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Fine structural ribonucleoprotein components of the cell nucleus visualized after spreading and high resolution autoradiography

Stanislav Fakan and Marie E. Hughes*
Centre of Electron Microscopy, University of Lausanne, Bugnon 27, CH-1005 Lausanne, Switzerland

Abstract. The fine structure of the nuclear components was studied following mild lysis of mouse or *Drosophila* tissue culture cells and spreading of nuclear material. Particular attention was paid to nuclear ribonucleoprotein (RNP) constituents, which were analysed by high resolution autoradiography after [^{3}H]uridine pulse labelling of cells. Comparison with the labelling kinetics of various in situ nuclear RNP constituents described previously suggests strong similarities between in situ constituents and structures observed within spread nuclear components. The present observations suggest that the nucleolar dense fibrillar component, shown previously in ultrathin sections of [^{3}H]uridine-labelled intact cells as carrying rapidly labelled pre-rRNA, in fact consists of highly compacted transcribing ribosomal genes. The growing RNP fibrils appearing in transcription complexes of extranucleolar active genes and the in situ observed perichromatin fibrils also show the same labelling properties. This confirms that the two structures indeed represent the same nucleoplasmic constituents. As for the nuclear structures involved in post-transcriptional events, our observations demonstrate the occurrence of a rapidly labelled RNP fibro-granular network. Its granular elements correspond, in size and perichromatin location, to the perichromatin granules seen in in situ preparations and suggest similarities between the two constituents. The results are discussed in the light of other data providing information on the role of various nuclear structural constituents.

Introduction

The ultrastructural morphology of the in situ nuclear components has been extensively studied during the last two decades. In addition to the structure and arrangement of chromatin, particular attention has been paid to the fine structure of the nuclear ribonucleoprotein (RNP) constituents including analysis of their cytochemical nature and of the possible roles they play in nuclear functions (for review see Fakan 1978; Fakan and Puvion 1980; Puvion and Moyne 1981). The introduction of new spreading techniques, allowing visualization of transcribing chromatin and detailed electron microscopical examination of the structure of active genes after lysis of nuclei, opened a new means of investigating the nuclear morphology (Miller and Bakken 1972; Miller and Beatty 1969). While the former approach provides information about the situation in situ in intact nuclei observed in ultrathin sections at the cellular level, the latter techniques allow functional processes taking place in the nucleus to be followed directly and the resulting constituents to be visualized at the molecular level. It is obvious that these two methodological approaches are complementary and that comparison of results from both makes it easier to analyse and understand the roles which different nuclear structural components play. In addition, high resolution autoradiography still represents the only way of investigating RNA labelling kinetics at the ultrastructural level and so far it has not been replaced by a non-radioactive probe detection system. Therefore analysing the incorporation of radioactive RNA precursors remains important for direct comparison of observed nuclear structures using different methods of visualization.

In the present work we investigated the fine structure of nuclear constituents after lysis of tissue culture cells and spreading of nuclear material. In particular, we paid attention to the different nuclear RNP constituents making use of radioactively labelled tissue culture cells incubated with [^{3}H]uridine for different periods of time preceding cell lysis, and of high resolution autoradiography. Since the labelling kinetics of in situ RNP constituents had been extensively studied in a number of earlier papers (for review see Fakan 1986), we compared the kinetic and cytochemical properties of some of the nuclear structural elements at the two above levels of visualization in parallel.

Materials and methods

Mouse P815 cells were cultured in Dulbecco's modification of Eagle's medium (Gibco) supplemented with 10% calf serum, penicillin and streptomycin, at 37° C, with a 5% CO_2-containing gas phase. *Drosophila* Schneider II cells were grown in Schneider's revised medium (Gibco) containing 10% foetal calf serum and 1% non-essential amino acids (Gibco), at 24°–25° C.

For radioactive labelling of RNA, 5–10 ml of exponentially growing P815 cells ($2–3 \times 10^5$/ml), or 1–2 ml of Schneider cells ($1–2 \times 10^6$/ml) were used, with continuous agitation. In short labelling experiments, the cells were incubated for 3 to 5 min with [^{3}H]5-uridine (80–100 μCi/ml, sp. act. 25–30 Ci/mmol, CEA or Amersham), while for longer incubation periods (30 or 60 min), the tritiated precursor was respectively at 50 or 30 μCi/ml. In some experi-

