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Nuclear Ultrastructures Associated With the RNA Synthesis and Processing

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Abstract Relatively a little is known about the spatial organization of RNA synthesis, processing, and transport in (mammalian) cell nuclei. This review summarizes results of electron microscopic mapping of RNA synthetic sites and macromolecules involved directly, or indirectly, in the metabolism of RNAs in somatic cell mammalian nuclei. Significance of these results will be discussed in the context of the molecular mechanisms underlying spatial arrangements of RNA metabolism. © 1995 Wiley-Liss, Inc.

Key words: electron microscopy, nuclear ultrastructures, RNA synthesis and processing, RNA, mRNA

One of the major tasks for contemporary cell biology is the determination of molecular mechanisms responsible for the nuclear organization. Much information about the cell nucleus has been gained in the past 15 years using bichemical or molecular biology techniques in vitro. Yet, such approaches usually provided the information about only partial processes taking place in this organelle. It appears that functional processes do not take place in the nuclear sap, but on structures of the highly organized structural framework, developed during evolution, which enables the optimal interaction of its constituents. The informational explosion due to the nucleic acid technology was, with a delay, complemented in the last 5 years by the appropriate in situ "follow-up." It was made possible by development of new morphological approaches, e.g., non-isotopic fluorescent) in situ hybridization (ISH) as well as new instrumentations, e.g., confocal microscopy.

The cell nucleus appears to be compartmentalized into structure-function domains [Spector, 1993]. The best example is the nucleolus, by far the most conspicious nuclear structure, in which rRNA is synthesized and preribosomes are formed [Raška et al., 1992; Scheer et al., 1993; Spector, 1993; Wachtler and Stahl, 1993]. There are other examples of nuclear domains such as the replication domains seen during the S phase [Nakayasu and Berezney, 1989; Raška et al., 1990; Spector, 1993], domains corresponding to fluorescent speckles rich in small nuclear ribonucleoprotein (snRNP) components [Lerner et al., 1981; Carmo-Fonseca et al., 1991; Raška et al., 1992; Spector, 1993; Wu et al., 1993], domains corresponding to coiled bodies (CBs) exhibiting the highest accumulation of snRNPs [Monneron and Bernhard, 1969; Carmo-Fonseca et al., 1991; Raška et al., 1992; Wu et al., 1993; Lamond and Carmo-Fonseca, 1993; Gall et al., 1994], or nuclear tracks, and/or interchromosomal channel network, corresponding to localization of specific precursor mRNAs [Lawrence et al., 1993; Xing and Lawrence, 1993; Rosbash and Singer, 1993; Cremer et al., 1993; Kramer et al., 1994]. The underlying molecular mechanisms involved in the regulation of RNA (and DNA) metabolism, which are responsible for the

Abbreviations used: BrU, 5-bromouridine; CB, coiled body; DFC, dense fibrillar component(s); FC, fibrillar center(s); EM, electron microscopy; EMAC, electron microscopic affinity cytochemistry; EMARG, electron microscopic autoradiography; GC, granular component(s); IG, interchromatin granules(s); IS, interchromatin space; ISH, in situ hybridization; NB, nuclear body; PF, perichromatin fibril(s); PG, perichromatin granule(s); snRNP, small nuclear ribonucleoprotein.

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existence of the above-mentioned nuclear domains, are more or less unknown.

Due to its enormous resolution power, the electron microscopic (EM) immunocytochemistry and in situ hybridization [EM affinity cytochemistry (EMAC)], used in conjunction with the colloidal gold adducts, represents a powerful tool that cannot be substituted by other approaches to the study of the structure-function organization of the cell nucleus. Results of EM in situ mapping of both RNA synthetic sites and macromolecules involved directly, or indirectly, in the metabolism of nuclear RNAs will be summarized here with an emphasis on somatic mammalian cells, and will be related to the knowledge of "spatial" metabolism of RNA.

ELECTRON MICROSCOPY AUGMENTED WITH CYTOCHEMIC AND AUTORADIOGRAPHIC APPROACHES IDENTIFIES ONLY A DOZEN STRUCTURAL COMPONENTS WITHIN NUCLEI

Disregarding the nuclear envelope with the adjacent layer of lamins, the nuclear interior, the nucleoplasm, of the somatic mammalian cell can be divided in three territories: (1) condensed chromatin composed basically of nucleohistones, (2) nucleolus, and (3) interchromatin space (IS) which encompasses the remaining intranuclear space in which most of the nuclear nonribosomal RNA metabolism takes place. This simplified picture of nuclear territories is seen in a cryosection of human HeLa cell shown in Figure 1a.

The structure-function organization of the chromatin will not be addressed in this study [see, e.g., Raška et al., 1990, 1992; Spector, 1993]. Morphologically speaking, only border chromatin regions next to IS, i.e., perichromatin areas, are of importance for the in situ EM description of RNA metabolism per se [Raška et al., 1992]. It is to be recalled, however, that depending on the cell type, metabolic and/or developmental state of the cell, and also on the chosen processing of cells or tissues for EM, the picture of chromatin structures, as well as that of other nuclear territories, may vary [Raška et al., 1990; Spector, 1993].

Three basic nucleolar components have been identified at the ultrastructural level (Figs. 1–2). They are: (1) relatively electron lucent fibrillar centers (FC) which are thought to be interphasic counterparts of nucleolar organizer regions of mitotic chromosomes; (2) dense fibrillar components (DFC) surrounding usually FC and apparently containing transcripts of rRNA genes; and (3) granular components which are believed to be, at least in part, preribosomes. Two additional components are found within nucleoli, nucleolar interstices of low electron density, and perinucleolar as well as intranucleolar clumps of condensed chromatin. The nucleoli contain highly active, tandem repeats of rRNA genes (400 genes per human diploid nucleus). By means of high resolution autoradiography (EMARG) employing tritiated uridine, rRNA synthesis is localized to DFC and/or to FC [Fakan and Pu-

Fig. 1. **a:** Thin cryosection of a HeLa cell (with the exception of Fig. 1b, HeLa cells are shown exclusively in the consecutive figures) labelled for the non-snRNP splicing factor SC35 of Fu and Maniatis [1990] and post-embedded in methylcellulose [Raška et al., 1990]. Three major territories are easily recognized within the cell nucleus: chromatin structures (C) with the highest electron density, nucleolus (Nu), and interchromatin space (IS) with, in average, the lowest density. IS has been called by other names such as nuclear RNP space, interchromosome channel network, or interchromosome territory. The 10 nm specific gold label due to SC35 (arrowheads) may be still increased (e.g., Fig. 3d) but I have chosen this particular picture to demonstrate the very differential SC35 labelling pattern within IS. Gold particles can be found all over the IS. They are specifically enriched within a few restricted domains which, at the light microscopical level, give rise to speckles. One such domain corresponds to a large cluster of IGs (IG), another corresponds to an NB associated closely with chromatin (arrows). Nucleolus and chromatin are devoid of gold particles. F, nucleolar FC; Cy, cytoplasm. ×31,000. **b:** Thin sectioned, plastic embedded rat hepatocyte labelled for DNA. The label observed in the chromatin can be considered as close-packed (anti-DNA monoclonal IgM antibody and secondary goat anti-mouse 5 nm gold adduct were used!), but the IS is essentially devoid of label. With pictures as this one, there is no in situ evidence for extended, transcriptionally active chromatin loops within IS [Raška et al., 1992]. Is the transcription then accompanied by a minor loosening of the chromatin only? In contrast, my opinion is that special, highly active gene clusters—similar to ribosomal genes within nucleolar territory—generate distinct nuclear domains such as nuclear bodies (Figs. 1a, 2a) or clumps of PFs (Fig. 2a,c). ×52,300. **c:** Ultrathin cryosection postembedded in polyvinylalcohol [Raška et al., 1992]. The cryosection was labelled for the factor SC35 (10 nm gold particles; larger arrowhead) and for a 100 kD protein of Szostecki and Guldner (5 nm particles; smaller arrowheads) depicting the annular nuclear body [e.g., Raška et al., 1992; Koken et al., 1994]. Without immunolabelling, it is sometimes impossible to find and identify such an NB in the thin sectioned nucleus. This body as well as some other nuclear domains (e.g., Fig. 3d) are not labelled with SC35 antibody [Raška et al., 1992]. ×68,200.

vion, 1980; Scheer et al., 1993; Wachtler and Stahl, 1993].

