA and protein, they are apparently not sites of ribosomal RNA synthesis (Brown and Gurdon, 1964); they are not capable of supporting growth and development of a normal embryo (Elsdale, Fischberg, and Smith, 1958; Beermann, 1960); and they lack the granular component discernible in electron micrographs of normal nucleoli (Jones, 1965; Hay and Gurdon, 1967; Swift and Stevens, 1966). Thus, the "blobs" formed in nuclei which lack a nucleolar organizer are to be distinguished from the multiple nucleoli of Chinese hamster cells which are apparently normal in structure and function.

The nucleoli found in the karyomeres of multinucleated Chinese hamster cells produced by colchicine treated appear to be true, functional nucleoli by the same criteria of structure, composition, RNA precursor incorporation, and response to actinomycin D applied to the nucleoli of uninuclear cells. This finding suggests that nucleolus-producing capacity is associated with most, and possibly all, 22 chromosomes of the Chinese hamster diploid complement. It cannot be stated with certainty that all chromosomes possess nucleolus-producing sites because it is sometimes difficult to observe a nucleolus in the smaller, more condensed micronuclei and even more difficult to determine whether or not some of the very small nucleoli are labeled in radioautographs. It is interesting that nucleoli in different micronuclei of the same cell usually display much morphological variation, whereas nucleoli in the same nucleus of a multimicronucleated cell, or in the single nucleus of control cells, are generally morphologically similar. This implies that the morphology and presumably the physiology of nucleoli is, at least in part, under nuclear control.

The hypothesis that nucleoli can be produced at

FIGURE 14 Low magnification electron micrograph of a multinucleated cell produced by 24-hr colchicine treatment. In any given section, nucleoli are generally seen in several micronuclei of a multinucleated cell. Nuclei in which nucleoli are not observed may possess nucleoli which lie above or below the plane of section. Arrows indicate nucleoli shown in Fig. 15. × 8,500.