* *Present address:* Pharmacia-LKB Biotechnology AB, S-75182 Uppsala, Sweden

Offprint requests to: S. Fakan

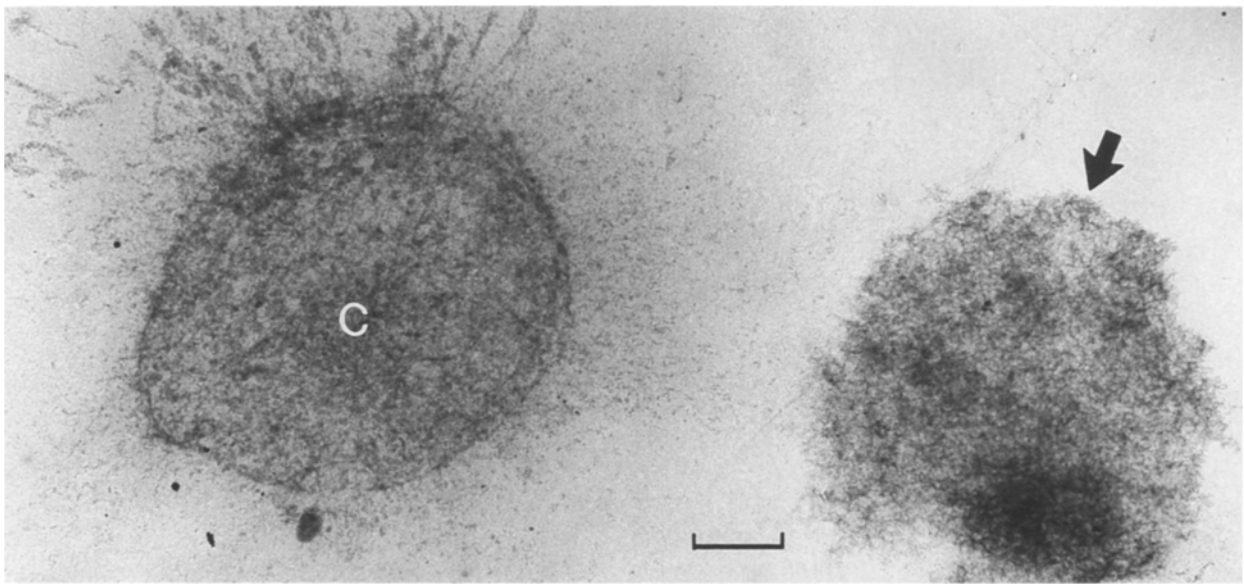


Fig. 1. A low magnification micrograph showing the spread nuclear contents of a cultured *Drosophila* cell. A round chromatin aggregate (*C*) can be seen with dispersed chromatin fibres at its periphery; some of the fibres are connected by a "basket-like structure" (*arrow*) consisting of a coarse fibrillar network. Bar represents 2 μm

ments, in order to increase the labelling intensity as much as possible, [^{3}H]5,6-uridine (Amersham, sp. act. 45–46 Ci/mmol) was used, at similar activities. At the end of the labelling period, the cultures were poured onto ice-cold phosphate-sucrose solution (0.2 mM KNa_2PO_4, 0.1 M RNase-free sucrose, pH 7.5) and processed for spreading in the usual way.

The details of the spreading technique, based on the method introduced by Miller and Beatty (1969), were described in earlier reports (Hughes et al. 1979; Villard and Fakan 1978). Briefly, the cells were first centrifuged at about 304 *g* for P815 cells, or at 580 *g* for *Drosophila* cells, and resuspended in 1 ml of the above-described phosphate-sucrose solution. To this 1 ml of 0.5% Nonidet P40 (NP40, Shell) in 0.2 mM EDTA, pH 7.4, was added dropwise with constant stirring. The resulting cell lysate was diluted in 30–50 ml of 0.2 mM EDTA, pH 7.5 and kept on ice. All the solutions used were ice cold.

In some experiments, phenylmethylsulphonyl fluoride (PMSF) was added to the cell suspension in phosphate-sucrose buffer before cell lysis, to a concentration of 2 mM.

For spreading, 30 μl of the EDTA-diluted lysate was layered on the top of 4% formaldehyde, 0.1 M sucrose (RNase free) solution, pH 8.5, in a translucide plastic chamber (Miller and Bakken 1972) containing on the bottom a freshly glow-discharged copper or gold electron microscope grid coated with Formvar-carbon membrane. Then the material was centrifuged at 2,400 *g*, 4° C, for 10 min. The grids were removed from the chambers, treated for 30 s in 0.4% Photo-flo 600 (Kodak), pH 7.5–7.9, and air dried. All the specimens were stained with 1% phosphotungstic acid (PTA) solution in 70% ethanol for 1 min, dehydrated in 95% ethanol for 20 s and air dried. Most grids, including all those subsequently submitted to the autoradiographic process, were rotary shadowed (angle 7–10°) to obtain a thin layer of platinum.

As a control for specificity of RNA labelling and of the RNA nature of the spread nuclear structural constituents, a series of grids containing spread material from 60 min-labelled cells were used for RNase digestion assays. In this case, the grids were removed from the chambers after centrifugation, rinsed with 0.2 mM EDTA, pH 7.5, and then floated on a solution of 10 μg/ml RNase (Sigma, type 1 A) in 0.2 mM EDTA, pH 7.5, for 60 min at 25° C. They were finally Photo-flo treated, stained and shadowed as above.

High resolution autoradiography was performed on grids which had been coated with a thin layer of carbon after platinum shadowing in order to avoid any undesirable interactions between the specimen and the photographic emulsion or the processing solutions. Ilford L4 emulsion was applied using the loop technique (Haase and Jung 1964) and the preparations were developed by the gold latensification – Elon ascorbic acid procedure (Wisse and Tates 1968) after 2 to 8 months exposure, according to a protocol described in detail recently (Fakan and Fakan 1987).