Besides nucleoli and chromatin structures, only a few morphologically defined components are seen in the remaining interchromatin space (Figs. 1–4). They are perichromatin granules (PGs), clusters of interchromatin granules (IGs), perichromatin fibrils (PFs), and nuclear bodies (NBs) [Fakan and Puvion, 1980].

Perichromatin RNP granules of about 50 nm diameter are surrounded by a clear halo. They are found in the perichromatin region and there are at most a few dozens of PGs per nuclear profile. On the basis of EMARG labelling data,

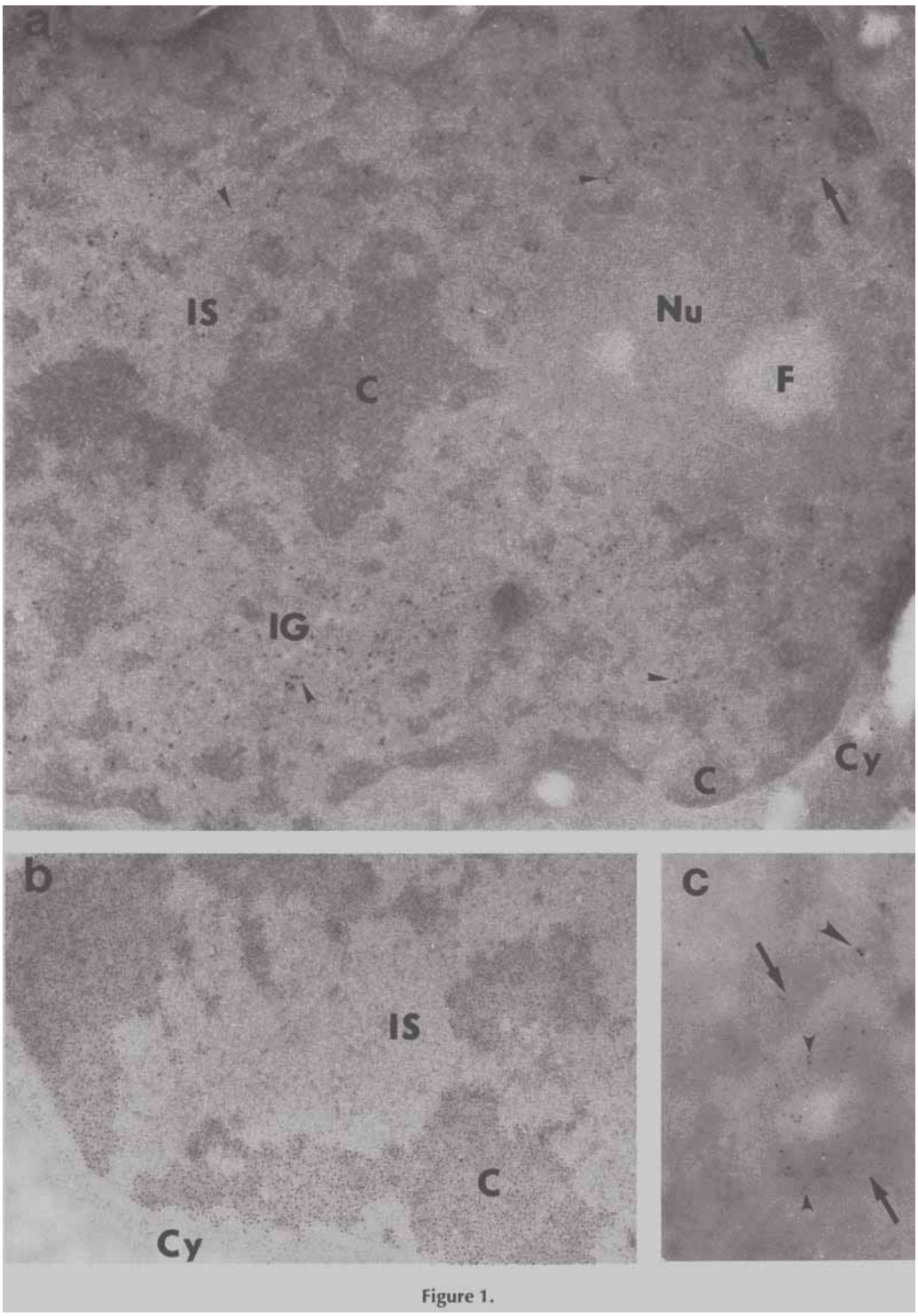

Figure 1.

which included experiments with altered RNA metabolism, it was speculated that these enigmatic nuclear components serve for the storage or transport of extranucleolar or nucleolar RNAs [Fakan and Puvion, 1980]. We recall that PGs are the only IS components the size of which corresponds to that of spliceosome particles [Spann et al., 1989].

Interchromatin RNP granules of about 25 nm diameter appear in clusters. Their number is at

