MORPHOLOGY AND REPLICATION OF INTRANUCLEOLAR DNA IN POLYTENE NUCLEI

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INTRODUCTION

This report is concerned with filamentous intranucleolar structures in the polytene salivary gland cells of *Drosophila melanogaster*. A number of studies on a variety of cells have provided evidence for the chromosomal association of such structures (e.g. references 6, 7), fortifying the concept of Lettré et al. (11) that the nucleolonema, of historic controversy, is not an autonomous structure of the nucleolus, but instead is derived from a portion of the nucleolar chromosome. Support for that concept and, further, a basis for the proposition that all of the filamentous structures of the nucleolus have a common identity, may be found in this study of a single cell type in which intranucleolar structures of diverse morphological conformations ranging from a dense mass to a much extended, ramified filament have been observed. A series of micrographs are presented as evidence that all forms are homologous and are alternate forms of the laterally extruded nucleolus organizer locus of the chromosome complement.

Radioautography of thymidine incorporation confirms that these structures contain replicating DNA and indicates that a negative correlation exists: the dynamic morphology is not related to replicative activity.

MATERIAL AND METHODS

Oregon-R, a wild type strain of *Drosophila melanogaster*, was used as the source of the larval salivary glands. Individuals within the last 10% of the duration of the third instar were selected to assure a fair number of nuclei of highest polyteny as well as those in all phases of replication (14).

1. The isolated gland was placed in a drop of aceto-orcein for 20 min, then squashed with a piece of food-wrap plastic between coverslip and tissue. The plastic was floated off in acetic alcohol, and the squash was restained in aceto-orcein for 10 min. After alcoholic dehydration, the preparation was washed in xylol and mounted. (The foregoing is referred to throughout this paper as the conventional aceto-orcein method of squash preparation.)

2. Some preparations, so noted, were immersed in 60% acetic acid for 60 min before restaining in aceto-orcein for 10 min.

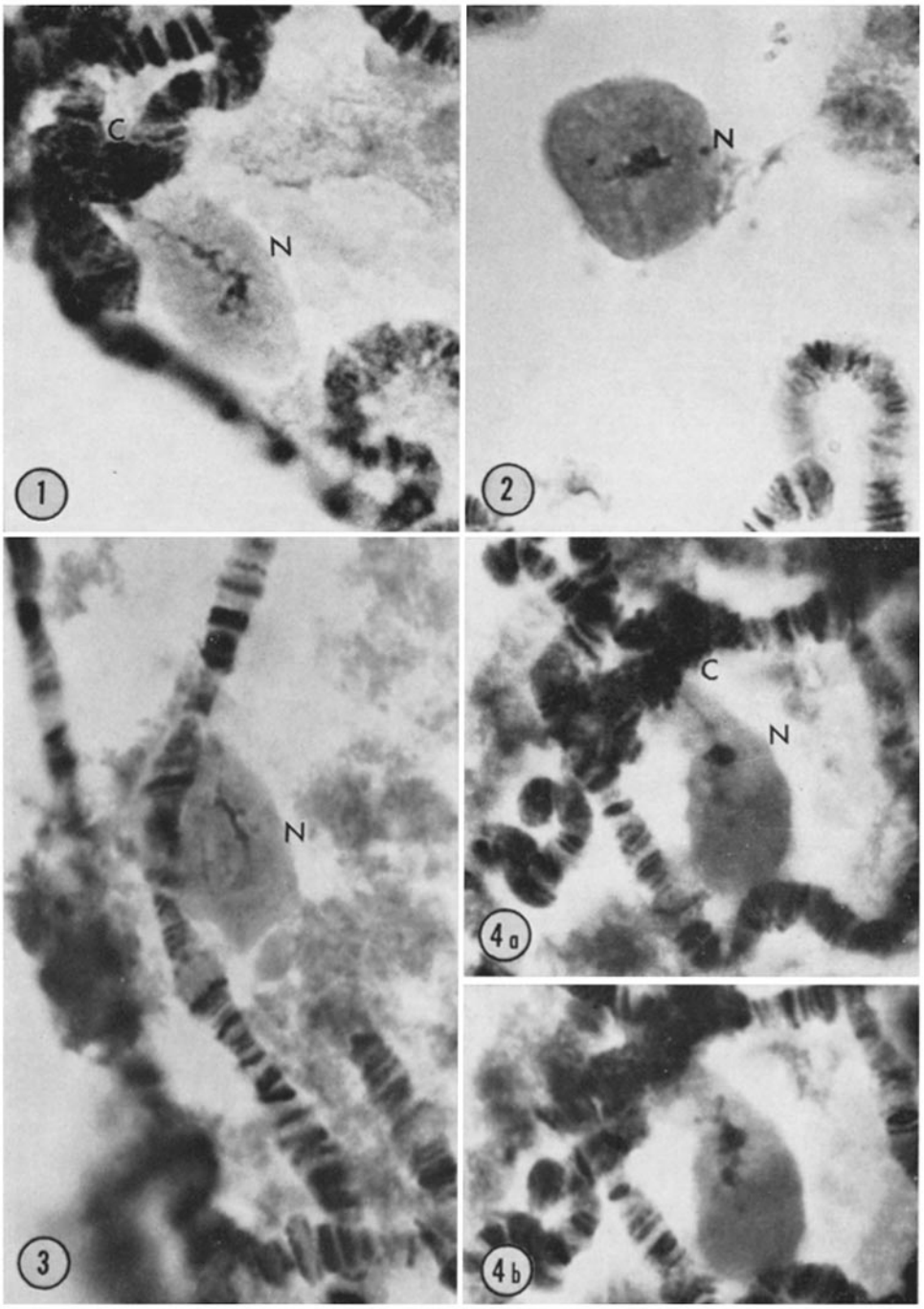
3. Some preparations, so noted, were immersed in 0.01 N NaOH for 30 sec and washed in 0.2 M phosphate buffer (pH = 7.2) before restaining in aceto-orcein.

