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The functional architecture of the nucleus as analysed by ultrastructural cytochemistry

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Abstract Ultrastructural cytochemistry has been, for many years now, a major tool for investigating structure–function relationships in the cell nucleus. It has been essential in approaching the roles which different nuclear structural constituents can play in nuclear functions. This article briefly summarises transmission electron microscopic studies aimed at characterising in situ nuclear architectural domains and their involvement in main nuclear functions, such as DNA replication, hnRNA transcription and pre-mRNA processing. It discusses the importance of ultrastructural cytochemistry in high resolution analyses of intranuclear distribution of chromatin domains and their topological relationships with other structural interphase nuclear constituents. It puts forward the central role of the perichromatin region as a functional nuclear domain. Finally, it attempts to critically evaluate some future applications of ultrastructural investigations of the nucleus and stresses the importance of combining them with light microscopic analyses of living cells.

Keywords Ultrastructural cytochemistry · Nuclear architecture · DNA replication · Transcription · Pre-mRNA processing

Introduction

The cell nucleus is defined by the nuclear envelope, consists of two main compartments, the nucleolus and the nucleoplasm, and its different internal structural constituents are not delimited by membranes (Fig. 1). Methods of ultrastructural cytochemistry, extensively used during the last 40 years to analyse the morpho-functional relationships in the cell nucleus in situ, have provided a major contribution in investigating the nuclear functional architecture. Long before the first confocal microscope became commercially available, the applications of various methods of electron microscopic cytochemistry gave rise to a number of essential observations allowing one to characterise the nature of nuclear structural domains and to approach their possible roles in nuclear functions.

The most studied nuclear compartment is probably the nucleolus. It is the site of ribosomal RNA synthesis and processing and ribosomal subunit assembly. The nucleolus consists of three structural subdomains: fibrillar centres, dense fibrillar component and granular component. A large number of studies has been devoted to the morphofunctional aspects in the nucleolus, leading to sometimes controversial findings. Most observations support the dense fibrillar component as the major site of pre-rRNA synthesis (see, for example, Granboulan and Granboulan 1965; Cmarko et al. 2000; for more details see Fakan and Puvion 1980; Goessens 1984; Hozak 1995; Biggiogera et al. 2001; Raska 2003).

In this short review article, we will mainly concentrate on the contributions of ultrastructural cytochemical analyses to characterising different structural domains in the nucleoplasm and to unravelling their roles in essential functions of the cell nucleus such as DNA replication, transcription, RNA processing and storage.

Methods of transmission electron microscopic cytochemistry, although giving rise to two-dimensional images, offer a resolving power that is about two orders of magnitude superior to light microscopy, including confocal laser scanning microscopy (CLSM; Stelzer 1995). Their application has made major contributions in obtaining basic information about the nuclear functional architecture. Early ultrastructural studies (see, for example, Swift 1962; Smetana et al. 1963; Monneron and Bernhard 1969) already provided a fundamental pool of data necessary for further rapid advancement in this im-

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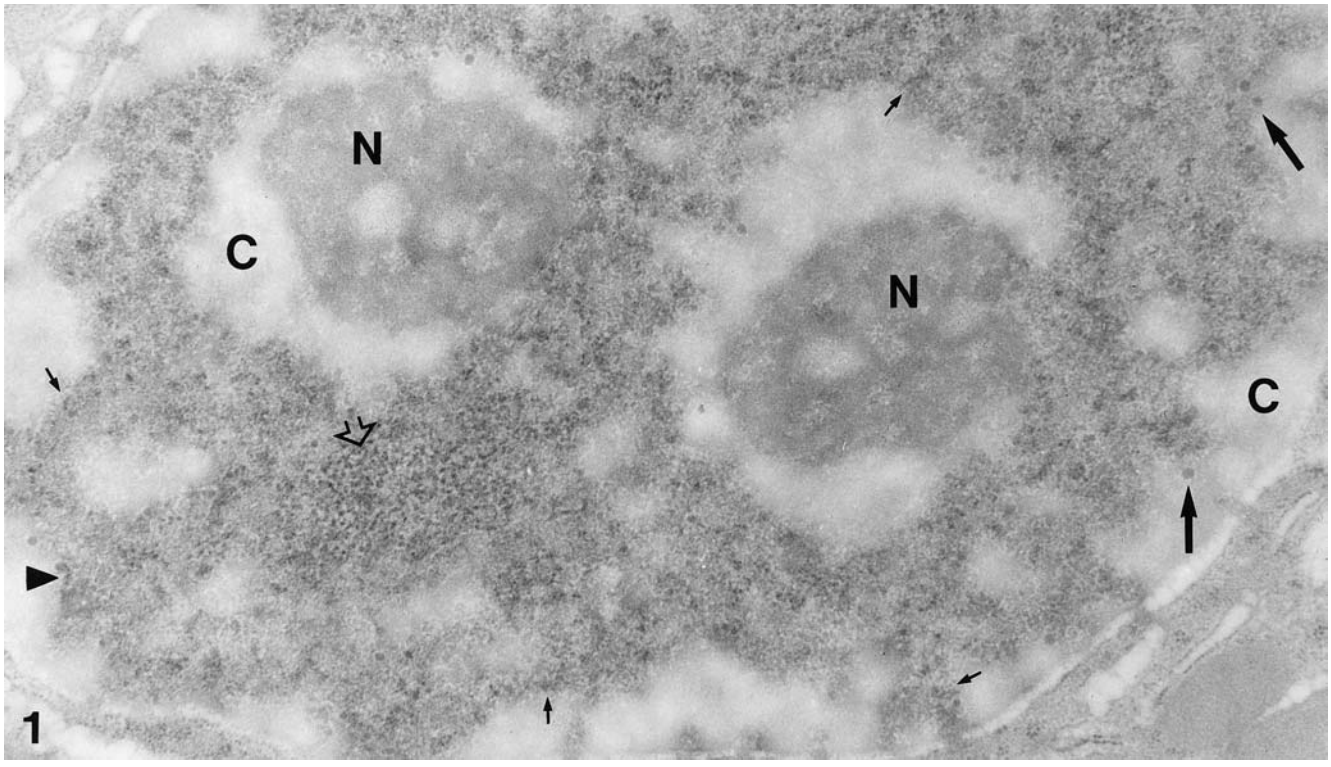