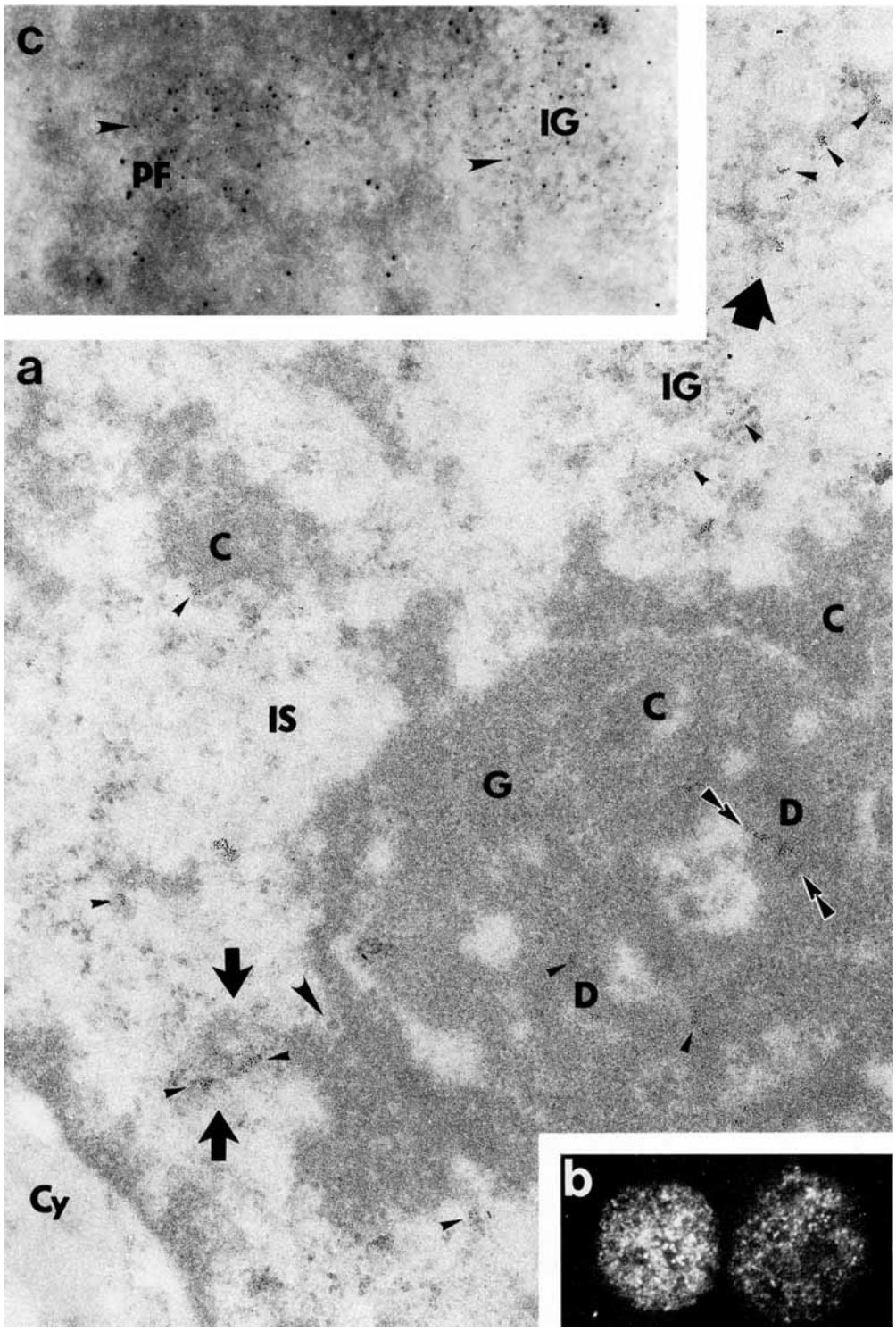

Figure 2.

least one order of magnitude higher than that of PGs which makes them a better target for EMAC probes. On the basis of more or less negative EMARG signal, only slowly labelled or very stable RNAs are believed to be present within IGs [Fakan and Puvion, 1980].

Perichromatin fibrils play a key role within the context of this communication as it has been unambiguously shown that the metabolism of extranucleolar RNA is, in time and space, associated mainly with these components [Fakan and Puvion, 1980; Fakan, 1994]. Yet, that created another ambiguity. PFs are not structurally defined at all and appear as a heterogenous mixture of stained RNP structures in the IS. In fact, any RNP structure within IS, which does not belong to either category of components mentioned in this section, should be identified as PF [Raška et al., 1990, 1992]. We should be reminded of a typical difficulty accompanying the ultrastructural description of the nucleus. IGs are recognized because they appear in clusters. I am unable (and congratulations to those who can!) to identify an individually appearing IG-like particle within IS as being *the* IG, and I include it among PFs.

The concept of PFs goes back to 1969 to Wilhelm Bernhard and his collaborators at Villejuif. At that time, the knowledge of mRNA maturation (e.g., splicing) was not existent, and only high resolution autoradiography (EMARG) and a limited number of cytochemical methods were available, particularly an EDTA method for the preferential visualization of RNA containing components [Monneron and Bernhard, 1969]. It was actually with this EDTA method that PFs were visualized and described. Wilhelm Bernhard's ingenious idea was to circumvent the difficulties arising from the empty information on precursor mRNA (hnRNA). He put all the difficulties in one basket and he named the basket "perichromatin fibrils representing the morphological expression of extranucleolar transcription" [Monneron and Bernhard, 1969]. His idea was ahead of the time by more than a decade. Bernhard's collaborators, particularly Fakan and Puvion [Fakan and Puvion, 1980; Fakan, 1994], exhaustively confirmed Bernhard's concept. According to the EMARG results, PFs appear to be the structural element containing transcribed RNA in the perichromatin region. After a chase with cold uridine, the radioactive RNA moves and is also contained within PFs of the "interior" part of IS [Puvion and Fakan, 1980].

Nuclear bodies are usually round structures of diameter, in most cases not exceeding 1 μm (Figs. 1–4) which are sometimes seen within the nucleoplasm [Raška et al., 1992; Brasch and Ochs, 1992]. Based on nuclear localization, fine structure, and cytochemical properties, many varieties of NBs have been described. In some cases it is difficult to distinguish between an accumulation of PFs and a nuclear body. Therefore, a domain with clearly defined structural features should be considered to belong to the category of NBs. According to the fine structure, simple nuclear bodies and CBs are by far most frequently observed. Simple nuclear bodies are usually round domains of homogenous texture which definitely encompass functionally heterogenous domains, concerning both the macromolecular composition and/or function. CBs are usually spherical aggregates of fibrils and gran-

Fig. 2. **a:** Localization of RNA synthetic sites (smaller arrowheads) in the thin sectioned, plastic embedded HeLa cell which was permeabilized by Triton X-100 and which incorporated BrU for 15 min. The nuclear ultrastructure looks like that of untreated cells. The signal (5 nm gold particles; smaller arrowheads) is within nucleoli associated mainly with DFC (D). One array of gold particles (double arrowheads) is found compatible with the occurrence of Miller's Christmas trees. In the IS, much of the label is found at the border of chromatin region. Two IS domains with increased incidence of gold particles are of interest. The domain in the proximity of the nucleolus (thinner arrows) corresponds to a nuclear body in which an array formed by gold particles is seen; the other domain (thicker arrows) are clumped PFs. Several gold particles are present within the cluster of IGs. A few PGs (larger arrowhead) present in this section are not labelled (compare with Fig. 4a), nevertheless, the efficiency of the postembedding immunocytochemistry is in the range of a few percent at best. G, nucleolar GC. ×50,100. **b:** RNA synthetic sites are depicted by BrU method in two HeLa cell nuclei by means of immunofluoresce. Both nucleolar and extranucleolar signals are observed. According to results of the differential inhibition assay by α-amanitin, the extranucleolar signal corresponds basically to the activity of RNA polymerase II. ×1,100. **c:** Cryosectioned HeLa cell postembedded in PVA and labelled for SC35 (5 nm gold particles; arrowheads) and snRNPs (10 nm particles). Both the IG cluster and clump of PFs (PF) exhibit a high labelling. Either of the two domains definitely generate a shining speckle at the fluorescence level for the two antigens. In addition to clumps of PFs, other kinds of NBs were identified to contribute to the speckled appearance of the cell nucleus (Fig. 1a) [Raška et al., 1990, 1992]. A clump of PFs shown in this figure is to be identified with the active process of RNA synthesis and processing. ×70,300.