FIGURE 15 Enlargements of the six nucleoli indicated by arrows in Fig. 14. These nucleoli contain granular, dense fibrous, and light fibrous components and, except for their smaller size, generally resemble nucleoli of the uninuclear cells of control cultures. × 31,000.
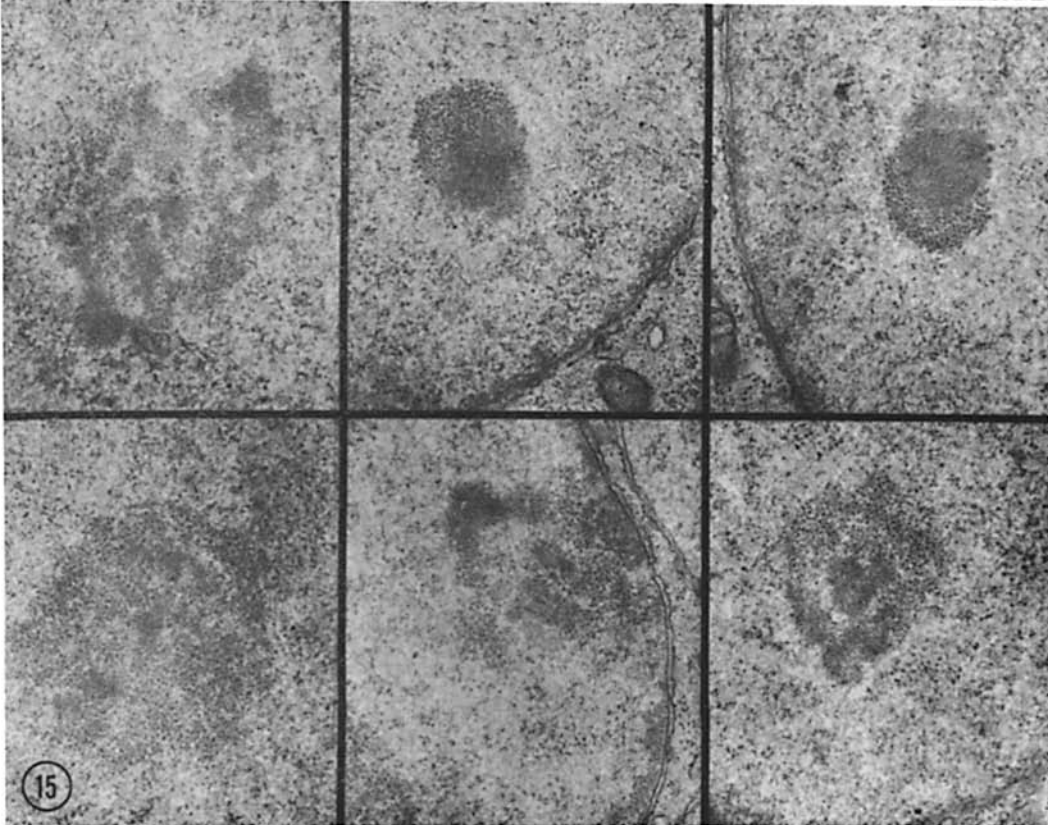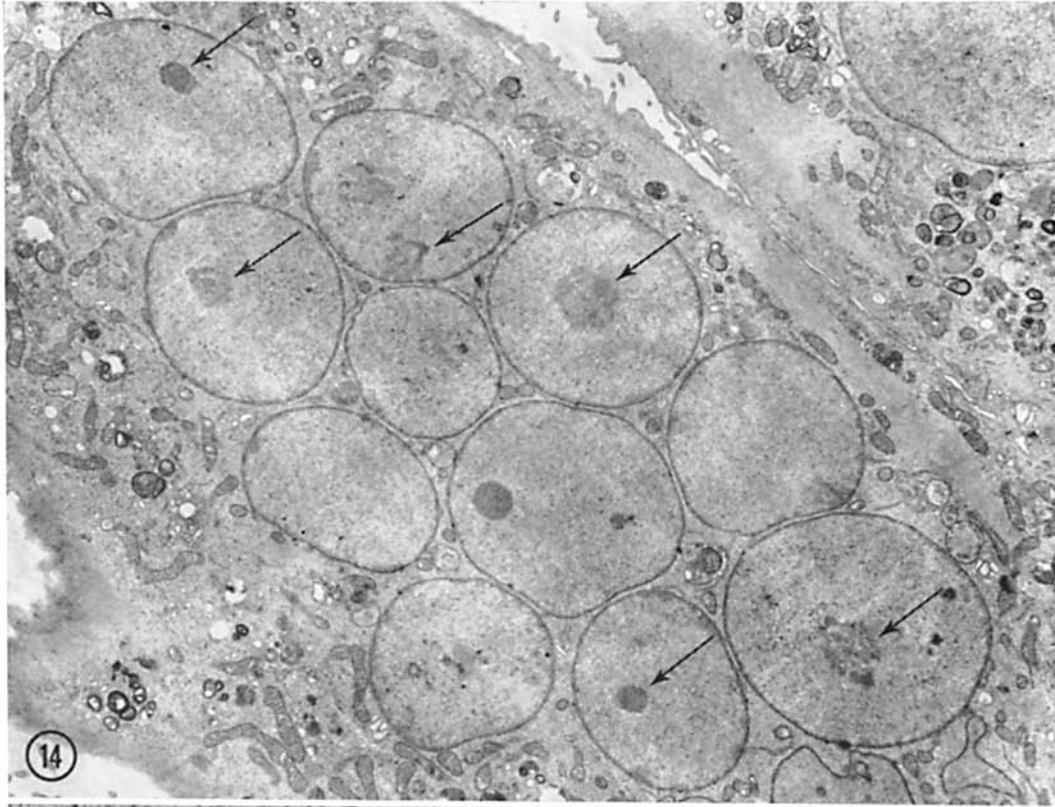
many chromosomal sites appears with considerable frequency in cytological literature. It is most often based on observations of telophase cells in which nucleoli appear to arise by coalescence of granules or matrix material coating the chromosomes (Heitz, 1931 b; Derman, 1933; McClintock, 1934; Jacob, 1940; Gates, 1942; Rattenbury and Serra, 1952; Lafontaine, 1958, Tandler, 1959; Lafontaine and Chouinard, 1963; Stevens, 1965). This hypothesis is supported, to some extent, by the findings that mitotic chromosomes studied in situ are coated with RNA (Kleinfeld and von Haam, 1959; Mazia, 1961; Feinendegen and Bond, 1963) and that RNA extracted from isolated mitotic chromosomes is similar to ribosomal RNA in composition (Salzman, Moore, and Mendelsohn, 1966; Huberman and Attardi, 1966; Maio and Schildkraut, 1967) suggesting that RNA associated with mitotic chromosomes may bear a relationship to nucleolar RNA. These observations might also be interpreted to mean that nucleolar material adheres nonspecifically to chromosomes during division and is reconstituted into nucleoli at telophase. Such a hypothesis would explain the appearance of nucleolus-like bodies in all the karyomeres formed by colchicine treatment. Indeed, Das and Alfert have described a proteinacious component of nucleoli which appears to behave in this manner in Vicia and Allium (Das and Alfert, 1961). They found, however, that, though nucleolus-like bodies initially are seen in all karyomeres of multimicronucleated cells arising after X-ray induced chromosome fragmentation, most of these bodies are inactive in RNA synthesis and disappear with time (Das 1962 a, 1962 b). This is not true, however, of the nucleoli formed in karyomeres of multimicronucleated Chinese hamster cells. The continued maintenance of RNA synthetic capacity in these bodies renders it unlikely that they are merely remnants of old nucleolar material sloughed nonspecifically from all the chromosomes.