4. The Feulgen reagent was made by the method of Stowell (16). Attention is directed particularly to the fact that the decolorized reagent should be water-white. The preparations were very lightly counterstained with 0.1% Light Green.

5. For those preparations that were to be analyzed by radioautography, the glands were incubated in tritium-labeled thymidine, as previously described (15), then subjected to one of the above procedures. Kodak AR 10 stripping film was applied, exposed for 18–21 days, and developed in D19b.

OBSERVATIONS

A recent report has attributed the varying morphological characteristics of nucleolar DNA in Dipteran salivary gland nuclei to species differences (1). However, a wide spectrum of morphological conformations of a densely staining structure within the nucleolar body was observed in aceto-orcein squash preparations of salivary glands of the single species of this study (Figs. 1–4). Confirmation that such structures are DNA-containing was made by means of radioautography of tritiated thymidine incorporation (Fig. 5), which further revealed that the capacity for replication was not restricted to any one morphological conformation. Occasionally, moreover, nucleoli were observed in conventional aceto-orcein preparations in which there was no differentially stained clump or thread-like structure, but in which replicating DNA could be detected (Fig. 6). On the supposition that, in such instances, the structure is present in a form so dispersed that it may not be revealed by conventional aceto-orcein staining, a method was sought whereby its presence might be unmasked. An important requirement for such a method was that the banding patterns of the chromosomes not be distorted since it could



FIGURES 1–4 Various conformations of intranucleolar structure as revealed by conventional aceto-orcein stain. Note attachment to chromosome in region of chromocenter in Fig. 4 (*a* and *b* are two levels of focus). *N*, nucleolus; *C*, chromocenter. × 1750.