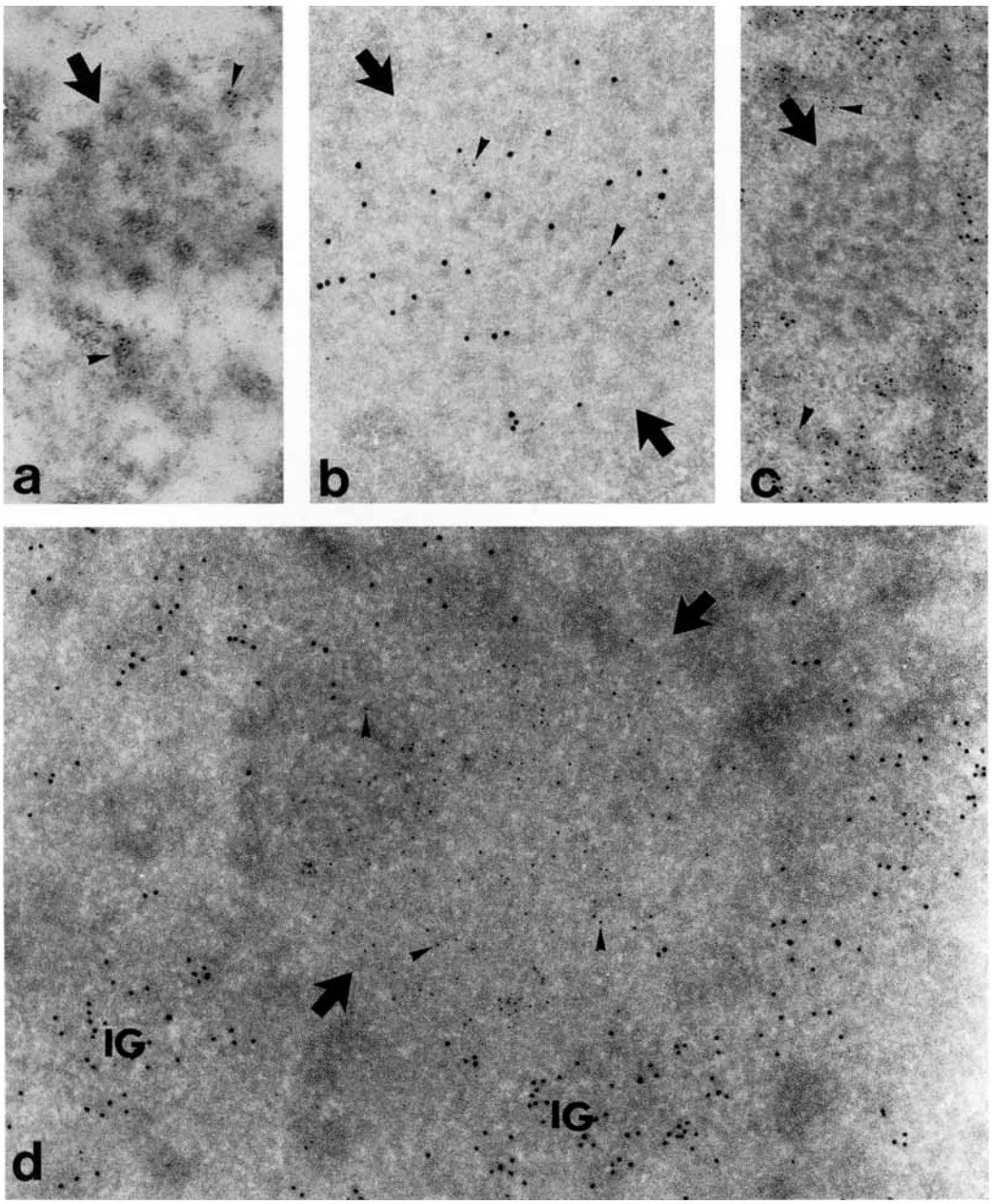

Fig. 3. Sectioned Hela cells. **a:** Plastic section, BrU incorporated for 10 min. Gold label (arrowheads; 5 nm particles) is absent in the CB (arrow) indicating that RNA synthesis does not take place in this nuclear organelle. ×78,000. **b:** Cryosection which was in situ hybridized for U3 snRNA with an antisense oligonucleotide tagged with biotin (5 nm particles; arrowheads) and then labelled for protein coilin (10 nm particles), a specific marker of the CBs. U3 snRNA is present in the CB (arrows). This result strengthens the claim that CB serves both the IS and the nucleolus [Raška et al., 1992; Brasch and Ochs, 1992; Gall et al., 1994]. ×70,400. **c:** Plastic section. DNA mapped by terminal deoxynucleotidyl transferase method (arrowheads; 5 nm particles) [Raška et al., 1994] followed by immunodetection of IGs by means of the specific antibody (10 nm particles). Note that neither 5 nm nor 10 nm particles are present in the CB. ×64,200. **d:** Methylcellulose embedded cryosection labelled for the protein coilin (arrowheads; 5 nm particles) and SC35 splicing factor (10 nm particles). Nucleoplasmic 10 nm particles are enriched in clusters of IGs, but are not present in the CB (arrows). ×72,300.

ules. Also CBs may be multifunctional as they serve both the nucleolus and interchromatin space [Raška et al., 1992; Brasch and Ochs, 1992; Wu et al., 1993; Gall et al., 1994; Fig 3]. The function of most NBs is unknown. It is believed that they are either of nucleolar origin, or that they are related to viral infection, or to some other pathological process, or that they appear in nuclei of hyperstimulated cells [Brasch and Ochs, 1992].

BOTH FUNCTIONAL AND STRUCTURAL CRITERIA ARE NEEDED FOR DESCRIPTION OF THE STRUCTURE-FUNCTION NUCLEAR COMPARTMENTALIZATION

It is evident that with just a dozen structural components or domains, we are not able to depict thousands of functional nuclear events related to the RNA metabolism. The EMAC picture of the cell nucleus has to be generated and, by inference, one has to ascribe the corresponding function to components or a domain, whether structurally defined or not, underlying the relevant affinity label. My opinion is that for many nuclear events, the spatial identity between a specific functional process and an ultrastructurally well-defined component or domain cannot be established. This applies particularly to the IS. Within nucleoli, the only known active genes are tandem repeats of ribosomal genes. Thousands of different RNAs are apparently synthesized within the IS.

Such a functionally, or even operationally, conceived ultrastructural perception of the cell nucleus, based on the dynamic changes of the labelling rather than on the structural features, is for a morphologist, particularly for an electron microscopist, not easily digestible. Nevertheless, EMAC results obtained during the last 4 years [for comparison see Raška et al., 1990] made it possible to define IS functional domains with distinct structural characteristics as well. Several such results will be pinpointed here with a message that the task is more difficult than anticipated. Leaving aside ISH probes for specific nucleic acid sequences, most antibody probes targeting IS are not specific for any given structure. The probes used react more or less with several nuclear components. A typical example are the antibodies to snRNPs or to a non-snRNP splicing factor SC35 (Fig. 1a) which, in our hands, label PGs, IGs, PFs, and several kinds of NBs. In a way, a more convenient approach is to identify domains which they do not label at all (e.g., Figs. 1c, 3d)! In addition, basic categories of macromolecules such as snRNPs, SR proteins, hnRNP proteins, or poly(A) sequences [Dundr, 1993] do show much of the overlap in distribution, but their concentration within different IS domains substantially varies. Moreover, the mapping of genes, transcripts, and various factors involved in RNA metabolism may identify but does not prove the localization of a given process such as transcription or splicing. We do not map the process, but only the structures related to the process. We also recall that with the negative localization result we cannot conclude that the method used is appropriate enough (e.g., a masking of the target epitope or of the sequence of bases, or a low number of target macromolecules [Raška et al., 1990]). In order to gain a deeper insight in structure-function relationships in IS, it is necessary to explore the following kinds of affinity cytochemistry probes: (1) probes specific for defined functional sites/structures in the metabolism of RNA. This includes the probes to 5-bromoudine-5′-triphosphate depicting the active process of RNA synthesis by means of incorporated bromouridine (BrU) within both extranucleolar and nucleolar RNA [Wansink et al., 1993; Raška et al., 1995], or, for example, the antibodies specific for active spliceosomes (Lührmann, personal communication). (2) Probes specific for defined molecules. Besides already mentioned ISH probes specific for a given species of mRNA, antibodies targeting a defined molecule confined to distinct nuclear domains, such as coiled bodies, should be mentioned. Screening of sera of patients with autoimmune diseases was valuable in providing autoantibody probes to new nuclear domains called at light microscopical level speckles, dots, foci, etc. [Raška et al., 1990, 1992].

FUNCTIONAL SUBCOMPARTMENTALIZATION OF THE NUCLEOLUS

With the non-isotopic detection of RNA synthetic sites, most of the gold particles are within nucleoli confined to DFC and also to the peripheral part of FC (Fig. 2) [Wansink, 1994; Raška et al., 1994]. The method is definitely superior to EMARG and the nuclear ultrastructure is approaching that of non-treated cells. It may not, however, be straightforward for the morphological description of further maturation steps of individual RNAs as incorporated BrU interferes with precursor RNA processing [Wansink, 1994].

