Ultrastructural Analysis of Transcription and Splicing in the Cell Nucleus after Bromo-UTP Microinjection

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In this study we demonstrate, at an ultrastructural level, the in situ distribution of heterogeneous nuclear RNA transcription sites after microinjection of 5-bromo-UTP (BrUTP) into the cytoplasm of living cells and subsequent postembedding immunoelectron microscopic visualization after different labeling periods. Moreover, immunocytochemical localization of several pre-mRNA transcription and processing factors has been carried out in the same cells. This high-resolution approach allowed us to reveal perichromatin regions as the most important sites of nucleoplasmic RNA transcription and the perichromatin fibrils (PFs) as in situ forms of nascent transcripts. Furthermore, we show that transcription takes place in a rather diffuse pattern, without notable local accumulation of transcription sites. RNA polymerase II, heterogeneous nuclear ribonucleoprotein (hnRNP) core proteins, general transcription factor TFIIF, poly(A) polymerase, splicing factor SC-35, and Sm complex of small nuclear ribonucleoproteins (snRNPs) are associated with PFs. This strongly supports the idea that PFs are also sites of major pre-mRNA processing events. The absence of nascent transcripts, RNA polymerase II, poly(A) polymerase, and hnRNPs within the clusters of interchromatin granules rules out the possibility that this domain plays a role in pre-mRNA transcription and polyadenylation; however, interchromatin granule-associated zones contain RNA polymerase II, TFIIF, and Sm complex of snRNPs and, after longer periods of BrUTP incubation, also Br-labeled RNA. Their role in nuclear functions still remains enigmatic. In the nucleolus, transcription sites occur in the dense fibrillar component. Our fine structural results show that PFs represent the major nucleoplasmic structural domain involved in active pre-mRNA transcriptional and processing events.

INTRODUCTION

RNA transcription and processing take place in association with discrete subnuclear structures. The functional organization of these nuclear substructures still remains an incompletely explored area of cell biology,

despite the fact that the first studies were performed years ago (e.g., Swift, 1962; Smetana *et al.*, 1963; Monneron and Bernhard, 1969).

A number of earlier studies identified, using radioactively labeled RNA and high-resolution autoradiography, the border of condensed chromatin as the morphological substrate of transcription. Moreover, these investigations have demonstrated that perichromatin

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fibrils (PFs) are the in situ form of the nascent transcripts and further indicated a migration of a portion of PFs toward the interchromatin space while their RNA was undergoing processing (for reviews, see Fakan and Puvion, 1980; Fakan, 1986; Puvion and Moyné, 1981). Furthermore, immunocytochemical (Fakan *et al.*, 1984; Puvion *et al.*, 1984; Spector *et al.*, 1991) and in situ hybridization (Visa *et al.*, 1993b) analyses strongly support the idea that splicing and polyadenylation of pre-mRNA also occur in association with PFs (for reviews, see Spector, 1993; Fakan, 1994; van Driel *et al.*, 1995; Puvion and Puvion-Dutilleul, 1996). These observations were later confirmed by means of nonradioactive RNA-labeling methods and immunofluorescence microscopy (Jackson *et al.*, 1993; Wansink *et al.*, 1993; Fay *et al.*, 1997).

Interchromatin granules (IGs), coiled bodies, and the recently described compartment called IG-associated zone (Visa *et al.*, 1993a) are considered to be initial sites for assembly of different splicing factors into processing complexes, which then move to the nuclear domains where RNA processing takes place (for reviews, see Fakan, 1994; Puvion and Puvion-Dutilleul, 1996; Spector, 1996). This favors previous indirect evidence showing cotranscriptional association of splicing factors (Fakan *et al.*, 1986) and suggesting that splicing can be a cotranscriptional event (Beyer and Osheim, 1988) using splicing complexes probably assembled elsewhere in the nucleus (Amero *et al.*, 1992). Whether IGs can act as recycling sites for some splicing factors remains unclear (Lamond and Carmo-Fonseca, 1993; Puvion and Puvion-Dutilleul, 1996).

Recent in vivo observations that focused on pre-mRNA processing events (Misteli *et al.*, 1997) are in agreement with previous reports (e.g., Malatesta *et al.*, 1994; Tamburini *et al.*, 1996) suggesting that the interphase nucleus is a dynamic cell compartment. Moreover, it has been proposed that an interaction between mRNA and different mRNA-processing machinery factors depends on a transcriptional status of the nucleus (Huang and Spector, 1996; Misteli *et al.*, 1997; Zeng *et al.*, 1997).