The possibility must then be considered that, though some organisms, such as Xenopus and Drosophila, possess only one nucleolar organizer locus per genome, in other organisms nucleolus-producing loci, presumably containing the ribosomal RNA cistrons, are scattered through the genome.

The nucleolar organizer locus in Xenopus and Drosophila is known to be a stretch of some 130–1600 redundant tandem repeats of the cistrons for ribosomal RNA (Ritossa, Atwood, Lindsley, and Spiegelman, 1966; Brown and Gurdon, 1964). It is reasonable to assume that such a locus could be broken up and scattered through the genome without disturbing its function. Indeed, translocations are known which break the nucleolar organizer locus into two parts, both of which then produce nucleoli (McClintock, 1934; Beermann, 1960).

The number of nucleolus-producing loci might be expected to be different in different species. The great many comparative studies of nucleolar number per cell in the literature would be expected to yield some information in this regard. However, simple counts of numbers of nucleoli per cell in material stained with basic dyes is not sufficient means to establish the number of such loci in an organism. Literally hundreds of reports of this sort in the early literature (see Montgomery, 1898 for review) are impossible to interpret because widely varying techniques of fixation and staining are employed for differentiating between various cellular components, and the biochemical bases for the staining differences achieved are not known. Thus, other nuclear structures such as blocks of heterochromatin are often confused with nucleoli. In addition, many early studies comparing numbers of nucleoli in various species do not take polyploidy into account, or they deal with oocytes, which are now known in several species to possess many excess copies of the nucleolar organizer region (Brown and Dawid, 1968; Gall, 1968). Even studies of nucleolar number per cell

FIGURE 16 More than 22 (2N) nuclei are visible in this section through a multinucleated cell. Therefore, colchicine may have arrested this cell in anaphase, or the cell may initially have been tetraploid. (Tetraploid cells occasionally arise spontaneously in culture.) Nucleoli are present even in some of the smaller nuclei. Arrows indicate nucleoli shown in Fig. 17. × 7,500.