BrU signal occurs in clusters of gold particles (Fig. 2). Such clusters reflect the likely presence of one (or at most a few) compacted transcribing gene(s). More than 50 clusters are easily identified in large, serially sectioned nucleoli of HeLa cells [Raška et al., 1994]. This number is within the order of magnitude of the number of ribosomal genes. Moreover, some of the arrays of

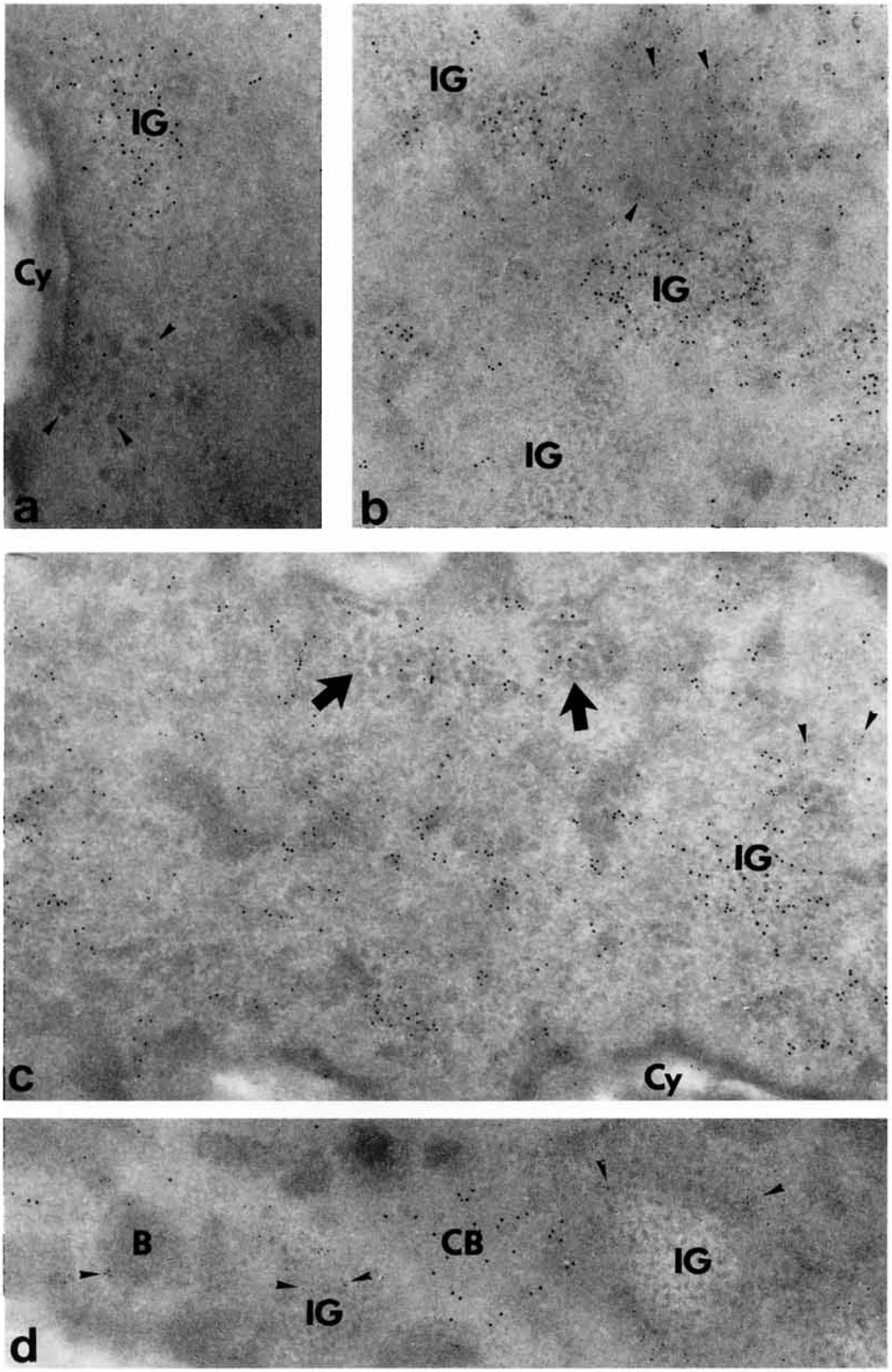

Figure 4.

gold particles seen within nucleoli are compatible with the picture of more or less compacted Miller's Christmas trees (Fig. 2).

It should be emphasized that by morphological approaches we depict rate limiting steps within the context of the given functional process. In most nucleolar sections, the number of gold particles present within DFC was much higher than that over FC. It can be argued that DFC represent the site of rRNA accumulation due to the rapid translocation of rRNA from its transcription site in FC towards DFC. Whereas the translocation might be a very rapid process, this is certainly not the case for rRNA (as well as mRNA) synthetic rates which are in the order of magnitude of 1 to 2 kb per minute and which we consider to be the rate limiting step in vivo. Therefore, by means of the non-isotopic method we identify both DFC and peripheral part of FC as the major sites of rRNA synthesis.

ISH experiments with various sequences to rDNA map ribosomal genes primarily to FC or to DFC [Scheer et al., 1993; Wachtler and Stahl, 1993; Raška et al., 1994]. Similarly, the localization results for important nucleolar macromolecules can be divided in two groups. First, the majority of label due to RNA polymerase I, the proper enzyme transcribing rDNA, and possibly to upstream binding factor, regulating the activity of RNA polymerase I, is usually observed over FC, whereas DFC exhibits a less intense label. The second group of results encompasses all other macromolecules which map either outside of FC, i.e., to DFC and GC, or show a higher incidence in DFC than in FC. They include various biotinylated probes to 18S, 5.8S, 28S, and 5S rRNA, RNA/DNA hybrids, DNA topoisomerases which unwind rDNA, proteins nucleolin, B23, and Nopp 140 which, apparently besides other functions, shuttle between the cytoplasm and the nucleolus, both protein fibrillarin and U3 snRNA which are components of the snoRNPs known to be involved in the processing of rRNA, as well as several ribosomal proteins [Meier and Blobel, 1992; Wachtler and Stahl, 1993; Testillano et al., 1994; Raška et al., 1995]. In addition, among the factors investigated, just RNA polymerase I and upstream binding factor remain associated with nucleolar organizer regions of mitotic chromosomes, all