Fig. 1 Ultrathin section of a nucleus from a rat hepatocyte fixed in glutaraldehyde and embedded in Epon. EDTA staining preferential for nuclear RNP constituents (Bernhard 1969). Nucleoplasmic structures, such as perichromatin fibrils (some indicated by *small arrows*), perichromatin granules (some indicated by *large arrows*; *arrowhead* points to a perichromatin fibril giving rise to a perichromatin granule) and interchromatin granules (*open arrow*), as well as nucleoli (*N*) are well contrasted, while chromatin (*C*) appears light grey (×30,000)

portant field of research. Later on, high resolution autoradiography and immunoelectron microscopy constituted major tools in investigating the involvement of various nuclear structural constituents in nuclear functions.

The nucleoplasm contains chromatin domains represented mainly by condensed chromatin areas observed on the periphery of the nucleus as well as throughout its interior. The border zone of condensed chromatin areas, termed the perichromatin region, represents an essential nuclear functional domain. Its importance appeared obvious as early as some 30 years ago and has been repeatedly confirmed as the major site of nuclear functions such as DNA replication, transcription and probably most steps of pre-mRNA processing. The internal nuclear compartment surrounding chromatin has been named the interchromatin space (see, for example, Fakan and Puvion 1980) or later also the interchromatin compartment (Cremer and Cremer 2001) and is mostly devoid of DNA. The interchromatin space contains various structural domains, some of them accumulating different types of RNA (Fig. 1).

Chromatin domains

High resolution distribution of chromatin throughout the nucleus can be investigated by virtue of a specific contrasting technique based on a Feulgen-type reaction with osmium ammine (Cogliati and Gautier 1973) allowing one to visualise DNA directly in ultrathin sections. This approach, extensively applied on different cell models (Biggiogera et al. 1996), can be combined with other methods such as immunocytochemistry and electron spectroscopic imaging (Biggiogera et al. 2001). Analysis of reconstructions from serial ultrathin sections of interphase nuclei revealed that about 50–60% of the nuclear space is filled by chromatin (Esquivel et al. 1989; López-Velázquez et al. 1996), while the remaining volume is occupied by the interchromatin space with its structural interchromatin domains. The interchromatin space is largely devoid of DNA (Fig. 2).

The development of fluorescence in situ hybridisation (FISH) and of molecular probes for specific chromosomes made it possible to localise territories of individual chromosomes within the interphase nucleus (for more details, see van der Ploeg 2000; Cremer and Cremer 2001). Chromosome painting, as well as the possibility to localise a particular gene with regards to chromosomes in mitosis, has proved to be a powerful tool in genetic diagnosis and in biomedical research. Keeping in mind the relatively low resolution of any light microscopic observation, the application of these techniques in investigating the interphase nuclear architecture and the interpretation of the results should be carried out with caution. In ad-

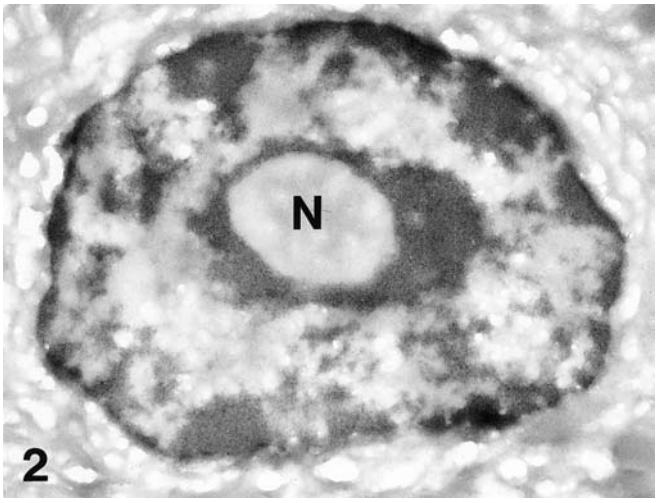

Fig. 2 Ultrathin frozen section of a mouse pancreas cell nucleus stained by the Feulgen-type reaction with osmium ammine (Cogliati and Gautier 1973) revealing specific distribution of DNA. *N* Nucleolus (×13,700)

dition, FISH procedures aiming at visualising the distribution of DNA sequences in the specimen, make use of rather harsh treatments in order to keep the target and the probe DNA denatured. Usually applied after a relatively mild specimen fixation, it gives rise to extensive DNA dispersion within the nucleus, leakage of DNA into the cytoplasm and general degradation of the nuclear architecture as shown by subsequent electron microscopic analysis of FISH-submitted cell samples (Solovei et al. 2002). The denaturation steps lead to alveolation of nuclear fine structure and the original distribution of various nuclear domains is considerably altered. This shows that FISH techniques, as presently available, are hardly suitable for higher resolution studies, such as localisation of genes with respect to particular nuclear structural domains. Since ultrastructural methods allowing one to carry out chromosome painting or ISH investigation of specific genome sequences providing satisfactory signal without significant deterioration of the nuclear structure are not yet available, other approaches avoiding the use of too deleterious preparative conditions have been explored. In order to study the relationships of a given nuclear domain or territory with its neighbourhood in the nucleus, Visser et al. (2000) made use of the possibility to label DNA in living cells by means of halogenated deoxyuridines (dUs), which can be detected at the ultrastructural level, using selected anti-dU antibodies and immunocytochemistry (Jaunin et al. 1998). Cultured cells labelled during one S-phase with BrdU and subsequently allowed to grow for several cell cycles, exhibit in their nuclei one to a few labelled chromatin domains obviously representing chromosome territories. This approach further enabled the investigation of the structural relationships between such labelled domains and their unlabelled neighbours and revealed three typical patterns of contacts. In some cases, domains were separated by interchromatin space, showed chromatin fibre continuation with a sharp