FIGURE 17 Enlargement of the six nucleoli indicated by arrows in Fig. 16. Nucleoli contain characteristic granular, dense fibrous, and light fibrous zones. × 33,000.
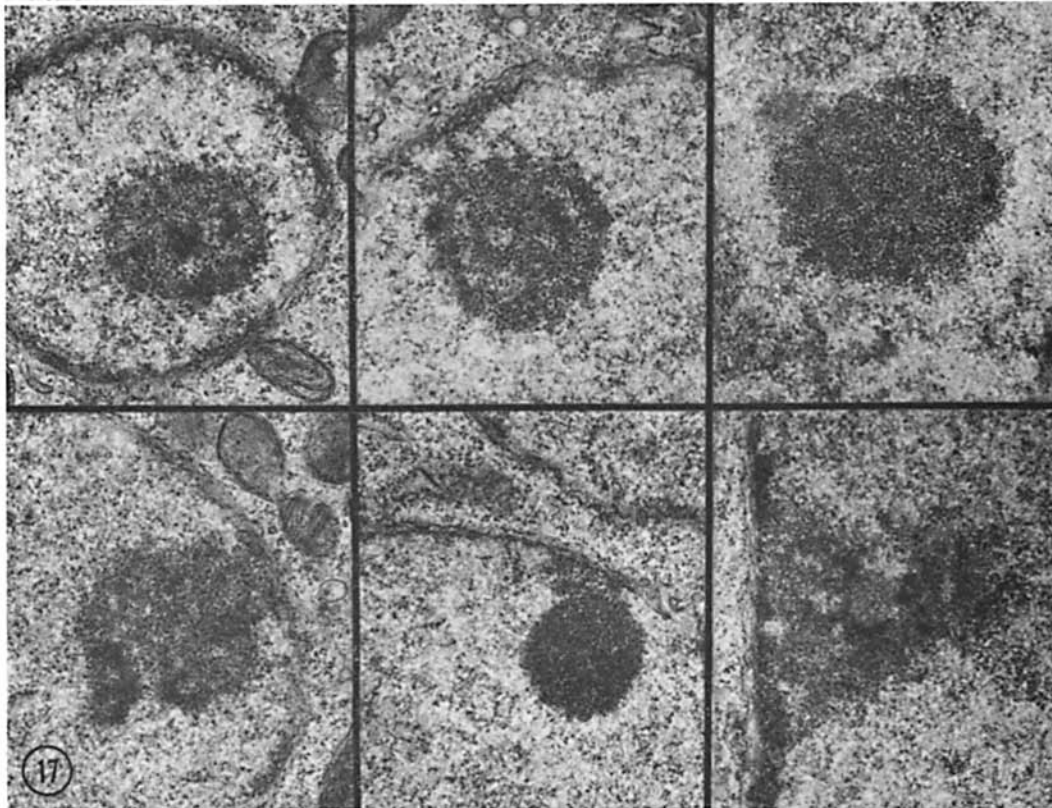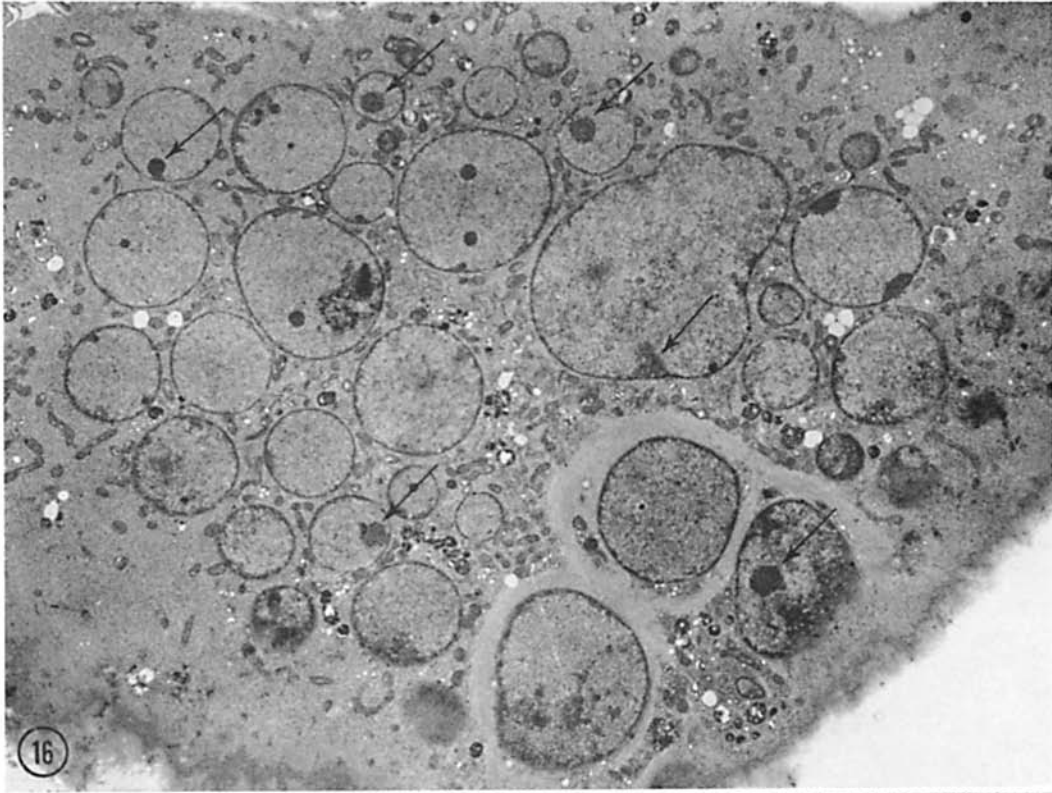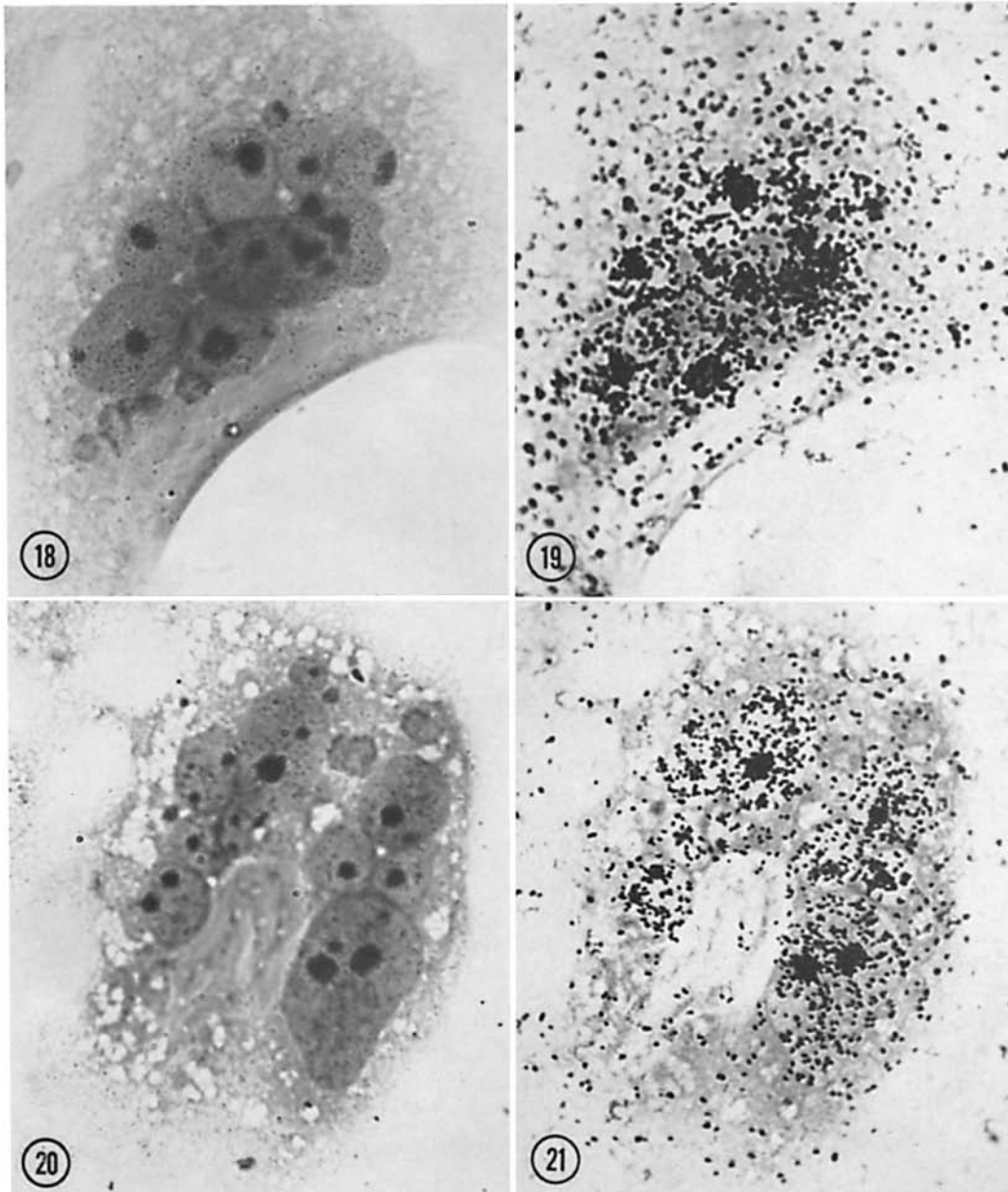
FIGURES 18–21 Multinucleated cells produced by 18-hr treatment with 5×10^{-5} M colchicine and subsequently incubated for 30 min with uridine-^{3}H and "chased" for 2 hr in 500-fold excess cold uridine. Where nucleoli appear in Figs. 18 and 20, from which silver grains