Fig. 4. Cryosectioned HeLa cells embedded in polyvinylalcohol. **a:** Transcriptionally hyperstimulated cell. Gold particles (10 nm) correspond to the localization of SC35 splicing factor. The chromatin structures are decondensed. Both IG cluster and accumulated PGs (arrowheads) are labelled and are seen next to the nuclear envelope. Under usual conditions of cell culturing, this localization is not common for the two nuclear components. Does the accumulation of PGs, due likely to the metabolic disbalance, correspond to the special kind of "traffic jam" discussed in the legend to Figure 4c? ×64,200. **b:** Heat shocked cell for 15 min was immunolabelled for coilin (5 nm particles; arrowheads) and SC35 (10 nm particles). Early changes in the labelling correspond to the non-homogenous label of coilin over CB (arrowheads) and the heterogeneity of IG clusters. The SC35 label is enriched in IGs next to the CB, but several IG domains are not labelled. ×64,100. **c:** Heat shocked cell for 40 minutes was labelled for SC35 (10 nm particles) and coilin (5 nm particles; arrowheads). Due to the environmental stress, the IS became rich in various RNP structures (compare with Fig. 1a as under physiological conditions of culturing, the IS has more homogenous appearance). Note the label over a remnant of the disintegrated CB (arrowheads) as well as over accumulated PG-like granules (arrows; 10 nm particles) at the nuclear envelope. A phenomenon is encountered which I call a "traffic jam" and which can arise because of various causes. In general, a massive redistribution of macromolecules, which is frequently differential for different macromolecules, accompanies experimental alterations, by biological, physical, or chemical factors, of DNA replication, of RNA synthesis and processing, and of protein synthesis [e.g., Fakan and Puvion, 1980; Raška et al., 1990; Spector, 1993]. For examples may serve virus infected cells, heat shocked cells, or experiments with inhibited RNA synthesis by drugs such as the treatment of cultured cells with 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Fig. 4d). The major impact of these results is that the distribution of snRNPs, SR proteins, hnRNP proteins, and poly(A) sequences depend on the transcription level and that after a prolongated inhibition of RNA synthesis, SC35 and poly(A) sequences, but not snRNPs and hnRNP proteins, accumulate in large "speckle-like" domains [Spector, 1993; Huang and Spector, 1994]. The direct structure-function relationship to a situation before the treatment may, however, become questionable. If already observable, my opinion is that then morphological changes reflect heavy functional (metabolic) changes. Therefore, the earliest changes should be studied, rather than the situation when everything is "jammed" after, e.g., several hours' treatment with "deadly" doses of RNA polymerase inhibitors [Raška et al., 1990]. In contrast, a different example of importance is the recent ultrastructural demonstration of the nucleolar association of transcripts for the EGF receptor [Sibon et al., 1994] which are not transported to the cytoplasm. I include the observed nucleolar accumulation of transcripts also under the "traffic jam" phenomenon. Interestingly, differentiating myoblasts do show a speckled fluorescence due to poly(A) mapping, but in newly formed myotubes large domains arranged in a distinct ring around the nucleolus are observed [Lawrence et al., 1993]. ×63,800. **d:** Extranucleolar RNA synthesis inhibition in this cell by a treatment with DRB resulted in a massive rearrangement of macromolecules. The cryosection was labelled for coilin (10 nm particles) and hnRNP proteins C1/C2 (5 nm particles; arrowheads). Note a different appearance of the two IG clusters. The central part of one cluster shows a less electron-dense texture. The simple nuclear body (B) is not labelled with either of the probes. ×65,100.

other macromolecules having a cytoplasmic localization during mitosis [Wachtler and Stahl, 1993]. All these results do not allow conclusions localizing the rRNA synthesis to either of the two components alone. I am of the opinion that FC and DFC generate a single functional unit involved in rRNA transcription, with individual rRNA genes becoming, through the active process of transcription, engulfed within DFC [Raška et al., 1994].

DFC and GC are involved in the further processing of precursor rRNA as all the relevant factors were localized to these two nucleolar components. GC of the nucleolus is considered to correspond to preribosomal particles. This concept, which is derived from the morphological granular appearance of both the nucleolar "granules" and cytoplasmic ribosomal "granules," is based on the time shift of the autoradiographic signal from DFC to GC [Fakan and Puvion, 1980; Raška et al., 1994]. Basic questions relative to an anticipated vectorial process of preribosome formation, however, remain unanswered. It is not clear whether all nucleolar granules correspond to preribosomes, and/or to what extent individual nucleolar granules represent preribosomes at various stages of assembly. As far as we know there is no morphological information available about the very different maturation pathway of small and large ribosomal subunits. Besides rRNA, other macromolecules which form an integral part of cytoplasmic ribosomes, i.e., several ribosomal proteins, 5S rRNA and 5S rRNP are mapped in DFC [Raška et al., 1994]. By inference DFC should also be considered to contain preribosomes. Of particular importance, in this respect, is the recent evidence that the terminal knob at the 5′ end of ribosomal transcripts depicted in Miller spreads is involved in the early processing of 45S rRNA [Mougey et al., 1993]. Since the terminal knob contains fibrillarin and fibrillarin maps almost entirely to DFC, the early rRNA processing should occur in DFC.

Little is known about the transport of preribosomes from nucleoli to cytoplasm. The nucleoplasmic tracks due to Nopp 140 may reflect this transport mechanism [Meier and Blobel, 1992]. Alternatively, it has also been assumed that nucleoli are situated at the nuclear envelope, but this is not confirmed in the case of serially sectioned quiescent human lymphocytes. The cytoplasmic protuberances, which disappear after the cells reach the confluency, being more or less in contact with nucleoli of metabolically active cultured cells were recently demonstrated [Vaux et al., 1994]. This finding strengthens the possibility of the direct contact between nucleoli and nuclear envelope in metabolically active cells. The concept of Harris [1972] should also be recalled as some mRNAs may be transported via the nucleolus and may take the rRNA pathway [Bond and Wold, 1993; Sibon et al., 1994] (see also legend to Fig. 4).

SUBCOMPARMENTALIZED AFFINITY CYTOCHEMISTRY PICTURE OF THE INTERCHROMATIN SPACE

With the BrU method, much of the extranucleolar non-isotopic label is found at the border of chromatin structures. This result is basically in agreement with the classical EMARG work [Fakan and Puvion, 1980]. The signal is seen in the form of clusters rather than individual gold particles and, similarly to transcribing ribosomal genes, individual, or at most a few, compacted transcribing genes may be identified with it. In serially sectioned individual HeLa cell nuclei, I appreciate the number of transcribing extranucleolar genes to be in the range of 10^3 to 1.5×10^4. Importantly, a number of macromolecules such as various snRNPs as well as hnRNP and SR proteins do show an overlap with RNA synthetic sites [Dundr, 1993].

Two observations have to be emphasized. Increased incidence of gold particles, sometimes in the form of elongated arrays, is associated with IS domains which can be identified as clumps of PFs and nuclear bodies, but not CBs (Fig. 3d). It could be that these domains are associated with highly expressed and/or repeated genes such as 5S rRNA, snRNAs or histone genes. The second observation is at variance with the classical EMARG observation and is related to the signal present in clustered IG. The signal is regularly observed in some clusters of IGs (Fig. 2), but it is relatively low to account for IG clusters to represent major nuclear transcription sites. EMARG results are taken for bona fide arbiters. The BrU label within IG clusters may then reflect a rate limiting step. It may correspond to an accumulation of transcripts which incorporated BrU and cannot be properly spliced and/or degraded. The results of Wang et al. [1991] who, after their microinjection to nuclei, observed an accumulation of fluorochrome conjugated mRNA in clusters of IGs may also reflect the incapability of

mRNAs to be properly spliced and/or degraded. On the other hand, there are indications for a structure-function heterogeneity of IGs as, within individual IG clusters of the same nucleus, differential patterns of both snRNP and SC35 signals are observed [Dundr, 1993] (see also Fig. 4).

As far as we know, data concerning the ultrastructural localization of either RNA polymerase II and III, or various transcription factors are scarce. RNA polymerase II is mapped to IS, with the exclusion of clusters of IGs and CBs [Spector et al., 1993; Dundr, 1993]. The overall label is relatively low, but is enriched in perichromatin areas. An alternative approach was introduced by Testillano et al. [1994] whose mapping of RNA/DNA hybrids by means of the specific antibody overlapped with that of RNA synthetic sites.

The maturation of synthesized RNA is associated with a number of events. They are cotranscriptional cap formation on the 5′ end, association of hnRNP proteins with nascent RNA, formation and binding of spliceosomes, splicing itself, degradation of introns, 3′ end endonucleolytic cleavage followed by polyadenylation (with the known exception of histone mRNAs), and RNA transport. Much has been learned about these processes from in vitro studies, but their localizations are ubiquitous. The mapping results relative to the cap formation and endonucleolytic cleavage are scarce [e.g., Takagaki et al., 1990; Izzauralde et al., 1994]. I shall limit the discussion to the splicing, polyadenylation, and transport.