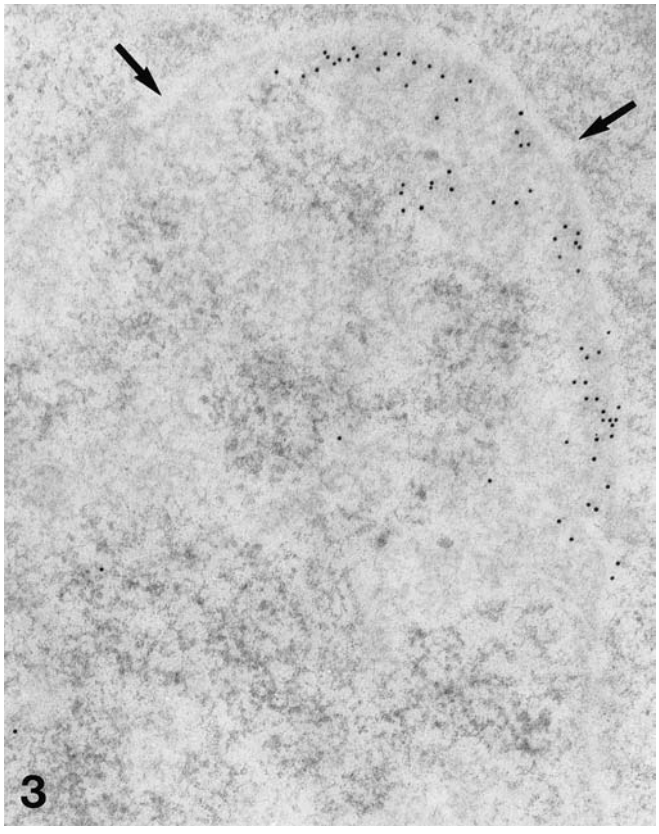

Fig. 3 Chinese hamster V79 cell labelled with BrdU for one S-phase and allowed to grow for a period of about five cell cycles. Ultrathin section stained with the EDTA method preferentially contrasting nuclear RNP constituents leaving chromatin light grey. *Arrows* indicate nuclear periphery. Gold grains label a chromatin domain probably belonging to the same interphase chromosome territory. For more details, see Visser et al. (2000). Micrograph by Françoise Jaunin. (×55,000)

limit of label, while sometimes the labelled DNA fibres were somewhat gradually interspersed with unlabelled fibres of the neighbouring domain, thus obviously favouring possible interphase interactions between chromosomes. Chromosome domains or territories are penetrated by numerous interconnecting channels as shown by reconstructions of small series of subsequent sections (Visser et al. 2000). Although this approach does not allow one to identify specific chromosomes, it proved to be a very useful alternative in analysing topological relationships between chromatin domains under conditions of excellent fine structural preservation (Fig. 3).

Perichromatin region

Thanks to the application of various ultrastructural cytochemical techniques in the investigation of functional nuclear architecture, it has become clear for more than 30 years now that the perichromatin region plays an important role in the expression of nuclear functions. Although rather difficult to be strictly defined, this nuclear structural domain is essentially determined by the border

of condensed chromatin areas where a layer of more dispersed chromatin including somewhat free adjacent chromatin fibres occurs. The definition of the perichromatin region has been facilitated by the introduction of a differential contrasting method for ultrathin sections using chelating agents, which gives rise to a high contrast of ribonucleoprotein-containing nuclear constituents, while chromatin remains weakly stained (Bernhard 1969).

Earlier studies making use of radioactive precursors of RNA or DNA and high resolution autoradiography revealed that sites of hnRNA transcription (Fakan and Bernhard 1971) and of DNA replication (Fakan and Hancock 1974) are both mainly limited to the border of condensed chromatin areas on EDTA-stained specimens of cultured cells after short incubation periods with tritiated precursors (see Fakan 1978 for extensive review of autoradiographic data). Such experiments pointed out the involvement of the perichromatin region in essential functions of the nucleus later confirmed by non-radioactive labelling approaches offering a better localisation resolution. The introduction of halogenated nucleic acid precursors visualised by means of secondary colloidal gold-coupled markers and immunoelectron microscopy, opened new possibilities in the localisation of synthetic processes in the nucleus.

DNA replication

When localising DNA replication sites, halogenated deoxyuridine is easily administered to living cells. However, the incorporation period needs to be restricted as much as possible, taking into account the rapid replication rate (1–2 μm/min; Huberman and Riggs 1968). In this way, several reports demonstrated predominant localisation of DNA synthesis in association with dispersed chromatin fibres occurring adjacent to or within the periphery of condensed chromatin areas (Sobczak-Thepot et al. 1993; Liu et al. 1995; Jaunin et al. 2000; Fig. 4). In addition, the perichromatin region also accumulated DNA polymerase α, cyclin A and PCNA (Sobczak-Thepot et al. 1993; Jaunin et al. 2000). When the kinetics of newly synthesised DNA (nascent DNA) was examined in V79 Chinese hamster cells after staining of ultrathin sections with a Feulgen-type reaction by means of osmium ammine (Cogliati and Gautier 1973) specifically visualising DNA, Jaunin et al. (2000) observed that fractions of DNA replicated within the perichromatin region were rapidly internalised into condensed chromatin domains. Moreover, such movement of DNA synthesised during a limited period of time was also obvious in pulse chase experiments using iodinated or chlorinated dU incorporated in a sequence of two incubation periods separated by unlabelled chase and differentially revealed by anti-BrdU antibodies exhibiting high affinity for one of the precursors. Together, these experiments support the conclusion that, during the synthetic phase, DNA moves rapidly between the perichromatin region where it is replicated and the interior of chromatin domains where it is internalised