To obtain new insights into the functional organization of the nucleus, we have investigated in this report nucleoplasmic RNA transcriptional events with relation to splicing and polyadenylation of pre-mRNA at the ultrastructural level. To localize the sites of transcription in situ by this high-resolution method, we microinjected 5-bromo-UTP (BrUTP) into living cells. Postembedding immunoelectron microscopy was used for visualizing both Br-labeled RNA (Br-RNA) and different factors involved in pre-mRNA transcription and processing. Moreover, we analyzed the possible colocalization of the nascent RNA with such factors within different nuclear subcompartments.

We demonstrate that PFs are the major in situ nucleoplasmic structural domains labeled after short

BrUTP pulses and that important pre-mRNA transcription and processing factors are preferentially associated with these structural constituents. Our observations support the idea that PFs are not only the in situ form of nascent transcripts but they also represent sites of pre-mRNA processing.

MATERIALS AND METHODS

Cell Culture

Human bladder carcinoma cells T24 (ATCCHB4) were grown at 37°C under a 10% CO₂ atmosphere in DMEM (Life Technologies, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim, Mannheim, Germany), 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). Cells were cultured on Alcian Blue-coated (Brink *et al.*, 1992) and microgridded Cellocate coverslips (Eppendorf, Hamburg, Germany); cells were used at 50–70% confluency.

Microinjection and Fixation

Microinjection of BrUTP (Sigma, St. Louis, MO) was performed as described previously (Wansink *et al.*, 1993, 1994a). Briefly, cells were injected into the cytoplasm of living cells with 100 mM BrUTP in 140 mM KCl and 2 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 7.4. Approximately 5% cell volume was injected. After microinjection, cells were cultured for 4–90 min at 37°C and fixed with 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer, pH 7.4, for 60 min on ice. For immunofluorescence, the microinjected cells were fixed with 4% paraformaldehyde in PBS for 10 min. To determine the exact time interval between injection and fixation, the injection time and the position of injected cells were recorded.

Immunofluorescence Labeling and Confocal Microscopy

The fixed cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS and incubated with PBS containing 100 mM glycine (Sigma) for 10 min. Subsequently, cells were incubated overnight at 4°C with a rat anti-bromodeoxyuridine (BrdU) antibody (Table 1) diluted 1:500 in PBS. Anti-BrdU antibodies were shown to recognize bromouridine with high specificity and affinity (Vanderlaan and Thomas, 1985). After washing in PBS, cells were incubated with a secondary anti-rat immunoglobulin G antibody coupled to biotin (Jackson, West Grove, PA) for 1 h at room temperature and subsequently for 30 min with either Cy3- or FITC-conjugated streptavidin (Jackson) or FITC-conjugated donkey anti-rat antibody (Jackson), diluted in PBS. For double-immunolabeling experiments, cells were incubated overnight simultaneously with anti-BrdU and a mouse monoclonal antibody against the proliferation-associated nuclear antigen (PANA) considered as a marker of IG (Clevenger and Epstein, 1984). Anti-PANA was detected with either FITC- or Cy3-conjugated donkey anti-mouse immunoglobulin M (Jackson) diluted in PBS. When anti-PANA and anti-BrdU were incubated separately, the same localization results were obtained. The specificity of each of the second antibodies was tested by omitting one of the primary antibodies. Slides were mounted in Vectashield (Vector, Burlingame, CA); they were kept at 4°C until evaluation and were observed within 24 h.

Images were recorded with a Leica confocal laser scanning microscope equipped with a 100×/1.23 NA oil immersion lens. A dual-wavelength argon ion laser was used to excite FITC and Cy3 fluorochromes simultaneously at 488 and 514 nm, respectively. Emitted fluorescence was detected using a 525 DF10 bandpass filter for FITC and a 550-nm longpass filter for Cy3. Pairs of images were collected simultaneously in the green and red channel. Three-di-