have been removed, there are corresponding dense accumulations of silver grains in the radioautographs of the same cells seen in Figs. 19 and 21. Figs. 18 and 19, $\times$ 2,800. Figs. 20 and 21, $\times$ 1,900.
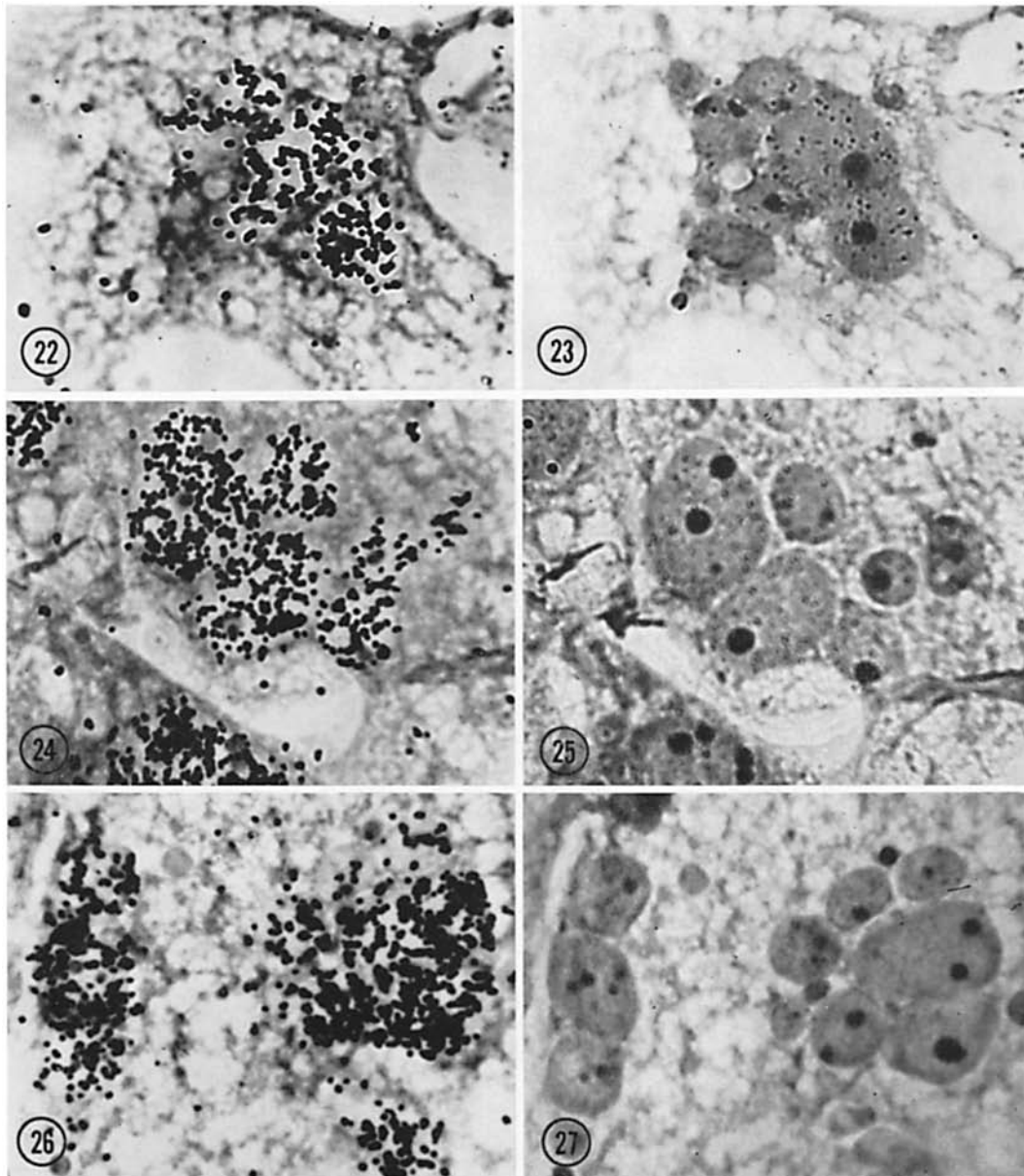
FIGURES 22, 24, and 26   Radioautographs (3-wk exposure) of multinucleated cells produced by 18-hr colchicine treatment. These cells were treated with a low level (0.04 µg/ml) of actinomycin D for 20 min prior to and during a 30-min incubation with uridine-^{3}H. sp. act. 21.0 c/mmole, 0.1 mc/ml. × 2,900.