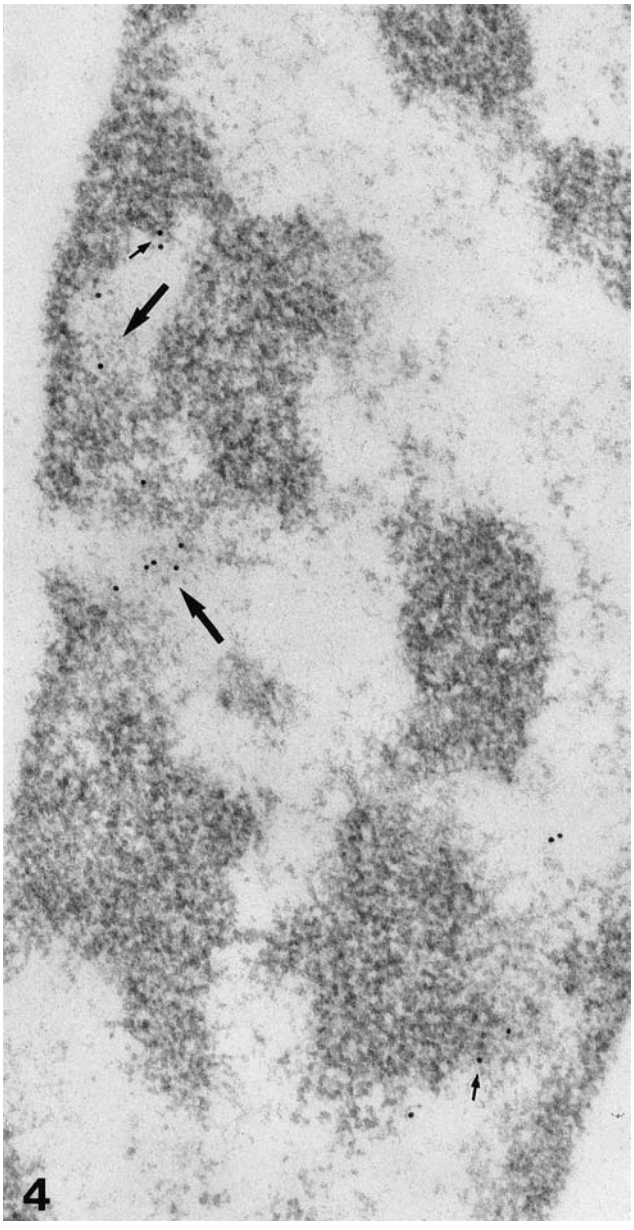

Fig. 4 Ultrathin section of a Chinese hamster V79 cell labelled with BrdU for 2 min, fixed with paraformaldehyde and embedded into Lowicryl K4 M. Osmium ammine staining specific for DNA. DNA replication sites are visualised on the periphery of condensed chromatin (*small arrows*) sometimes associated with fibres of dispersed chromatin (*large arrows*). For more details, see Jaunin et al. (2000). Micrograph by Françoise Jaunin. (×77,000)

(Jaunin et al. 2000). As to the pattern of chromatin domains during the DNA synthetic period, chromatin appears rather dispersed in numerous spots during early S-phase, while in late S-phase label occurs associated with larger condensed chromatin domains (O'Keefe et al. 1992; Leblond and el-Alfi 1996; Jaunin et al. 1998). However, the major replication pattern followed by internalisation of nascent DNA into the chromatin domains seems to be independent of S-phase period. A more detailed discussion about DNA replication topology studied

by both light and electron microscopic visualisation approaches was presented earlier (Jaunin and Fakan 2002).

Transcription sites

Similar to DNA replication analyses, earlier studies demonstrated transcription sites localised on the border of condensed chromatin areas after short pulses of different cells in culture with ^{3}H-uridine and revealing of rapidly labelled RNA by means of high resolution autoradiography (Fakan and Bernhard 1971; Fakan et al. 1976; for review see Fakan 1978; Fakan and Puvion 1980). Moreover, the application of the EDTA regressive staining (Bernhard 1969) allowed Monneron and Bernhard (1969) to describe a novel ribonucleoprotein-containing structural constituent abundant within the perichromatin region of nucleoplasm, the perichromatin fibril (PF). Later, autoradiographic investigations of hnRNA kinetics demonstrated the occurrence of rapidly labelled RNA in perichromatin fibrils (Nash et al. 1975; Fakan et al. 1976), thus confirming that they represent in situ forms of hnRNA transcripts (Fakan 1994). Furthermore, pulse chase experiments revealed that a significant fraction of labelled fibrils migrated, after their formation, into the interchromatin space (Fakan et al. 1976; Puvion and Moyne 1978).

The introduction of non-radioactive labelling methods of RNA by means of brominated precursors provided a new high resolution tool for investigating the in situ transcription features. Similar to halogenated DNA, incorporated brominated UTP can be visualised by means of anti-dU antibodies and immunocytochemistry. Original fluorescence microscopic studies of cells shortly labelled with Br-UTP (Jackson et al. 1993; Wansink et al. 1993) demonstrated the occurrence of signal either in a form of multiple small spots or as diffused fluorescence distributed throughout the nucleoplasm (Fig. 5). Immunoelectron microscopic analysis of ultrathin sections of cultured cells microinjected with Br-UTP (Cmarko et al. 1999) or incubated with Br-uridine (Trentani et al. 2003) revealed the perichromatin region as the main transcription site in the nucleoplasm containing perichromatin fibrils as rapidly labelled RNP constituents (Fig. 6). These observations, confirming earlier autoradiographic data, further showed that Br-labelled RNA resulting from short incubation periods is mostly represented by individual gold particles. Moreover, when combined with a specific staining of RNA using terbium citrate (Biggiogera and Fakan 1998), they ascertained direct association of signal with RNA fibrils (Trentani et al. 2003). Finally, immunocytochemical localisation to perichromatin fibrils of hnRNP core proteins by means of specific monoclonal (Leser et al. 1984) or polyclonal (Jones et al. 1980) antibodies (Fakan et al. 1984; Malatesta et al. 1994a; Cmarko et al. 1999) brought additional evidence about the hnRNP (or pre-mRNP) nature of this nuclear structural constituent. Perichromatin fibrils visualised in the perichromatin region are also major sites of association with pre-mRNA transcription factors, such as RNA polymerase II or TFIIH (Cmarko et al. 1999).