Nascent precursor mRNAs associate with snRNPs, splicing non-snRNP factors, and hnRNP proteins [Spector, 1993; Pinol-Roma and Dreyfuss, 1993]. Splicing of mRNA takes place in spliceosomes which are composed snRNPs containing U1, U2, U4, U5, and U6 snRNAs, many snRNP proteins, and several non-snRNP splicing factors, particularly SR proteins [Zahler et al., 1992; Spector, 1993; Pinol-Roma and Dreyfuss, 1993]. hnRNP proteins are involved in the packaging of hnRNA as well as its further processing and transport [Pinol-Roma and Dreyfuss, 1993]. The splicing begins on nascent transcripts, but some of the subsequent splicing reactions may be separated in time and space from the synthetic site [Beyer and Osheim, 1991; Kopczynski and Muskavitch, 1992; Baurén and Wieslander, 1994]. In fact, if late (?) splicing steps, at least for some of the transcripts, take place in association with IGs, this would explain the observed BrU label over these nuclear components.

Concerning the spatial or temporal relations between sites of transcription and in fact any further maturation step in RNA metabolism, the localization of snRNPs should be taken as the reference basis for further discussion. The fluorescent snRNP image due to protein moieties, as well as that of U2, U4, U5, and U6 snRNA, is unique and consists of a few dozens of shining speckles together with some less intense, diffuse label within IS. For U1 snRNA, the difference in the intensity of staining between speckles and other IS domains is not as pronounced as it is for the mentioned snRNAs. At the ultrastructural level, shining speckles can be definitely identified with clusters of IGs. Besides it, however, several kinds of NBs, including simple nuclear bodies and CBs, and clumps of PFs give rise to speckles (Fig. 2c) [Raška et al., 1990, 1992]. The diffuse fluorescent label corresponds to the widespread gold label within IS associated with RNP structures, i.e., basically with PFs, but also with PGs. Collectively, with the exception of distinct domains such as the body seen in Figure 1c, the label due to snRNP is found widespread over the IS.

SR proteins constitute a group of several proteins (e.g., SC35, U2 snRNA auxilliary factor [U2AF]) which are highly conserved [Zahler et al., 1992]. Besides other motifs, they bear RNA-recognition motif apparently for the mRNA binding and a region called RS domain rich in arginine and serine. The RS domain appears to be a multifunctional protein-protein interaction domain that is critical for the localization of SR proteins to nuclear speckles, and for the role these factors play in the splicing reaction. In both cases this domain appears to mediate the assembly of multicomponent complexes, in one case on the nuclear matrix, in the other on nascent pre-mRNA (Maniatis, personal communication). With the exception of U2AF, the ultrastructural localization of SR proteins is reminiscent of that of snRNPs. U2AF is widespread within IS with both PFs and PGs being labelled; its highest concentration is in CBs. In contrast to SC35, it does not give rise to speckles as its concentration within clusters of IGs is not increased. SC35 protein is highly enriched in speckles, i.e., in clusters of IGs, in clumps of PFs, and in several kinds of nuclear bodies, but not in CBs [Raška et al., 1992; Spector, 1993; Dundr,

1993]. It is widely distributed within IS and it does label PGs [Raška et al., 1992].

More than 20 different hnRNP proteins designated by letters A to U were identified. At the light microscopical level, these proteins show, with a few exceptions, a more or less homogenous IS distribution. The ultrastructural mapping of hnRNP proteins provides an overall IS label with exclusion of CBs and to some extent clusters of IGs. Gold particles label PGs, PFs, and several kinds of NBs, they are associated with the periphery of IG clusters, the label within the clusters being exceptional. One of the exceptions to this rule are the hnRNP proteins of group L which in nuclei generate 2 to 5 speckle-like domains. These domains are identified as clumps of PFs [Raška et al., 1992]. The hnRNP protein A1 was shown to shuttle at very high rates between the nucleus and the cytoplasm [Pinol-Roma and Dreyfuss, 1993]. Whereas the accumulation of gold particles could be observed in the cytoplasm after the inhibition of RNA synthesis, the cytoplasmic label due to A1 proteins could not be put in evidence in cells cultured under physiological conditions [Dundr, 1993]. The negative result apparently reflects the lower sensitivity of the EM approach. I should mention here that a light microscopical distribution of the cleavage stimulating factor involved in the processing of the 3′ mRNA [Takagaki et al., 1990] is widespread within the IS and is similar to the distribution of most hnRNP proteins.

A great majority of mRNAs is polyadenylated. Poly(A) positive domains were therefore called by Lawrence and collaborators "transcript domains." Surprisingly, the poly(A) sequences generate at the fluorescent level a speckled distribution that more or less collocalizes with snRNPs [Lawrence et al., 1993]. As mRNA is rapidly labelled and speckles correspond primarily to clusters of IGs in which most RNAs are stable, this result indicates that there may be at least two populations of poly(A) sequences. One population could correspond to a rather stable species associated mainly with IGs where it may play a structural role. The other population may correspond to polyadenylated mRNA which is associated with PFs and which is exported to the cytoplasm. This explanation for the speckled pattern of poly(A) distribution received support in the recent paper of Huang et al. [1994] who at the (light and) ultrastructural level provided evidence for the stable population of poly(A). By means of a new photooxidation method they visualized polyadenylated RNA in the process of being transported through all of the observed nuclear pores. IGs, PFs, but not CBs, were shown to contain poly(A) sequences. Interestingly, the regions of polyadenylated RNA which reached the nuclear pore appeared as narrow concentrations of RNA, suggesting a limited or directed pathway of movement [Huang et al., 1994]. Due to the high concentration of both poly(A) and SR proteins in speckles, their interplay, together with other (matrix?) proteins, may have an anchoring role. One could then speculatively consider the polyadenylation of mRNA, besides other functions, as a sort of "vaccination" against its possible anchoring in speckles during its transport through the IS. Coming back to the BrU signal seen in IG clusters, it is to be recalled that BrU likely interferes with the polyadenylation as uridines are contained both in upstream and downstream regulative cleavage sequences at the 3′ end.

There is an increasing amount of evidence that besides poly(A) RNAs, other species of RNAs, including mRNAs, are not transported to the cytoplasm [Lawrence et al., 1993; Hogan et al., 1994; Huang et al., 1994]. There is only a little ultrastructural information available about their storage [e.g., mRNAs see Bond and Wold, 1993; Sibon et al., 1994]. The information about the fate of excised introns is also limited. It is usually claimed that the excised introns are rapidly degraded [Lawrence et al., 1993], but contrary examples also exist [e.g., Kopczynski and Muskavitch, 1992; Quian et al., 1992]. Even though there is no direct evidence, it has been speculated that IG clusters and CBs are involved in RNA and/or intron degradation. Interestingly, at the ultrastructural level exons and introns of the EGF receptor transcripts are localized within paranucleolar domains which consist of subdomains exhibiting label due to either exons only, or introns only, or both exons and introns (Sibon, personal communication).