FIGURES 23, 25, and 27   The same cells with silver grains removed. The nucleoli appear to be unlabeled. Compare these cells with those in Figs. 18–21 which are not treated with actinomycin D. × 2,900.
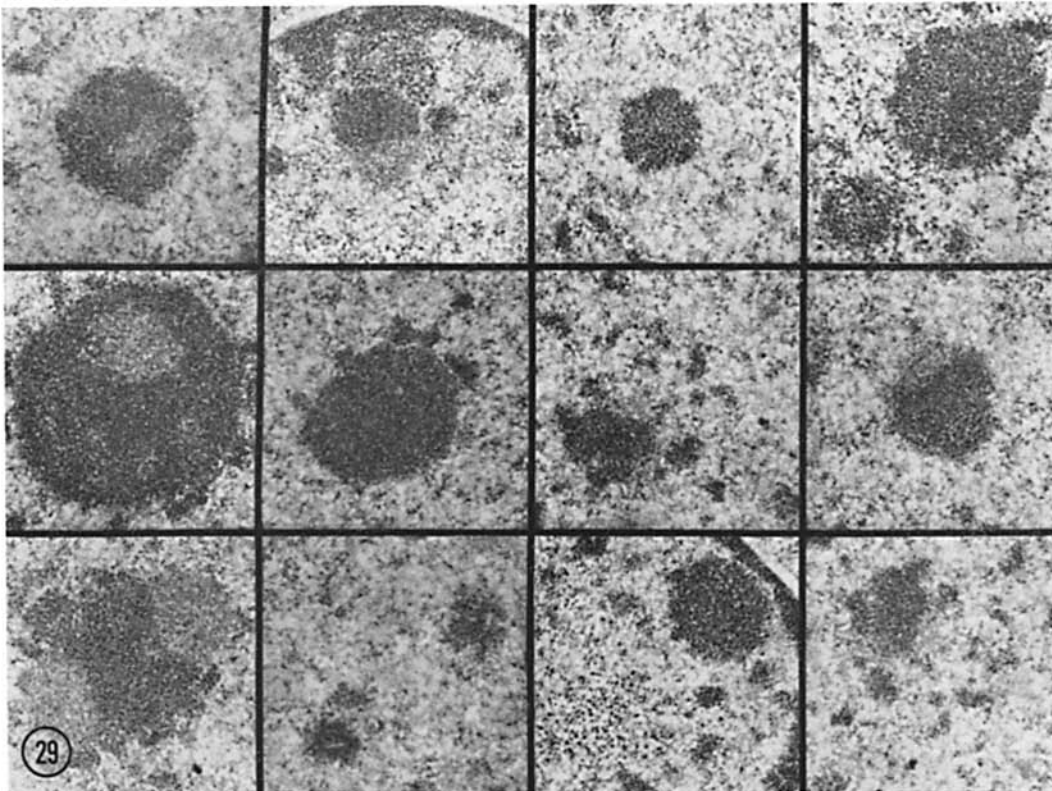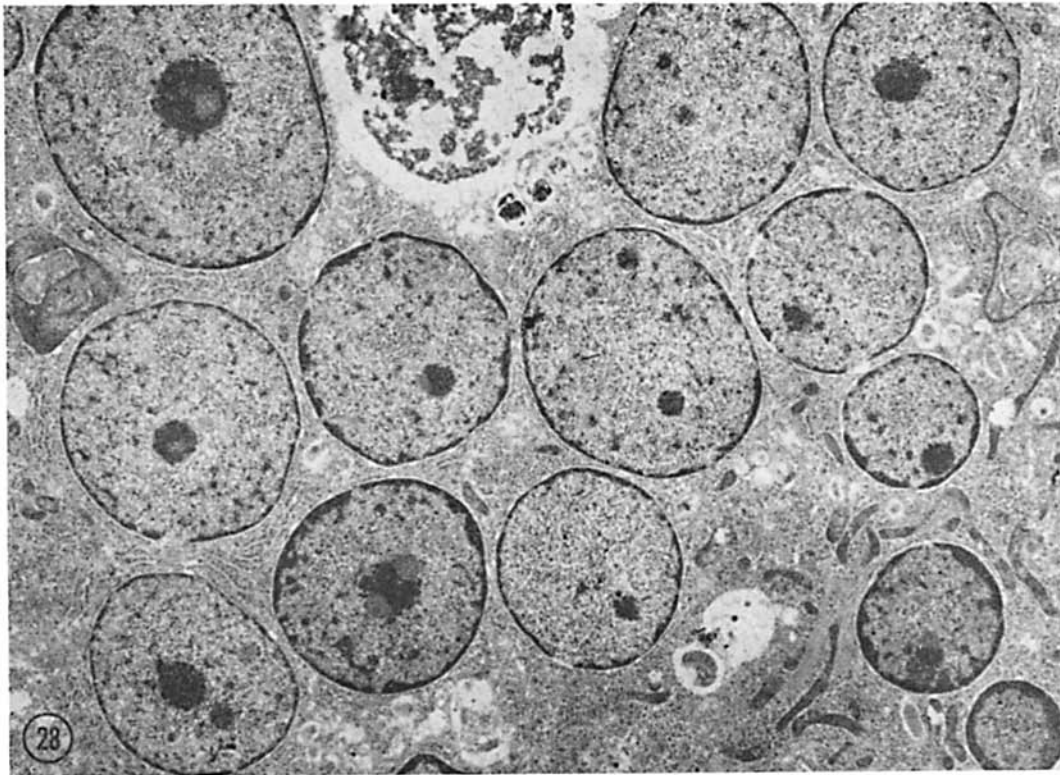
FIGURE 28   Multimicronucleate cell treated with 0.04 µg/ml actinomycin D. Nucleoli appear more spherical than in untreated multimicronucleate cells. × 1,700.