All grids were observed in a Philips EM 400 or a Zeiss EM 10 electron microscope, at 40 or 60 kV, using a 30 or 40 μm objective aperture.

Results

The general appearance of the spread nuclear material following the lysis of cells with NP40 is represented mostly by round-shaped chromatin aggregates which exhibit more or less dispersed chromatin fibres at their periphery. Bundles of chromatin sometimes connect these bulk chromatin masses or clusters to "basket-like structures" always occurring outside the chromatin regions and which resemble the structures described previously as the "nuclear shell" or "cortex" structures (Bouvier et al. 1980; Hubert et al. 1979). The basket-like structures, whenever observed, occur in the vicinity of one large round-shaped chromatin aggregate, suggesting that both arise from one lysed nucleus (Fig. 1).

Analysis of autoradiographs of spread [^{3}H]uridine-labelled nuclear contents shows a similar pattern of distribution of radioactive labelling for both cell types used in these

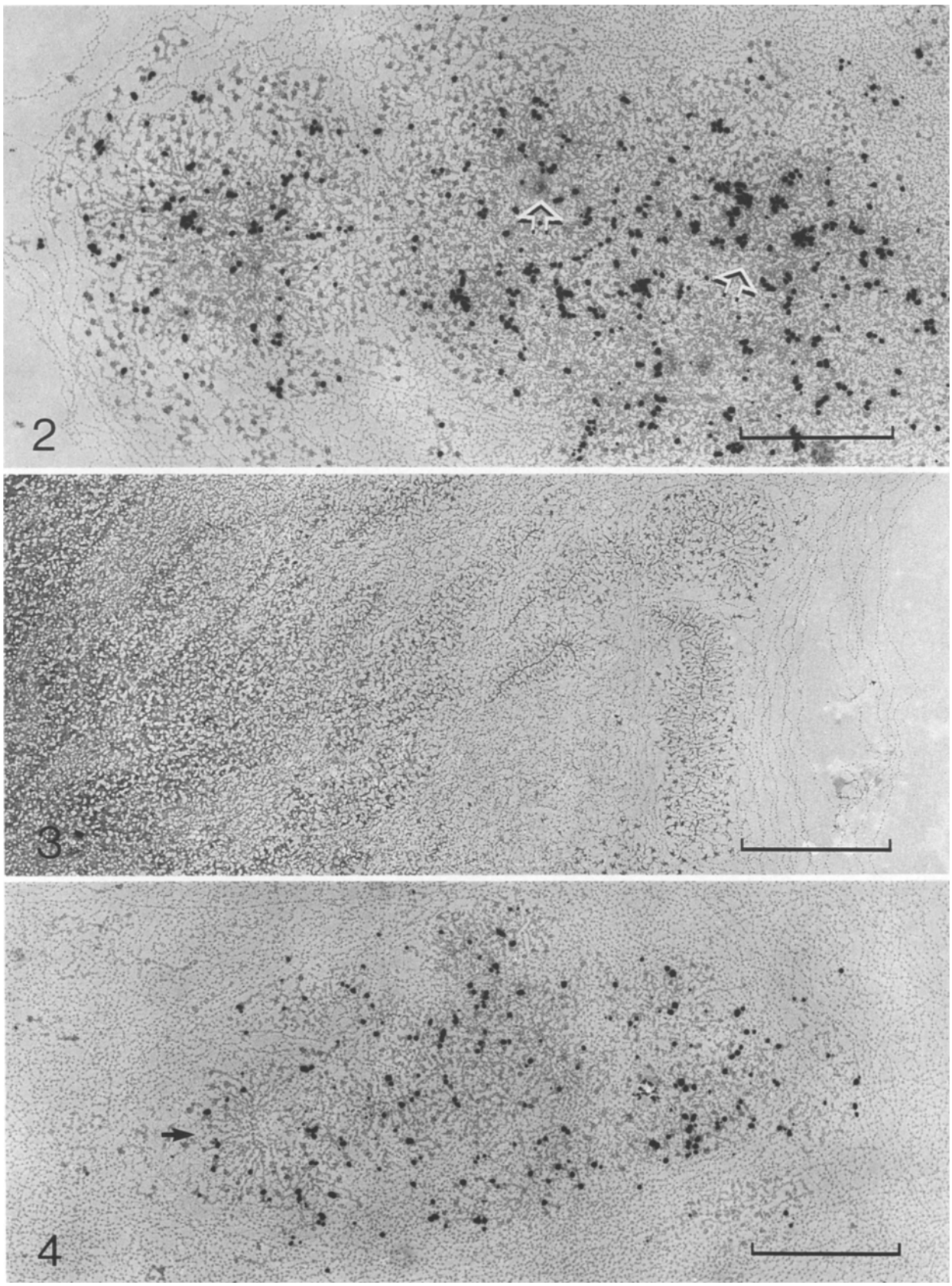


Fig. 2. Autoradiograph of spread nuclear material of a mouse P815 cell after 5 min incubation with [^{3}H]uridine. A strongly labelled, gradually dispersing fibrillar cluster can be seen revealing highly compacted portions in the central regions (*arrows*), and dispersing fibrils at its periphery. Bar represents 1 μm