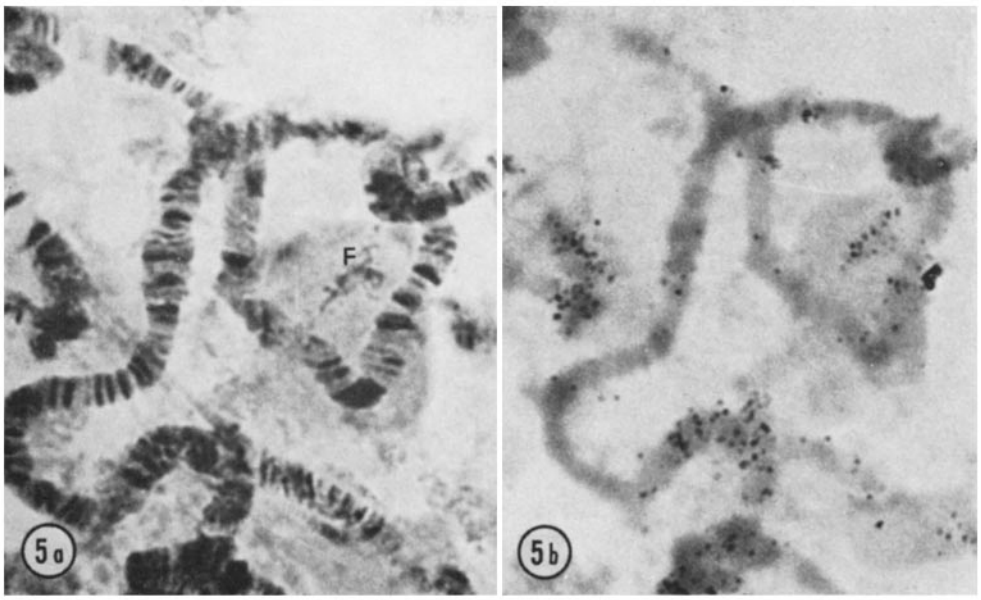


FIGURE 5 Intranucleolar filament showing label after short pulse of Thymidine-^{3}H. *a*—before, *b*—after radioautography. Conventional aceto-orcein stain. *F*, filament. × 1450.

be argued that, if the integrity of chromosomal morphology was maintained, the revealed intranucleolar structure could be assumed to be real and not artefactual.

Those criteria were satisfied by the intercalation of a controlled acid or alkaline extraction step into the conventional aceto-orcein staining procedure (see 2 and 3 of Methods). When the aceto-orcein staining of the squashed preparation was preceded by 1 hr of immersion in 60% acetic acid, the matrix of most nucleoli was "thinned out" (Figs. 7–9); the typical compact thread-like structure was seen in many nucleoli (Fig. 7), while in others a ramified filamentous structure of a type not observed in nucleoli of the conventional aceto-orcein preparations was revealed (Fig. 8). Radioautography of thymidine incorporation demonstrated that the filamentous material contains replicating DNA. Such extended filamentous structures within some nucleoli were equally well displayed after the short treatment with 0.01 N NaOH (Fig. 10). When the typical clumped thread conformation was seen in NaOH-treated preparations, it appeared, as did the chromosome bands, to be stained far more intensely than with the conventional aceto-orcein stain. In such preparations, also, the ramified filamentous form of the structure was identified as replicating DNA.

The continuity of the intranucleolar structure with the chromosome is clearly seen in a representative specimen of each of the methods of preparation (Figs. 1, 4, 9, 10). The nucleolar organizer locus in *Drosophila melanogaster* has been found by genetic mapping to be located very close to the centromere on both the X and the Y chromosomes (5, 8) and, by cytological localization (9), between 20 A3 and 20 D1 on the salivary gland chromosome map. What appears to be the extension of a single band embedded in the matrix of the nucleolus may be seen originating from the chromocenter, which, in *Drosophila melanogaster*, includes the coalesced centromeres, and is distinguishable from the banded portions of the chromosomes by its vesicular structure (Figs. 1, 4).

The chromosomal locus of origin of the laterally extruded band, clearly seen in Fig. 10, may be precisely identified on the cytologic map (2, 10) (see insert, Fig. 10 *a*) to occur within the region of the X chromosome designated by Kaufmann (9) to contain the nucleolar organizer. Fig. 10 *a* also suggests that the continuum of the chromosomal axis is maintained in the lateral extension around which the nucleolus has formed. Furthermore, if the structure is observed at several levels of focus (Figs. 10 *a*, *b*), it is apparent that the ramifying filamentous material, revealed by the alkaline