Two models were proposed to describe the transport of mRNA from sites of synthesis to nuclear pores. The first model called "track" model [Lawrence et al., 1993; Xing and Lawrence, 1993] was originally described in cells carrying two integrated viral genomes. Using a thoroughly elaborated ISH technique, fluorescent nuclear tracks several micrometers long corresponding to viral transcripts were visualized. The tracks could be later demonstrated for

a few other genes [Lawrence et al., 1993; Xing and Lawrence, 1993; Spector, 1993], including the ultrastructural visualization of c-fos transcripts which, after the gene induction, were shown to form an elongated track from the site of synthesis to the nuclear envelope [Spector, 1993]. Besides the tracks, however, a dot-like signal was observed for transcripts of several other genes, suggesting that only an accumulation of transcripts at the site of synthesis is being depicted in these cases [Kopczynski and Muskavitch, 1992; Lawrence et al., 1993; Xing and Lawrence, 1993; Spector, 1993]. An exciting result was obtained during the mapping of the fibronectin gene transcripts [Lawrence et al., 1993; Xing and Lawrence, 1993]. The fibronection gene is localized at one end of a long track formed by transcripts, and in the proximity of a poly(A) speckle. Exon probes generate a longer track than intron probes, indicating that the splicing occurs while the RNA is transported in a directed manner along the track. The directed model of RNA movement along the tracks raised, however, a criticism based on the claim that tracks correspond entirely to (preparation induced?) looped Christmas trees of nascent transcripts [Rosbash and Singer, 1993; Kramer et al., 1994]. Several facts cannot be adequately explained with the track model: The tracks do not end at the nuclear membrane and the time of RNA synthesis should be considered to be a time limiting step, i.e., RNA synthesis is depicted rather than its transport and/or splicing. On the other hand, Lawrence and collaborators [1993] do visualize the corresponding genes as dots only, irrespectively of the preparation used, and there is no in situ ultrastructural argument for the existence of extended (5 or even more micrometers) transcribing genes.

The second model was established while mapping overexpressed RNA constructs in polytene nuclei of Drosophila [Kramer et al., 1994]. High concentration of transcripts is found at the transcription sites, their lower concentration is found widespread in the extrachromosomal channel network which we call here IS. This result is interpreted as if just an "isotropic" diffusion within the channel network is responsible for the transport of RNA, the processing of RNA being carried out progressively within this compartment. The diffusion model is attractive, particularly because of its simplicity. It remains to be established whether the situation in polytene nuclei can be transposed to mammalian cells, but those mappings generating just the dot-like signal for transcripts [Lawrence et al., 1993] may apparently enter the diffusion scheme.

NUCLEAR ARCHITECTURES ASSOCIATED WITH THE RNA SYNTHESIS AND PROCESSING

There are probably hundreds of structure-function processes taking place within nucleoli at a given time and there are basically only three ultrastructural components identified in typical mammalian nucleoli. It is clear that each component has to accomodate not one, but dozens of functional processes. Surprisingly little is known about the spatial organization of preribosome formation and transport to the cytoplasm. I am of the opinion that this situation will rapidly change in the near future [e.g., Vaux et al., 1994]. In addition, specific antibodies to many ribosomal proteins are available for the differential nucleolar mapping from which clues for the preribosome formation may arise.

The nucleolus is an organelle accounting for almost 50% of synthesized RNA in cultured cells. A massive import and efflux of macromolecules accompanies such functional events within the nucleolar factory. It can be speculated that sometimes at the beginning of the G1 phase of the cell cycle, the steady state of the import and efflux is reached. This steady state has to be somehow modulated during the S phase and probably reaches another niveau of the steady state after the replication of nucleolus-associated DNA is completed. I propose that the RNA synthesis is initiated in FC, in some form of association with DFC. As the cell proceeds during the cell cycle, and reaches the steady state mentioned, the major site of RNA synthesis becomes DFC. In the future, two questions should definitely be studied. The first is the establishment of the morphological correlate of the activity of (individual) rRNA genes, or rather the identification of inactive and underexpressed rRNA genes, since it has not been excluded that such genes may have a different localization from that of the fully active genes seen in classical Miller's spreads. This problem could be approached by a collocalization of rDNA with incorporated BrU (Schöfer, personal communication). The second is the mapping of transcription sites in synchronized cells because I expect definite changes in their localization associated with the cell cycle related changes.

The problem of biological diversity (cell type, cell developmental stage, cell cycle stage, and

actual metabolic state of the cell) has to be kept in mind, although I consider it to be to a large extent only a quantitative factor with respect to rRNA synthesis. A different situation is found in the IS, as qualitative differences are encountered in quiescent lymphocytes, metabolically active hepatocytes, or cultured HeLa cells. At present, it is impossible to unambigously associate various functional steps in the metabolism of extranucleolar RNA with one or another of the ultrastructural components within IS. Moreover, as far as I know there are no ultrastructural data available which differentiate between RNA polymerases II and III mediated processes, respectively. Bearing these limitations in mind, I give here my speculative perception of the fate of extranucleolar mRNA. The gene is transcribed by RNA polymerase II in the perichromatin region, with the anticipated battery of transcription factors being present. The corresponding structural substrate should be PFs or RNP structures within distinct domains which I call clumps of PFs and nuclear bodies. snRNPs, SR, and hnRNP proteins then bind to nascent transcripts and processing of mRNA starts. Highly expressed and/or repeated genes are confined to the distinct domains which effectively correspond to transcript domains of Lawrence et al. We map to these domains poly(A) as well. The postembedding ISH signal of poly(A) provides specific, but too low signal [Dundr, 1993] to be compared with the shining speckles [Lawrence et al., 1993] or with heavy ultrastructural label obtained in the preembedding technique [Huang et al., 1994]. Such transcript domains should be differentiated from those characterized by the high concentration of poly(A) only. Do PGs enter mRNA synthetic step? We map to these components snRNPs, several SR proteins, hnRNP proteins, occasionally even poly(A) sequences, but a great majority of PGs are devoid of label in the BrU method. If PGs are on the spot during the transcription process, I expect a clearly detectable signal associated with the sizeable portion of PGs. Unless PGs are affected by the cell processing for EM, it is then difficult to identify enigmatic PGs with active spliceosomes. But then where are 50 nm spliceosomes? In my opinion, the genuine hints have to come from the detailed analysis of the postembedding affinity label distribution in the perichromatin region. During the elongation process, some, or maybe all, of the introns are removed. The mRNA cleavage takes place shortly after the relevant sequences are synthesized at the 3′ end, and polyadenylation starts. Some transcripts diffuse towards the nuclear envelope, others are transported, at least along a certain distance, in a directed manner along the tracks. Anyway, the RNA transport is a good model situation for EM to play a good arbiter. As far as I know, no EM mapping has yet been performed for single copy genes.

How do all the remaining ultrastructural components enter this scheme? With the exception of CBs, I shall not address the category of NBs. The reason is that they constitute a very heterogenous category of structures. According to their nuclear localization and labelling properties, several variants of simple nuclear bodies are seen [e.g., Raška et al., 1990]. Concerning IG clusters, despite being highly enriched in snRNPs, SR proteins, and poly(A) sequences, I consider them as only indirectly related to the RNA processing in most cases. I identify them with the storage and recycling of factors involved the splicing, eventually with the degradation of RNA. According to Spector [1993], the necessary factors are recruited from IG domains to serve the neighbouring IS. Similarly, in agreement with Gall's view [Gall et al., 1994], I do not consider the some CB's to be directly involved in the processing of RNA (Fig. 3). As U3 snRNA is also localized to some CBs, they have to be involved in the metabolism of both snRNAs and small nucleolar RNAs, and likely in the maturation and assembly of snRNPs. Such a function does not exclude a possible transport role played by CBs that would explain their association either with clusters of IGs, with the DFC part of the nucleolus, or with the nuclear envelope in most cases [Dundr, 1993]. The transport role is supported by the presence of Nopp 140 in CBs (Meier, personal communication). In addition, U7 snRNA has been demonstrated in sphere organelles of amphibian oocytes, which are equivalent nuclear structures to CBs [Gall et al., 1994], specifically implicating CBs in the metabolism of histone mRNA. I consider CBs as sites of assembly of RNP complexes, particularly snRNPs, and as indirect metabolic markers of active processes, such as the locally ongoing intense RNA synthesis, situated nearby.

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