Fig. 3. A portion of spread nucleolar material from a *Drosophila* cell showing a highly compacted area on the left side of the picture gradually dispersing to easily recognizable pre-rRNA transcription complexes on the right. Bar represents 1 μm

Fig. 4. Autoradiograph of the same type of specimen as in Figure 2, showing an aggregate of relatively dispersed strongly labelled material, exhibiting gradient-like feature (*arrow*) characteristic of pre-rRNA transcription complexes. Bar represents 1 μm

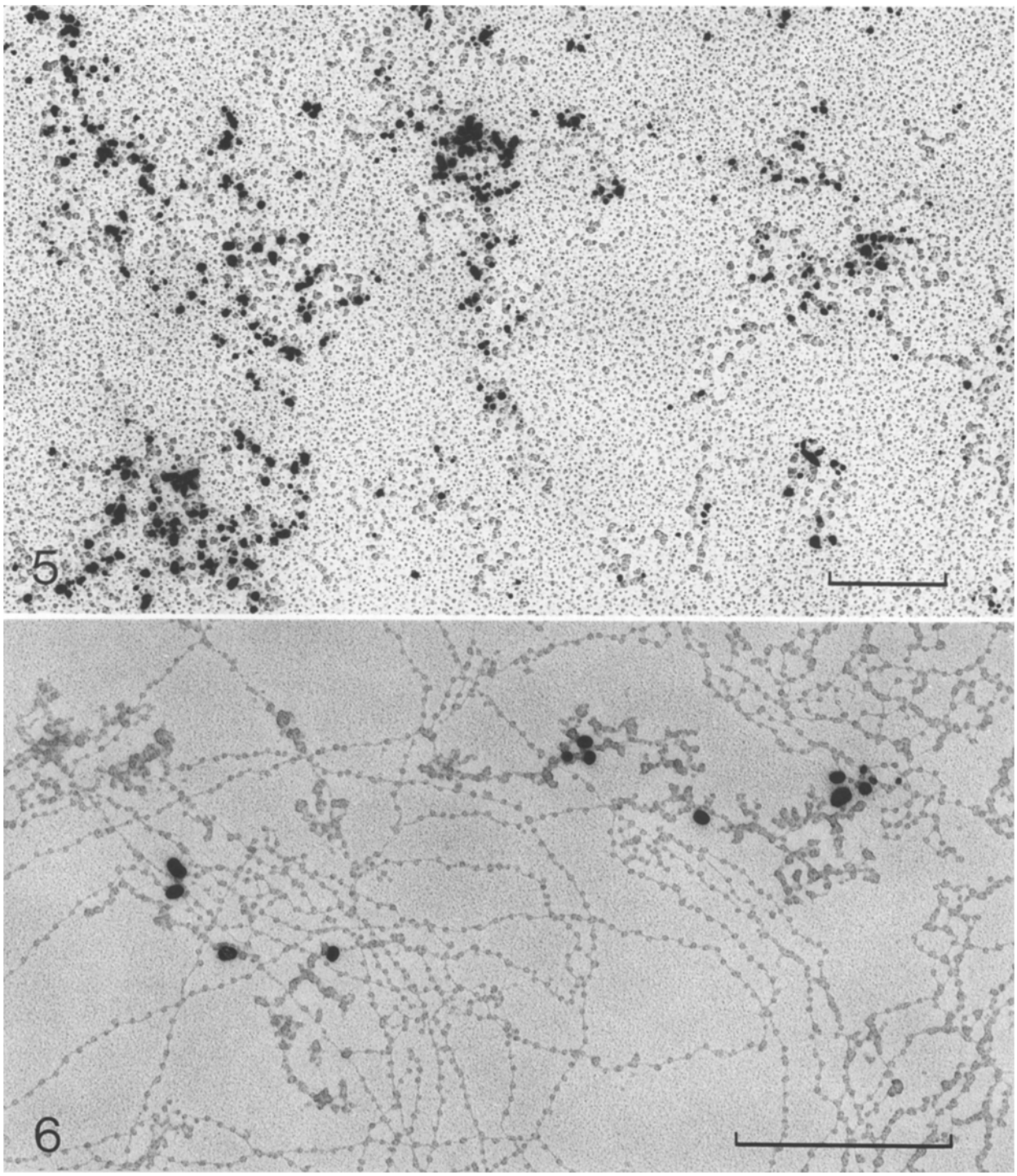


Fig. 5. A poorly dispersed chromatin area from a P815 cell labelled with [^{3}H]uridine for 30 min. Regions of strongly labelled prominent ribonucleoprotein (RNP) fibrils are surrounded by chromatin. Bar represents 0.5 μm

Fig. 6. After 5 min labelling of P815 cells with [^{3}H]uridine, autoradiography reveals radioactivity associated with growing RNP fibrils on mostly individual transcription complexes of non-nucleolar RNA. Bar represents 0.5 μm

experiments. When examined at low magnification after short incubation periods with [^{3}H]uridine (3 or 5 min), the spread material reveals, within areas of poorly dispersed chromatin, regions appearing as foci of strong radioactivity (Fig. 2). These areas correspond to the more or less densely packed fibrillar regions observed on unlabelled spread specimens (Fig. 3). At a higher degree of dispersion, transcription complexes of the ribosomal type, often strongly labelled on autoradiographs (Fig. 4), can be recognized.