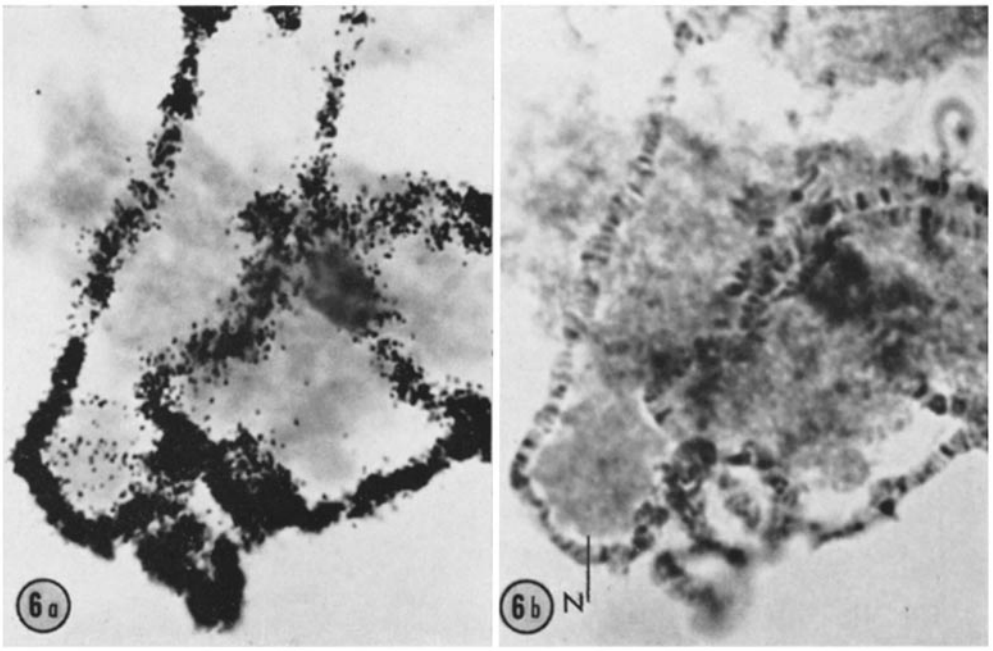


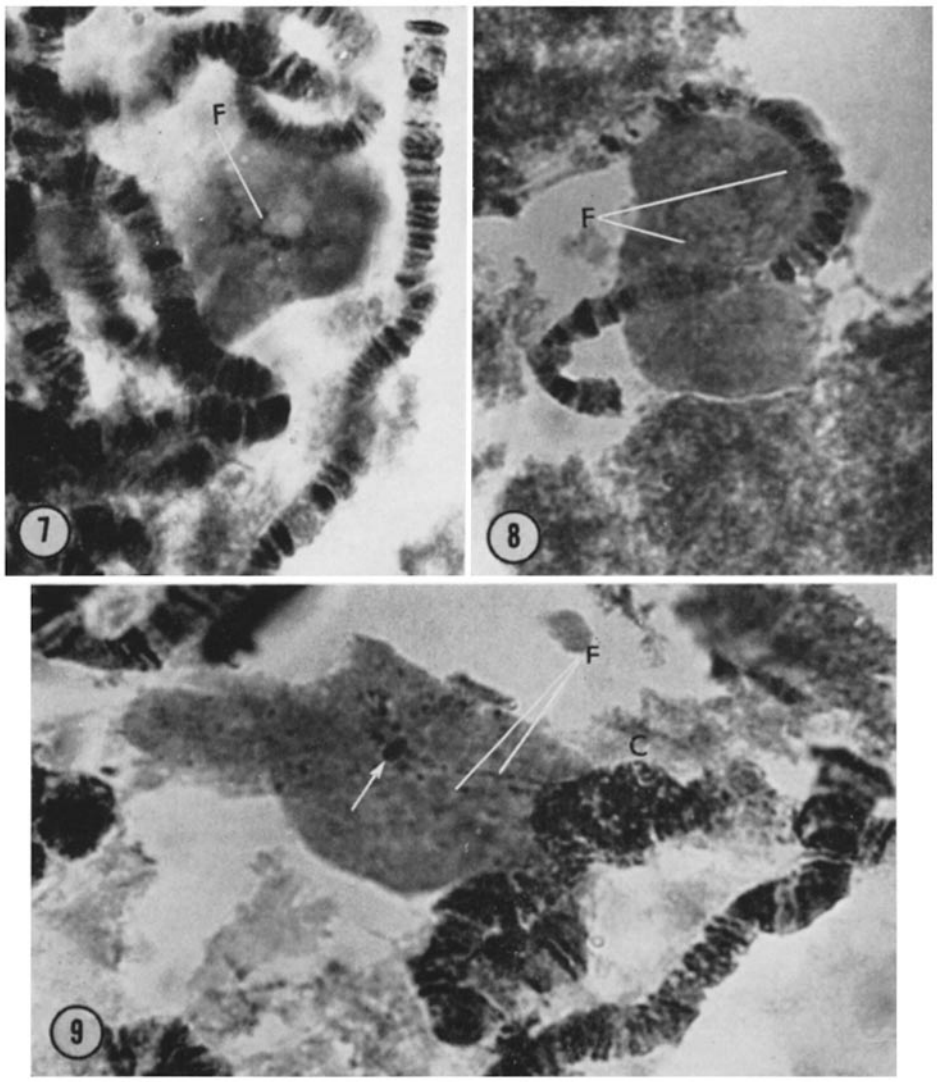
FIGURE 6 Nucleolus is labeled with Thymidine-^{3}H, but no filament is visible within it. *a*—before, *b*—after radioautography. Conventional aceto-orcein stain. *N*, nucleolus. × 1450.