FIGURE 29   Enlargements of 12 of the nucleoli seen in Fig. 28. Segregation of nucleolar components is observed in the favorably transected nucleoli. Some of the nucleoli display only fibrous or only granular zones; presumably the other nucleolar components lie above or below the plane of section. × 53,000.

in which nucleoli are carefully identified by specific staining techniques (Shea and Leblond, 1966) cannot always predict the number of nucleolar organizer sites per genome, due to complicating factors such as the tendency for nucleoli to fuse after their initial appearance at telophase (Heitz, 1931 a; Gates, 1942; Swift, 1959) and the possibility that some nucleolar organizers present in the genome are not being expressed (Nawaschin, 1927; Crosby, 1957; Ritossa, 1968). Thus, nucleolar number per cell alone is not a reliable index of the number of nucleolar organizers per genome. Stringent biochemical and genetic criteria have been used to establish that *Xenopus* and *Drosophila* have one nucleolar organizer per genome (Wallace and Birnstiel, 1966; Ritossa and Spiegelman, 1965). In two other species, *Chironomus tentans* and wheat (*Triticum sativum*), careful analysis has revealed more than one nucleolar organizer per genome. In *Chironomus*, nucleolar organizers may be visualized in squashes of larval salivary gland chromosomes as the sites of attachment of the nucleoli. Beermann (1960) produced fertile hybrids of *Chironomus tentans* and *C. pallidivittatus*, whose single nucleolus occupies a site not homologous to the site of either of the two nucleoli of *C. tentans*. Matings of hybrids produce 25% nucleolus-less offspring which die as embryos. A different approach was taken by Crosby (1957) to demonstrate nucleolar organizer sites on four different chromosomes of wheat. She obtained plants which were aneuploid with repect to each of the 21 chromosomes and studied the micronuclei formed as a result of the lagging of the unpaired chromosome in meiosis. Aneuploids for four of the chromosomes produced micronuclei which contained nucleoli; the other 17 chromosomes did not support a nucleolus.