Another labelling pattern is presented by areas of poorly dispersed chromatin within which are distributed thicker contrasted fibrils of variable length. The majority of these fibrils are labelled during short pulses (3 or 5 min) as well as after longer incubation with [^{3}H]uridine (30 or 60 min)

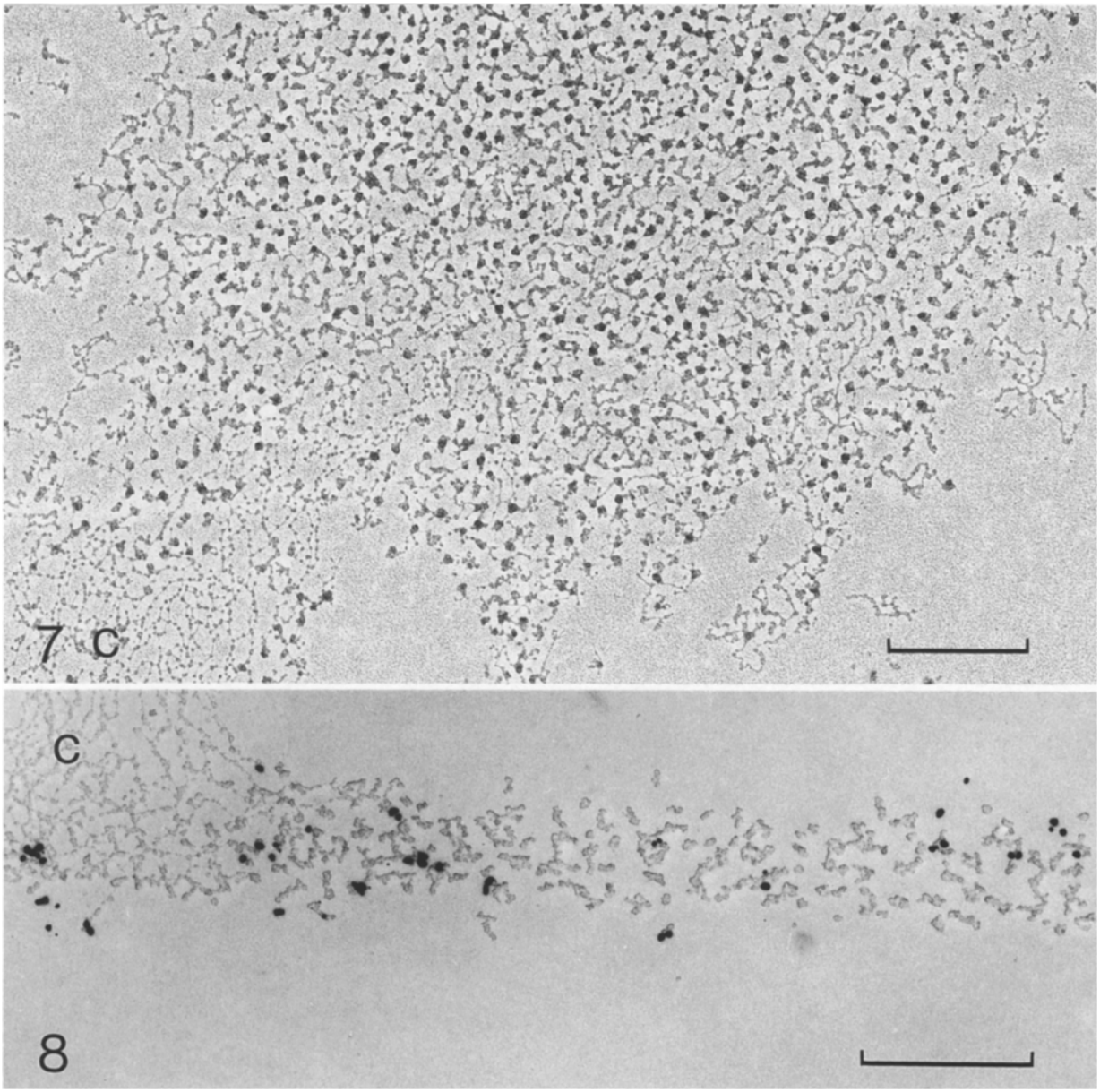


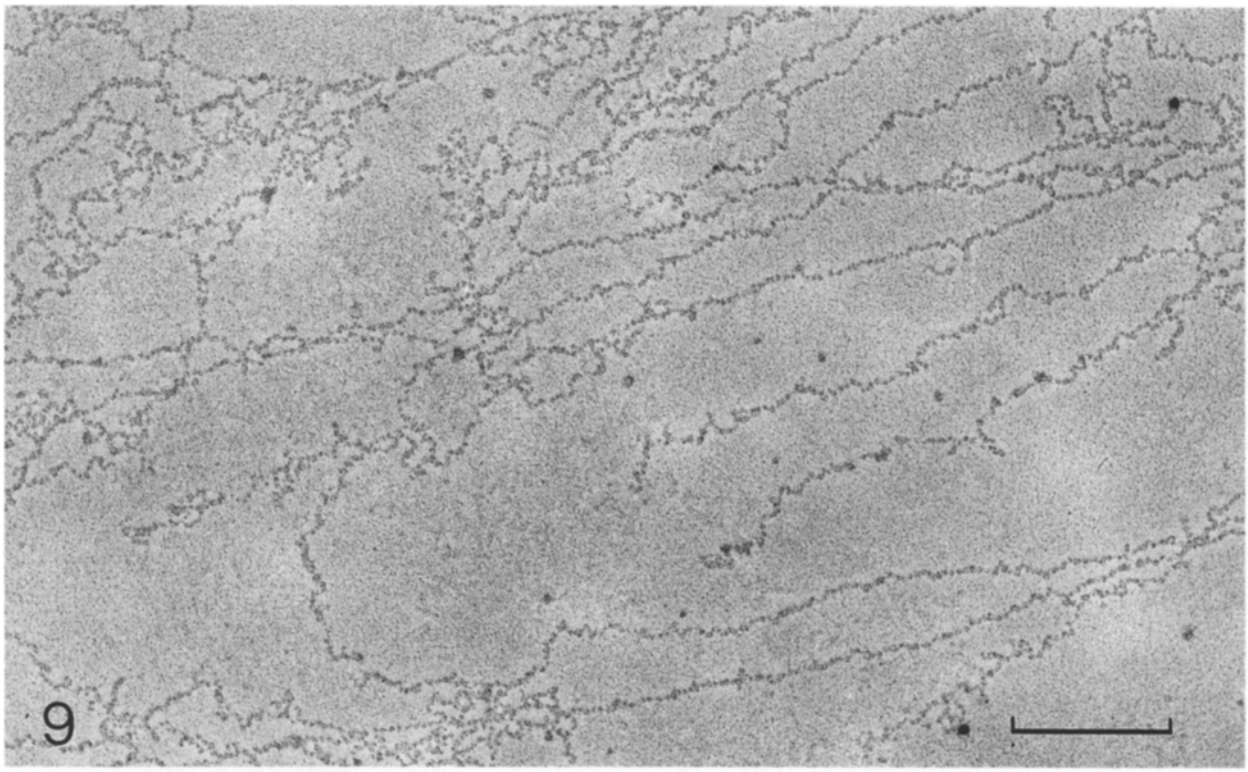
Fig. 7. A fibro-granular network appearing at the edge of a poorly dispersed chromatin aggregate (*C*) of a P815 mouse cell. Bar represents 0.5 μm

Fig. 8. Autoradiography of a *Drosophila* cell spread revealing labelling of fibro-granular material occurring at the edge of a chromatin cluster (*C*), after as little as 5 min of incubation with [^{3}H]uridine. Bar represents 1 μm

(Fig. 5). When chromatin areas of this type exhibit regions containing well-dispersed chromatin fibres, transcriptional complexes with mainly individual transcripts but sometimes also in the form of gradients of lateral fibrils of variable frequency, can be identified. The lateral fibrils often appear labelled (Fig. 6) regardless of the incubation time with the radioactive precursor.

On the periphery of some large aggregates of inactive chromatin fibres, a layer of fibro-granular densely contrasted material, sometimes extending from the chromatin aggregates, can be observed. This material consists of granules of 30–50 nm in diameter which are often interconnected with fibrils of variable length forming a network-like arrangement (Fig. 7). After incubation of cells with [^{3}H]uridine for different periods of time, this type of material becomes labelled (Fig. 8). All the above structures, exhibiting radioactive labelling, can be observed independently of the presence of PMSF during the lysis of the cells and, therefore, they do not result from endogeneous protease action. These structures as well as the radioactivity associated with them are virtually removed after RNase treatment of the spread preparations (Fig. 9). This is also the case for the growing RNP fibrils occurring in transcription complexes.

It is interesting to note that in some preparations treated with RNase, a homogeneous layer of moderately contrasted material, detaching from the periphery of large aggregates of chromatin is observed. This phenomenon has never been detected on preparations incubated in parallel under control conditions. The layer of homogeneous material is sometimes connected with the basket-like structures found occasionally on the periphery of the main chromatin masses (Fig. 10).