treatment, is a continuation of that structure. A similar relationship of the intranucleolar thread to the chromosome is suggested in Fig. 9 where the bipartite connection of that thread to the chromosome is clearly displayed.

Various types of intranucleolar filaments have frequently been described as Feulgen negative (11). However, it was possible, in this study (with little modification of the conventional basic-fuchsin Schiff reagent) to demonstrate positive Feulgen stain in the intranucleolar bodies, which, like their aceto-orcein-stained counterparts, were of varying forms (Figs. 11, 12). The density of the stain was low, particularly in the much ramified type of structure, but it could be seen, even in bright field; and in a few favorable preparations the Feulgen-positive thread was identified as replicating DNA by thymidine radioautography. Radioautography further revealed that, in general, label over the nucleolus parallels density of label over its related chromosome set (Table I) and that, without exception in 167 salivary gland preparations, no nucleolus was labeled when the chromosomes were not.

DISCUSSION

The extraction procedures, as modifications of the aceto-orcein squash technique, were arrived at empirically, and, as such, were serviceable for the purpose of this study. However, only a tentative explanation for their cytochemical activity may be offered at this time. Although the mechanism of orcein staining has not been established, the appearance of orcein preparations is well known—all structures are pink or red, with the DNA-rich structures being most densely stained. It may be that the DNA concentration of the much extended filamentous structure is so low that its orcein stain is not visibly differentiated; the structure, therefore, is revealed only when some of the surrounding amorphous material, supposedly protein, has been removed. In the nucleolus of Fig. 10, the portion of the thread at its chromosomal origin, only partially uncoiled, is densely stained, while the extended portion is far less densely stained, but, as a result of the extraction procedure, is revealed. That interpretation is supported by the observation that, after either one of the extraction procedures, the nucleolar matrix appears to be vacuolated while the structure of the thread



FIGURES 7–9 Nucleolar filaments after acetic acid extraction. Fig. 7—Compact structure similar to the structures in Figs. 1–4. Fig. 8—Extended ramified filament. Fig. 9—Note bipartite attachment at chromocenter (piece of debris at arrow). *F*, filament; *C*, chromocenter. × 1750.

and of the associated chromosomes is retained (e.g., Figs. 7–10). Since the acid proteins present in abundance in the nucleolar matrix are separable into fractions that are either alkali- or acid-soluble (4), the vacuolated appearance of the nucleolar body may well have resulted from the removal of either fraction.

It was possible, therefore, by such treatments of the squash preparations, to demonstrate thread-like nucleolar inclusions of various morphologic forms and to establish, by circumstantial evidence, at least, that all may be homologous, representing different conformations of the nucleolar organizer differentially extended within the nucleolar body.

The squash technique is essentially a blunderbuss method of tissue preparation and may rightfully be suspected of producing certain artefacts. For instance, the array of conformations of the intranucleolar structure depicted in Figs. 1–4 may all be considered to be the same basic form. The