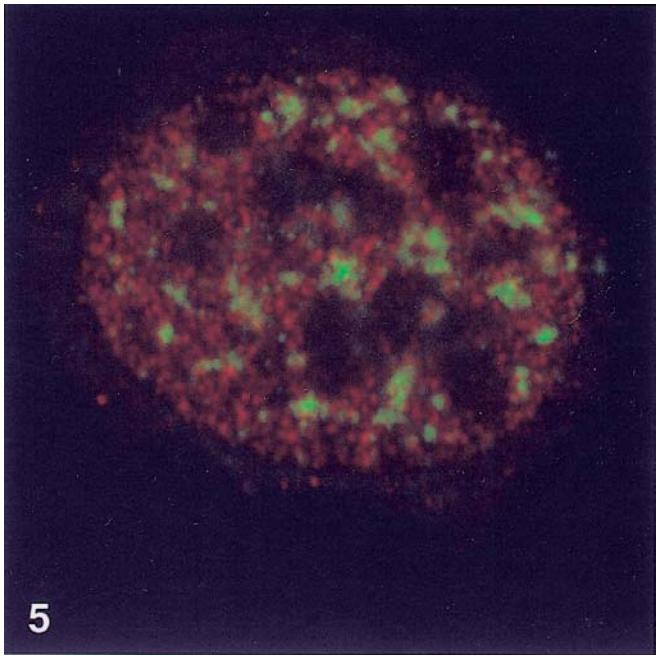

Fig. 5 Confocal optical section of a T24 cell labelled for 10 min following microinjection of Br-UTP. This figure shows a merge after a double labelling of newly synthesised RNA (*red colour*) and of a PANA protein marker of interchromatin granules clusters (*green*). It shows very little overlapping of the two labels. Fore more details, see Cmarko et al. (1999). Micrograph courtesy of Pernette Verschure (x 4,000)

Pre-mRNA processing

Perichromatin fibrils located within the perichromatin region have been demonstrated as accumulating all so far probed factors involved in or associated with pre-mRNA processing. These are namely snRNPs (Fakan et al. 1984; Puvion et al. 1984; Fig. 7), m_3G cap structure of snRNAs (Malatesta et al. 1994a; Trentani et al. 2003), SC-35 protein (Spector et al. 1991), poly(A) polymerase (Cmarko et al. 1999) and survival of motor neuron (SMN) protein (Malatesta et al. 2004). Similarly, ISH experiments using oligo-dT probes identified poly(A)-containing RNA in perichromatin fibrils (Visa et al. 1993a; Huang et al. 1994). The fact that perichromatin fibrils are the only nucleoplasmic structural constituent containing rapidly labelled RNA, as well as all transcription and processing factors, strongly support that they are also the site where most pre-mRNA processing steps take place. This is also in line with previously reported observations (see, for example, Fakan et al. 1986; Beyer and Osheim 1988; Wieslander et al. 1996) suggesting that pre-mRNA splicing occurs co-transcriptionally.

Immunoelectron microscopic assays also demonstrated the presence of hnRNP core proteins in perichromatin granules, structural constituents located almost exclu-

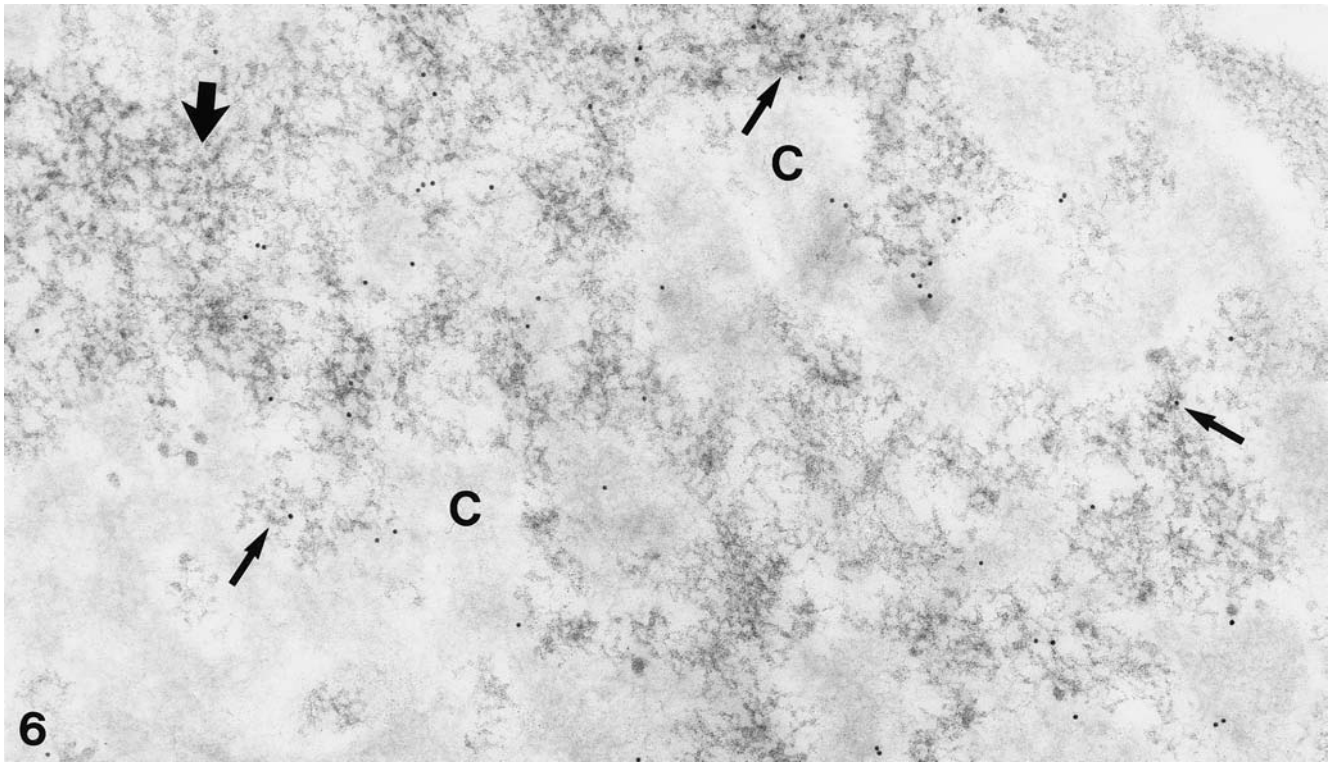

Fig. 6 Ultrathin section of an HTC cell nucleus labelled for 10 min after microinjection of Br-UTP. EDTA staining preferential for nuclear RNPs. Newly synthesised RNA is mostly associated with perichromatin fibrils (some indicated by *small arrows*) occurring on the periphery of condensed chromatin areas (*C*). Interchromatin granule cluster (*large arrow*) remains unlabelled. For more details, see Cmarko et al. (1999). Micrograph by Dusan Cmarko. (×63,000)