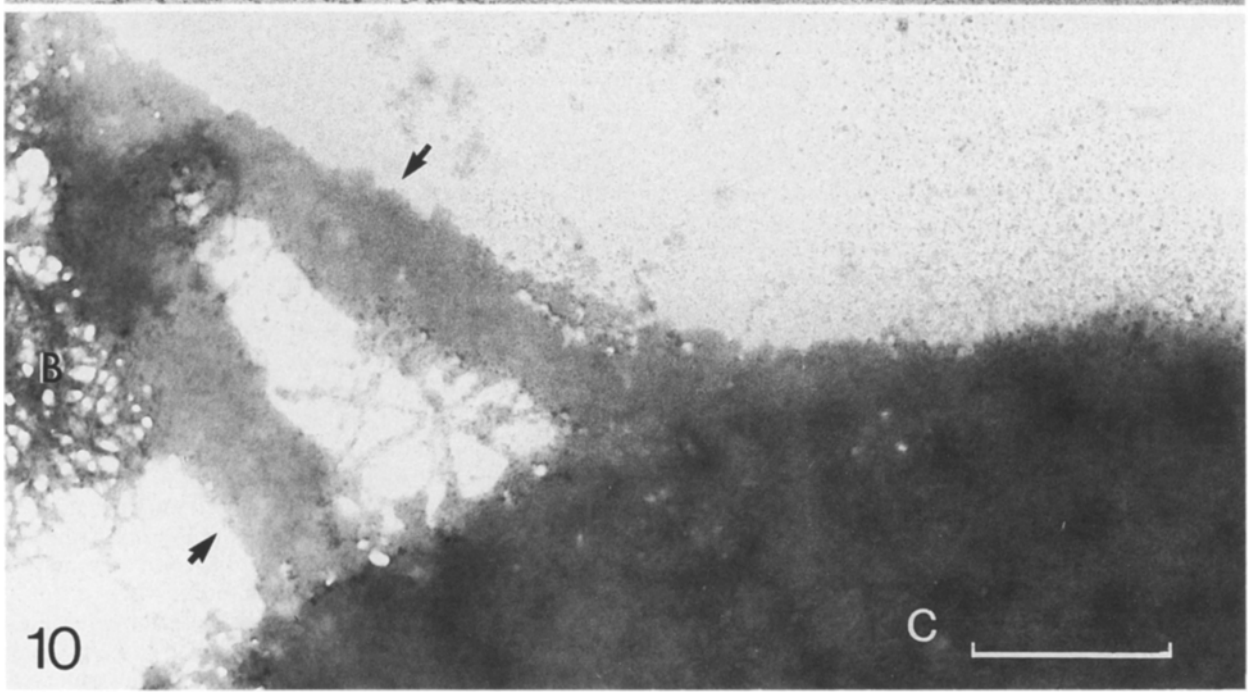


Fig. 9. Autoradiograph of mouse cell chromatin after 60 min of labelling with [^{3}H]uridine and RNase digestion of the spread preparation. Radioactive label as well as fibrillar or granular RNP structures are virtually removed. Bar represents 0.5 μm

Fig. 10. After RNase treatment of a *Drosophila* cell spread, a layer of rather homogeneous material (*arrows*), sometimes connected with the basket-like structures (*B*) is occasionally observed on the periphery of undispersed chromatin aggregates (*C*). Bar represents 0.5 μm

Discussion

The main purpose of this paper was to examine similarities in morphology and labelling kinetics between the RNP-containing nuclear structural constituents previously described in situ (for review see Fakan 1986; Fakan and Puvion 1980; Puvion and Moyne 1981), and the structures observed after lysis of cells and spreading of nuclear material. The following main conclusions, based on the present observations, can be drawn:

1. The labelling kinetics of the nuclear dense fibrillar component detected in situ corresponds to that observed, after

spreading, for the dense fibrillar aggregates, which gradually decondense during chromatin dispersion giving rise to the rRNA transcription units. This observation suggests that the dense nucleolar fibrillar component consists of a highly condensed and concentrated assembly of transcription complexes of pre-rRNA. It suggests at the same time a high degree of compaction of these complexes in the nucleolus.

2. The labelling of individually growing RNP chains visualized on non-nucleolar transcription complexes, and that of perichromatin fibrils observed in situ, show similarities which strongly suggest that these two structural constituents are identical.

Concerning the in situ visualization of the sites of nucleolar RNA synthesis, it has been established for a long time, thanks mainly to studies using short pulses of radioactive labelling of RNA and high resolution autoradiography, that the dense fibrillar component contains pre-rRNA. Following pulse-chase experiments, the label progressively migrates from the fibrillar towards the granular component confirming a precursor-product relationship between these two nucleolar structural constituents (Granboulan and Granboulan 1965; for review see Fakan 1978, 1986; Goessens 1984; Stahl 1982). Several reports support the idea that the dense fibrillar component is indeed constituted of rRNA transcription units. When the nuclear material is mildly loosened in situ and then observed in ultrathin sections, highly contrasted fibrillar clusters, obviously originating from the nucleolar fibrillar component, are detected. These clusters become rapidly labelled after incubation of cells with [^{3}H]uridine and it has been concluded that they correspond to sections of nucleolar transcription complexes (Puvion-Dutilleul and Puvion 1980). After a biochemical and in situ morphological analysis of rat hepatocyte nucleoli following D-galactosamine treatment, Dimova et al. (1979) have proposed transcription units as being the main constituents of the dense fibrillar component. Recently, an attempt to visualize nucleolar transcription complexes directly on sections of the dense nucleolar component in early prophase stages of multinucleate cells has been reported (Ghosh and Paweletz 1987).