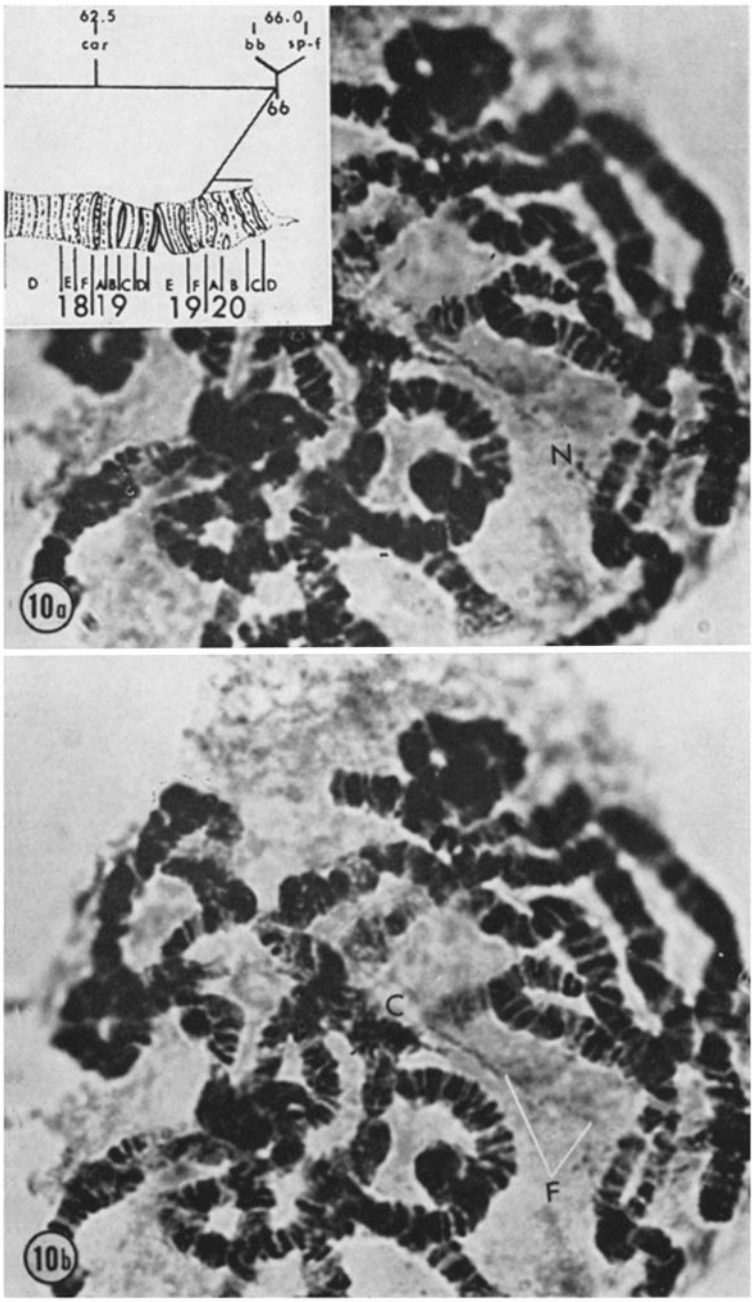
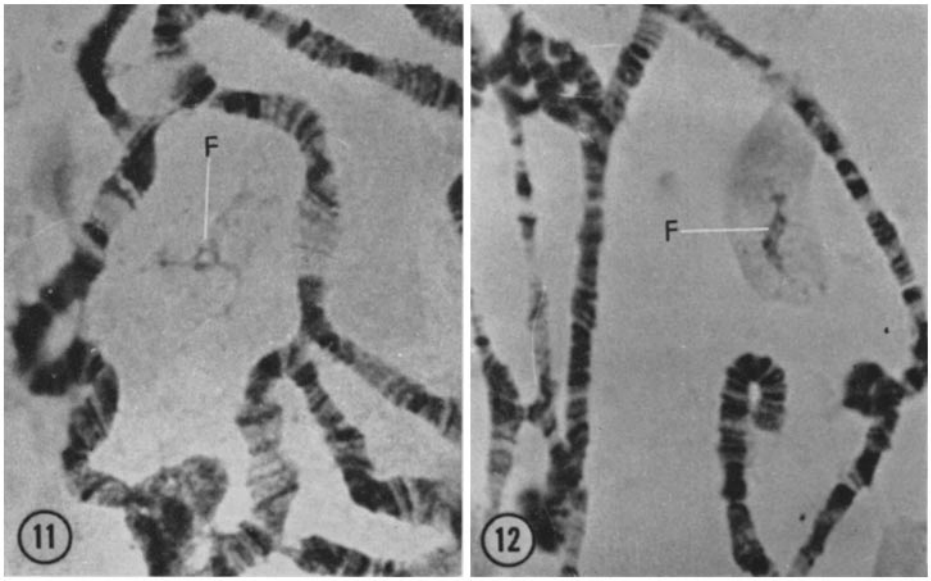