sively in perichromatin regions (Fakan and Puvion 1980). Similar to Balbiani ring granules of *Chironomus* polytene nuclei by their morphology and cytochemical nature (Vazquez-Nin and Bernhard 1971), as well as by RNA arrangement within the granule (Vazquez-Nin et al. 1996, 1997a), perichromatin granules have been shown as being involved in intranuclear pre-mRNA storage and transport in hormone target cells (Vazquez-Nin et al. 1997b). This is in agreement with extensively documented evidence about Balbiani ring granules as intranuclear vectors of large already processed pre-mRNA molecules (for review see Daneholt 2001), while splicing factor association was reported only within the Balbiani ring during the granule formation from the transcript (Vazquez-Nin et al. 1990). Perichromatin granules occur individually and can occasionally be observed as winding from a perichromatin fibril (Fig. 1). It is, therefore, obvious that at least some transcripts give rise to perichromatin granules which are then vehicled in the nucleus.

Interchromatin space

Two major structural domains are observed in the interchromatin space of mammalian and most eukaryotic cells: interchromatin granule clusters (IGs) and the Cajal (coiled) bodies (CBs). Cytochemical analyses demonstrated the presence of RNA in both these constituents (Monneron and Bernhard 1969).

Interchromatin granules

This remarkable nucleoplasmic domain has been shown to accumulate pre-mRNA splicing factors such as snRNPs (Spector et al. 1983; Fakan et al. 1984; Puvion et al. 1984; Fig. 7), the splicing factor SC-35 (Spector et al. 1991) and the m_3G cap of snRNAs (Malatesta et al. 1994a; Trentani et al. 2003). Autoradiographic evidence of ^{3}H-uridine incorporation into cultured cells or later of Br-UTP or Br-uridine labelling showed that IGs are virtually devoid of newly synthesised RNA (Fakan and Bernhard 1971; Fakan et al. 1976; Cmarko et al. 1999; Trentani et al. 2003) as well as of hnRNP core proteins (Fakan et al. 1984). These observations were corroborated by immunofluorescence assays (for review see Spector 2003), using different anti-splicing factor antibodies, usually giving rise to a diffuse labelling together with a number of "speckles" (see Spector 2003; Lamond and Spector 2003 for review), some of them corresponding to IGs, while the diffuse signal and some of the small speckles obviously represent labelling of PFs. The application of anti-hnRNP antibodies leads only to a diffuse labelling pattern (Martin and Okamura 1981). In situ hybridisation assays using oligo-dT probes at both light and electron microscopic

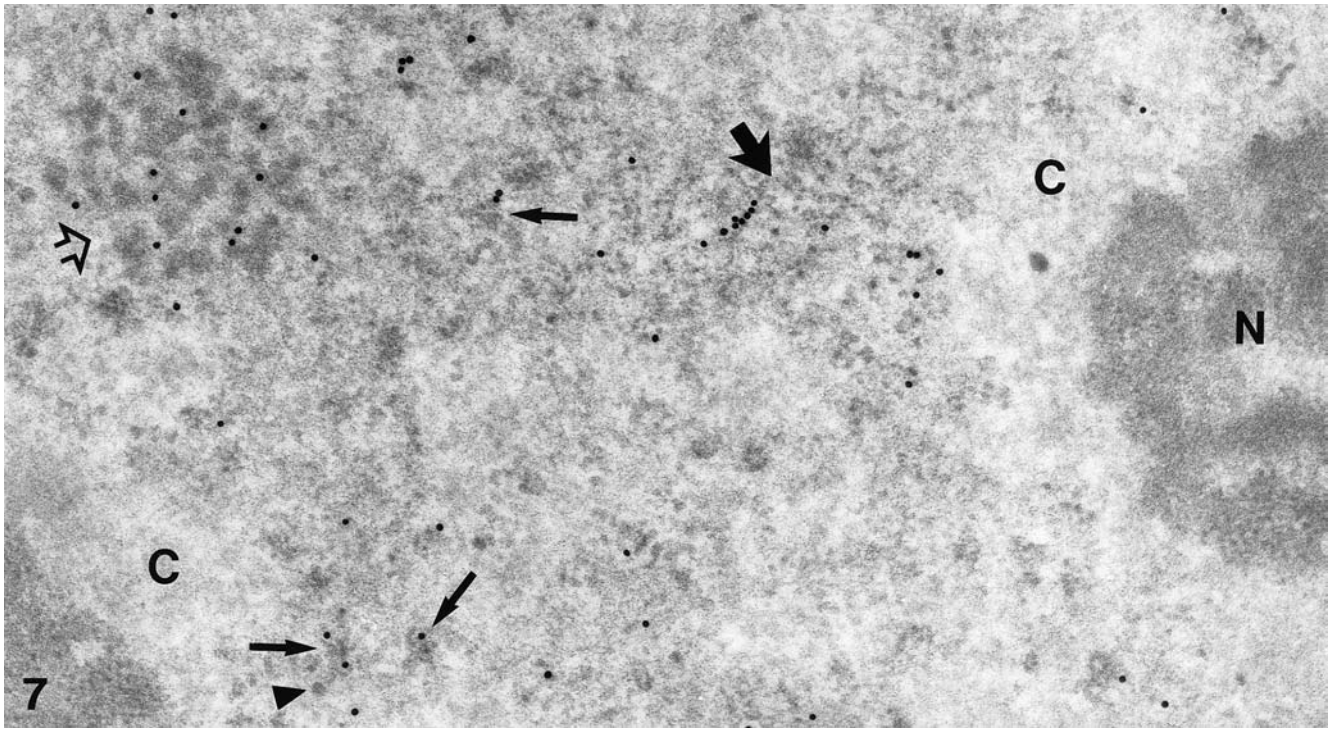

Fig. 7 Ultrathin section of a rat hepatocyte labelled with an autoimmune antibody recognising the Sm antigen of snRNPs. The signal is mainly associated with perichromatin fibrils (*small arrows*), a cluster of interchromatin granules (*large arrow*) and a Cajal (coiled) body (*open arrow*). *Arrowhead* Perichromatin granule, *C* condensed chromatin, *N* nucleolus. (×53,750)