It has been reported that nucleoli of Chinese hamster cell lines often persist during mitosis and may then be found in association with several chromosomal loci (Hsu, Arrighi, Klevecz and Brinkley, 1965; Heneen and Nichols, 1966). However, since nucleoli normally become inactive and disappear during mitosis, it is difficult to decide what significance to attach to associations of left-over nucleolar fragments which ultimately will be discarded into the cytoplasm. The results of the present study make it fairly clear that in the Chinese hamster, most, if not all, of the 11 chromosomes possess the capacity to produce nucleoli. There are indications that scattered nucleolus-

producing sites may also be characteristic of other mammals, including man. Diploid mouse liver cells are reported to contain more than two nucleoli (Bisele, Poyner, and Painter, 1942). From counts of nucleolar number per cell in a wide variety of tissues stained with Azure B after DNase extraction, Shea and Leblond (1966) estimate the number of nucleolar organizers in mouse to be at least six per cell. This finding seems to contradict earlier studies of meiotic prophase in mouse testis (Sachs, 1955; Ohno, Kaplan, and Kinosita, 1957) indicating that nucleoli are associated with only the sex chromosomes. However, studies of interphase somatic cells are probably more reliable than studies of meiotic prophase since the nucleoli seen at prophase are small, no longer active in RNA synthesis (Utakoji; 1965, personal observation), and fewer in number than in many diploid somatic cells of the same organism. Indeed, in Chinese hamster testis only the X and Y chromosomes show evidence of a nucleolar association at meiotic prophase (Yerganian, Kato, Leonard, Gagnon, and Grodzins, 1960). Studies of meiotic prophase in rat testis reveal nucleolus-associated sites on two autosomal bivalents (Ohno, Kaplan, and Kinosita, 1959), which might be considered a minimum in light of the observations in mouse and Chinese hamster testis. Cytogenetic analyses of human somatic and testis cells suggest possible associations of nucleoli with five of the smaller chromosomes (Ferguson-Smith and Handmaker, 1961; Yerganian, 1963). McConkey and Hopkins found that isolated nucleoli of human (HeLa) cells contained only 15% of the chromosomal sites capable of hybridizing with ribosomal RNA (McConkey and Hopkins, 1964). This result could be explained by assuming that many sites containing ribosomal RNA cistrons exist in an inactive state. The ideal approach to this problem would be to isolate the chromosomes of the complement, separate them into classes, and test each chromosomal class for its ability to anneal ribosomal RNA. This type of analysis has been pioneered by Huberman and Attardi (1967) with isolated chromosomes from HeLa cells. They find that at least 50% of the sites annealing ribosomal RNA are associated with the smaller chromosomes of the human complement which have been postulated, on cytogenetic grounds, to carry nucleolar organizers. The technique is not yet refined enough either to exclude or to implicate any particular

chromosome as the bearer of ribosomal RNA cistrons.

In summary, in *Xenopus* and *Drosophila*, the most carefully analyzed organisms in this regard, the ribosomol RNA cistrons are localized at a single chromosomal site which is also associated with the production of a nucleolus. However, it should not be forgotten that these two organisms were specially selected for analysis because of pre-existing genetic studies indicating the presence of a single nucleolar organizer locus. Evidence is presented here that the Chinese hamster genome possesses many nucleolus-producing sites. Perhaps

the generally accepted view that one nucleolus-producing site per genome is the rule among animal species (Brachet, 1957; Brown, 1966) should be carefully re-examined.

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