FIGURE 10 Nucleolar filament after alkaline extraction. *a* and *b*—two levels of focus showing laterally extruded chromosome band continuous with extended ramified filament. Band is identified as 20 A, B or C, with reference to map (inset). *N*, nucleolus; *F*, filament; *C*, chromocenter. × 1750. (except map inset).

tightly massed structure of Fig. 3 could have resulted from recoil of an outstretched band, such as those in Figs. 1 and 4, as it was broken at its point of chromosomal attachment. It is not likely, however, that the difference between those structures (Figs. 1–4) and the much ramified filaments revealed by the modified staining procedures (Figs. 8–10) could be due to the distorting effects



FIGURES 11–12 Feulgen-stained nucleolar filaments. *F*, filament. × 1750.

TABLE I

Relationship of Density of Thymidine-^3H Label Over the Nucleolus to that Over the Chromosomes

Chromosomal label	Total no. of nuclei	Frequency of nucleolar label		
		+	++	+++
3C	8	2	23	75
3D	16	12	26	62
2C	20	25	15	60
2D	30	61	14	26
1D	41	85	10	5

Designations of label over chromosomes are in accordance with those previously assigned (15). C = continuous; D = discontinuous. Ascending numerical value represents greater density. Designations of label over nucleolus are: + = 2–4 grains; ++ = 4–8 grains; +++ = clump or more than 8 grains.

of squashing. Those two groups clearly represent different states of extension of the intranucleolar thread. There are two ways in which the filament with multiple loops could arise from a laterally extruded band. It might result from considerable linear extension of the composite polytene structure, in which case it would be analogous to the outloopings of each of the diploid components of the lampbrush chromosome; or, in the manner of puff formation, it could result from the separation of the multiple chromosome strands together with some degree of linear extension or uncoiling. Fig. 10 clearly shows that the band corresponding to the nucleolar organizer may extrude as a polytene unit. It is probable, then, that the band is linearly extended within the nucleolar body when the conformations such as those seen in Figs. 1–4 are present. Further uncoiling as well as asynapsing of the individual units of the polytene structure may occur as that portion of the genome is engaged in or increases its functional activity.

With respect to the two possible functions of DNA, it is unlikely that the variant morphology of the intranucleolar structure is related to replicative activity, since it was observed that both the compact and extended forms are capable of replication and that the density of label over the nucleolus is related to that over the chromosomes (Table I) rather than to the compactness of the thread. Differential patterns of thymidine label over the chromosomes are believed to reflect asynchrony of replication among the replicative units (12, 15). Similarly, then, variable density of label over nucleoli may indicate that there is more than one replicating unit in the nucleolar DNA. Since the biochemical evidence implies that the final nucleolar product, ribosomes, consists of at least two separate units of RNA, it is not illogical to speculate that the two corresponding genetic

units (13) may have different rates or timing of replication. Reservation must also be made for the possibility that there is a nucleolar product other than ribosomes the DNA template of which would constitute a separate replicating unit.

Thus far, no certain conclusion can be made as to whether or not the polytene nucleolus is dispersed and reformed cyclically, as in the case of conventional mitotic nuclei. However, the radioautography of this study indicates that nucleoli are present in the intersynthetic period as well as in all phases of the endomitotic replication cycle, as previously defined by density and distribution of thymidine label (15). It is possible that a basic structure of the nucleolus is retained, as is that of the polytene chromosomes, and that the production and release of nucleolar product occurs intermittently and is correlated with the dynamic morphology of the nucleolar organizer locus.

A number of recent studies (e.g. reference 3) have shown that increase in the capacity for ribosome synthesis is achieved at specific times in oocyte maturation by multiple independent replications of the corresponding chromosome region, presumably the nucleolar organizer. It may be that, in somatic cell nuclei, amplification of the transcriptional capacity of that chromosome segment is effected by differentially controlled extension. The observations of this study point to a system in which that conjecture may be tested.

Investigation into the relationship between the morphologic variation of filamentous intranucleolar bodies and transcriptional activity in the polytene nucleolus is indicated and is under way in these laboratories.

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