levels show more or less significant labelling associated with IGs (speckles) (see, for example, Visa et al. 1993a; Huang et al. 1994) suggesting the occurrence of poly(A)-containing RNA within IGs. A detailed immunofluorescence microscopic quantitative digital analysis of nascent Br-RNA, SC-35 factor and poly(A) distribution in nuclei of cultured cells reported that, for all probes examined, most of the signal was diffusely spread throughout the nucleoplasm. SC-35 label was especially diffusely distributed in the nucleoplasm (70–80%), whereas only a minor portion was concentrated in IGs (speckles) (Fay et al. 1997). This also clearly argues that splicing factors are not markers of speckles as erroneously reported in a number of fluorescence microscopic investigations. However, speckles can be specifically revealed by virtue of marker proteins, such as PANA (Clevenger and Epstein 1984; Fig. 5). Together, these different findings show that IG clusters are not sites of hnRNA transcription. This is also supported by the absence of significant signal for RNA polymerase II in this nucleoplasmic domain (Cmarko et al. 1999), some previous immunofluorescence observations of RNA polymerase II in speckles being recently explained as preparative artefacts (Guillot et al. 2004). The absence of poly(A) polymerase also rules out IGs as sites of polyadenylation (Cmarko et al. 1999). Taking into account the above observations, the question arises as to the role IGs can play in pre-mRNA metabolism. Original immunoelectron microscopic data suggested that this nucleoplasmic domain could be a storage site for pre-mRNA splicing factors (Fakan et al. 1984) and strong evidence for this hypothesis came from an elegant in vivo demonstration by time-lapse microscopy of a splicing factor delivery from speckles towards sites of pre-mRNA transcription (Misteli et al. 1997). Further information about a possible involvement of IGs in transcription and pre-mRNA splicing was provided by experiments making use of the serine-arginine (SR) protein kinase cdc2-like kinase Clk/STY to manipulate the integrity of IG clusters in vivo. Immunofluorescence, as well as transmission electron microscopic analyses, revealed that overexpression of Clk/STY gives rise to dispersion of IGs within the nucleoplasm, whereas this phenomenon was not observed with an inactive mutant. Thus, it seems that hyperphosphorylation of SR proteins provokes IG dispersion. Moreover, these observations also indicated that cells lacking IG clusters continued to synthesise pre-mRNA, while accumulation of splicing factors at sites of pre-mRNA synthesis and splicing itself were strongly reduced, pointing out that the integrity of IG clusters was important for coordination of transcription and splicing (Sacco-Bubulya and Spector 2002). While the role of IGs in splicing factor storage and/or assembly of pre-spliceosome seems now well established, the nature of polyadenylated RNA occurring in IG clusters and its possible role in this nucleoplasmic domain remain enigmatic. Ultrastructural ISH assays suggested that some viral RNAs occur in IGs of HSV-1 infected cells (Puvion and Puvion-Dutilleul 1996), whereas HIV-1 intron-containing pre-mRNA sequences accumulated only on the periphery of IG clusters in nuclei of cells

transfected with a *rev*-minus construct (Cmarko et al. 2002). Fluorescence microscopic observations reported from in vivo ISH trials provided contradictory results indicating either diffused distribution of poly(A) RNA without special accumulation when using oligo(dT) (Politz et al. 1999) or rather strong accumulation of poly(A) RNA in speckles after microinjection of 2′0-methyl oligoribonucleotide probes complementary to poly(A) tail (Molenaar et al. 2004). Taking into account that pre-mRNA forms complexes with hnRNP core proteins which accompany the RNA on its nuclear pathway (Dreyfuss et al. 2002) and the fact that IGs appear to be mostly devoid of hnRNP core proteins probed so far (see above) makes it difficult to accept that pre-mRNA accumulates in IG clusters. If so, the poly(A) RNA fraction present there should be able to dissociate from its core proteins before entering the IG clusters and reassociate with them again in case of transient interaction with speckles. However, it is also possible that IG clusters represent a domain involved in RNA decay mechanism, where previously modified hnRNA–protein complexes or other types of nuclear RNA migrate. Keeping in mind that most nuclear RNA represents non-protein-coding RNAs, many of them being polyadenylated (Mattick 2003), this possibility must be taken into consideration.

A domain named IG-associated zones (IGAZ) can be observed close to IG clusters (Visa et al. 1993b). Exhibiting a finely fibrillar rather homogeneous texture, it has been shown to contain U1 but not U2 snRNA, Sm antigen of snRNPs (Visa et al. 1993b), some transcription factors, as well as moderate amounts of newly synthesised RNA after longer labelling periods (Cmarko et al. 1999) and SMN protein (Malatesta et al. 2004). Originally proposed as possible maturation sites of the U1 snRNP particle (Visa et al. 1993b), its involvement in pre-mRNA processing pathways still remains obscure.