The results of all in situ studies aiming to localize the sites of nucleolar RNA synthesis by means of radioactive RNA precursors and high resolution autoradiography published so far, demonstrate the dense fibrillar component as the nucleolar compartment containing rapidly labelled RNA, regardless of whether the fibrillar component occurs on the periphery of the clusters of intranucleolar condensed chromatin clumps (Fakan and Bernhard 1971) or around or even inside the fibrillar centres (Thiry et al. 1985). In view of these results the dense fibrillar component represents the morphological expression of nucleolar transcription. Considering all the data obtained from different types of in situ autoradiographic analysis, together with our observations, ones reaches the conclusion that the dense fibrillar component of the nucleolus consists of an assembly of highly compacted rDNA transcription units. The way in which they are compacted and their arrangement within such a dense in situ constituent is however yet unclear.

In this context it is pertinent to mention previous studies aiming to detect RNA polymerase I distribution within the nucleolus in situ. In isolated rat liver nuclei incubated with radioactive nucleotide triphosphates, high resolution autoradiography has shown that the polymerase activity is associated mainly with the dense fibrillar component (Gruca et al. 1978). However, when an anti-RNA polymerase I antibody was localized by means of immuno-electron microscopy, the label indicating the distribution of the polymerase was detected essentially within the fibrillar centres (Scheer and Raska 1987; Scheer and Rose 1984). The fact that a polymerase molecule can be detected by means of a specific antibody, of course, does not mean that it is just engaged in the transcription process. Nevertheless, the discrepancy between the above results remains, at present, difficult to explain and its clarification will require further in situ experimentation.

As for the extranucleolar transcription sites, earlier work has shown that these are located in situ predominantly within the border zone of condensed chromatin areas (Fakan and Bernhard 1971), and that perichromatin fibrils are the constituent bearing newly synthesized pre-mRNA (Fakan et al. 1976; Nash et al. 1975; for review see Fakan 1978, 1986; Fakan and Puvion 1980; Puvion and Moyne 1981). More recently, using specific anti-RNP antibodies and ultrastructural immunocytochemistry it has been demonstrated that perichromatin fibrils contain heterogeneous (hn)RNP and small nuclear (sn)RNP (Fakan et al. 1984). In addition, immunocytochemistry applied to lysed and spread nuclear material revealed association of anti-hnRNP and -snRNP antibodies with growing RNP fibrils occurring on transcription complexes of non-nucleolar type (Fakan et al. 1986). These data together with our present observations based on the features of radioactive labelling kinetics confirm the idea that perichromatin fibrils are indeed the in situ morphological expression of extranucleolar RNA synthesis.

Little is yet known about the topological pattern of the intranuclear pathwys of RNA following its synthesis or about the structural constituents which fulfil the role of vehicle or storage during the postsynthetic process. Perichromatin granules have been considered as possible candidates for these functions for hnRNP (Monneron and Bernhard 1969). It has also been shown that at least a part of pre-mRNA migrates from its sites of synthesis in the perichromatin regions towards the internal interchromatin space. This migration of labelled RNA takes place in association with perichromatin fibrils, and RNA processing occurs during this period (Fakan et al. 1976; Puvion and Moyne 1978). Our observations on spread nuclear material often reveal high numbers of RNP granules occurring on the periphery of clusters of poorly dispersed chromatin. These granules, 30–50 nm in diameter, occur within a fibrogranular network which becomes labelled after as little as 5 min of incubation of cells with [^{3}H]uridine (Fig. 8). They suggest a comparison with perichromatin granules observed in ultrathin sections of practically all types of nuclei so far examined. One might be astonished by the high density of these granules in spread preparations. If, however, one realizes that the spread specimen represents a sort of "whole mount" preparation, it is not surprising to find a relatively high local concentration of granules. Considering an average number of 50 perichromatin granules per ultrathin section of a rat hepatocyte nucleus (Monneron and Bernhard 1969), we can imagine, in the case of mouse P815 cells exhibiting an average nuclear diameter of 7–8 μm, a total of about 2×10^3 granules per nucleus. This would account for the high local frequency of granules found in our spread material. In addition, the granules and

other RNP structures appearing on the spread specimens are sensitive to RNase digestion following the spreading process. Further studies, including the use of anti-RNP antibody immunolabelling, will however be necessary to ascertain the precise nature and origin of these granules.

Finally, we were surprised by the occurrence of a layer of homogeneous material, sometimes connecting the bulk chromatin mass with the basket-like structures, appearing occasionally after RNase treatment of spread preparations. This phenomenon has not been observed in untreated specimens. It suggests a possible similarity of this material with the nuclear peripheral lamina. Following removal of some RNP constituents, this material would start to detach as fine strips from the underlying chromatin. This finding favours the importance of RNA for maintaining the structural integrity of the cell nucleus (Bouvier et al. 1982; Herman et al. 1978; Kaufmann et al. 1981; Müller et al. 1983). We are attempting to assay this system using anti-lamin antibodies in order to analyse the nature of different non-chromatin structural constituents occurring under our experimental conditions.

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