Cajal (coiled) body

The CB is a mostly nucleoplasmic domain, of which fine structural features and cytochemical nature were examined in detail by ultrastructural methods (Monneron and Bernhard 1969). Containing both nucleoplasmic (snRNP and snRNA but not SR-rich splicing factors) (Fig. 7) and nucleolar (fibrillarin, U3 snRNA) processing components, it behaves as a dynamic structure (for review see Gall 2003). Observed either free in the nucleoplasm or associated with nucleoli (see, for example, Malatesta et al. 1994b), the CB is abundant in nuclei of various tissues of hibernating dormice (Malatesta et al. 1994a). The occurrence of the marker protein p80-coilin in CBs (Raska et al. 1991) made it possible to follow reticulated structures giving rise to CBs at the onset of hibernation, and the dispersion and disappearance of its constituents in the nucleoplasm and nucleoli during the animal arousal. In vivo fluorescence microscopic analysis of a cell line stably expressing a fusion protein of p80-coilin with GFP suggested movement of CBs throughout the nucleoplasm and between the nucleoplasm and the nucleoli (Platani et al. 2000).

Recent ultrastructural investigation analysed the structural and topological relationships between CBs and a newly described nuclear domain accumulating SMN and Gemin2 proteins and named electron-dense fibrogranular clusters (EFGCs; Malatesta et al. 2004). This obviously corresponds to the domain previously reported by fluorescence microscopy as gemini of CBs (gems; Liu and Dreyfuss 1996). EFGCs and CBs were occasionally found closely associated but usually distant from each other (Malatesta et al. 2004). These two domains seem to be involved in splicing factor storage and assembly and a recent detailed fluorescence microscopic analysis suggested that they are kinetically independent structures (Dundr et al. 2004). The elucidation of their role in nuclear functions will require further investigations.

Concluding remarks and perspectives

The different data discussed above point out essential contributions made by ultrastructural cytochemistry in analysing the functional nuclear architecture. In spite of the two-dimensional nature of EM images, various cytochemical methods adapted to transmission electron microscopic application represent a major high resolution tool for investigating structure–function relationships in the cell. Presently offering a rather large scale of preparative techniques mostly consisting in different chemical fixation approaches combined with resin embedding or cryoultramicrotomy, they also involve cryofixation and cryosubstitution procedures excluding conventional fixatives (von Schack and Fakan 1993). Contrary to modern EM cytochemical methods mainly applied on specimens embedded into different resins stabilising the cellular fine structural features, many light microscopic techniques require harsh treatments of cells, such as permeabilisation and denaturation, which can give rise to extraction or displacement of nuclear proteins or nucleic acids. This must be kept in mind, in addition to the limits of resolution, when interpreting light microscopic data. Recent developments of live cell microscopy opened a new way of investigation of cellular processes including real-time studies of inducible gene expression systems (see, for example, Janicki et al. 2004; Roix and Misteli 2002). Such observations combined with subsequent ultrastructural cytochemical analysis of the same cells offers an ideal complementary approach to the understanding of functional nuclear architecture and represents a major direction for the future.

Most of the above-mentioned ultrastructural investigations of the topology of nuclear functions, such as DNA replication, transcription and pre-mRNA processing, point out the central role played by the perichromatin region as a functional domain in the nucleus. The localisation of transcription sites reflects the location of most active genes in the perichromatin region. Evidence about the diffusion of relatively large molecules or molecular

complexes within compact nuclear compartments (Verschure et al. 2003) suggests that the organisation of the perichromatin region situated on the border of rather compact condensed chromatin areas is likely to be independent of accessibility problems. Further questions can arise as to whether silenced or momentarily inactive genes also occur within the perichromatin region. Data provided by an ultrastructural study on the intranuclear distribution of polycomb group gene silencing proteins indicate that these proteins concentrate on the border of condensed chromatin (Cmarko et al. 2003), thus suggesting that some epigenetically silenced genes are also located in this nucleoplasmic domain. Another question related to possible functions of the perichromatin region regards pre-mRNA movement in the nucleus. Assuming that a portion of pre-mRNA is packed into perichromatin granules and knowing that the granules mostly occur within perichromatin regions, one can expect that these granules are vehicled along perichromatin regions towards the nuclear pores. Whether such a movement corresponds to a kind of channelled diffusion as previously suggested (Kramer et al. 1994) remains an open question. Free diffusion has been favoured as a model for RNA movement throughout the nucleoplasm (see, for example, Singh et al. 1999; Politz and Pederson 2000), while others proposed an energy-dependent mechanism (Molenaar et al. 2004). The pathways of different types of RNA with regards to nuclear compartments remains a major issue to be studied in the future. In this context, it may be interesting to mention the association of proteins involved in cellular mobility, such as actin and myosin with transcriptionally active genes (Fomproix and Percipalle 2004; Pestic-Dragovich et al. 2000), as well as to question a possible function of intranuclear lamins (Hozak et al. 1995; Shumaker et al. 2003).

Structural domains occurring in the interchromatin space such as IG clusters or CBs are obviously involved in storage of pre-mRNA processing factors and their delivery to sites of pre-mRNA transcription and processing. We do not know yet the structural turnover of these domains. In addition, more information is needed about the formation of CBs and the relationship between those observed in the nucleolus and in the nucleoplasm.

We also lack major information about the internal organisation of the nucleus and the way in which different nuclear domains are generated. Self organisation of nuclear structure would promote both architectural stability and dynamic behaviour of its components (Misteli 2001). The contribution of macromolecular crowding effects has recently been suggested as playing a role in driving the formation and maintaining the functions of nuclear compartments (Hancock 2004). The nuclear matrix, an operational term for residual intranuclear fibrogranular, predominantly protein network was proposed as a scaffolding system in the nuclear architecture. However, no clear evidence about the existence of such a structural network in the intact cell nucleus in situ has been provided so far (for detailed discussion, see Hancock 2000; Pederson 2000).

Finally, we still lack information about relationships between nuclear architecture and regulation of gene expression, including modifications at the level of DNA or histones. Regulatory mechanisms integrated at different levels (sequence to chromatin to three-dimensional spatial organisation of the genome in the nucleus) are poorly understood in the topological context of nuclear architecture (for detailed discussion, see van Driel et al. 2003) and will certainly represent a major topic of investigation in the future.

In conclusion, ultrastructural cytochemistry continues to contribute extensively to unravelling important questions regarding functional nuclear architecture. It is essential to combine different high standard microscopic approaches in order to take up such a complex challenge.

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