Table 1. Antibodies used for immunoelectron microscopy

Primary antibody	Dilution	Origin	Gold marker (nm)
Mouse anti-BrdU	1:50	Partec (Münster, Germany)	GAM 5, 6, 12, or 15
Rat anti-BrdU	1:50	Sera-Lab (Crawley Down, UK)	GARa 6
Chicken anti-hnRNP core protein ^a	1:10000	Jones <i>et al.</i> (1980)	GAR 15
Rabbit anti-RNA polymerase II ^a	1:2	Kim and Dahmus (1986)	GAR 15
Chicken anti-RNA polymerase II ^a	1:10000	Carroll and Stollar (1983)	GAR 15
Rabbit anti-p80-coilin	1:50–200	Andrade <i>et al.</i> (1991)	GAR 15
Rabbit anti-poly(A)-polymerase ^a _b	1:3	Martin and Keller (unpublished observations)	GAR 15
Mouse anti-SC35 splicing factor	1:50	Fu and Maniatis (1990)	GAM 15
Mouse anti-Sm complex snRNPs ^b	1:25	Lerner <i>et al.</i> (1981)	GAM 15
Mouse anti-transcription factor TFIIF ^b	1:50	Schaeffer <i>et al.</i> (1994)	GAM 15

GAM, goat anti-mouse secondary antibody conjugated with colloidal gold (Aurion, Wageningen, The Netherlands); GAR, goat anti-rabbit secondary antibody conjugated with colloidal gold (Aurion); GARa, goat anti-rat secondary antibody conjugated with colloidal gold (Aurion).

^a Antibodies used for double-labeling with mouse anti-BrdU.

^b Antibodies used for double-labeling with rat anti-BrdU.

mensional images were scanned as $512 \times 512 \times 32$ voxel images (sampling rate 49 nm lateral and 208 nm axial). Optical cross talk was quantified, and images were corrected (Manders *et al.*, 1992). Image analysis was performed using SCIL-IMAGE software (Ten Kate *et al.*, 1990; van Balen *et al.*, 1994). Images were subjected to a restoration procedure to correct for diffraction-induced distortion using a measured point-spread function (van der Voort and Straster, 1995).

Immunoelectron Microscopy

After fixation, the cells were repeatedly washed in Sörensen phosphate buffer and PBS to remove free aldehydic groups as well as nonincorporated BrUTP. The cells were then dehydrated in ethanol and embedded in LRWhite resin that was polymerized by heat. The embedded cells were separated from the gridded coverslips after short treatment with liquid nitrogen. The injected

cells were localized by the α -numeric imprint on the surface of the block and cut parallel to the substrate using a Leica Ultracut UCT ultramicrotome. The ultrathin sections were mounted on Formvar/carbon-coated nickel grids and processed, with minor changes, for postembedding immunogold labeling as described previously (Biggiogera *et al.*, 1989; Malatesta *et al.*, 1994). Antibodies used for immunoelectron microscopy are listed in Table 1. Briefly, the grids with sections were pretreated with 10% normal goat serum in PBS for 10 min and then reacted, for 17 h at 4°C, with a mixture of primary antibodies diluted in PBS containing 0.05% Tween 20 (Sigma) and 1% BSA (Fluka, Buchs, Switzerland). After washing with PBS-Tween and PBS alone, followed by a repeated treatment with normal goat serum for 10 min, the sections were reacted with a mixture of colloidal gold-conjugated secondary antibodies in PBS at room temperature for 30 min. When chicken primary antibodies were used, a rabbit anti-

Figure 1. Confocal optical section of a microinjected T24 cell showing immunocytochemical signal for Br-RNA, after 10 min incubation, and PANA marker of clusters of IGs. Large unlabeled areas correspond to nucleoli. (A) The typical diffuse distribution of sites of nascent RNA is demonstrated. (B) Several anti-PANA-labeled speckled regions correspond to clusters of IGs. (C) Merged colocalization image shows very little overlapping between anti-PANA-labeled regions (green) and Br-RNA (red). Bar, 2.0 μ m.

Table 2. Summary of immunoelectron microscopic distribution of individual antibodies

Antibody	Nuclear area	4–5 min	6–7 min	8–9 min	10–11 min	12–13 min	14–15 min	20–25 min	30–35 min	90 min
Anti-BrdU	PF	x	xx	xx	xx	xx	xx	xxx	xxx	xxx
	IG	o	o	o	o	o	x	x	x	x
	IGAZ	o	o	o	o	x	x	xxx	xxx	xxx
Anti-RNA pol II	PF	xx	xx	xx	xx	xx	xx	xx	xx	xx
	IG	o	o	o	o	o	o	o	o	o
	IGAZ	x	x	x	x	x	x	x	x	x
Anti-hnRNP	PF	xx	xx	xx	xx	xx	xx	xx	xx	—
	IG	o	o	o	o	o	o	o	o	—
	IGAZ	o	o	o	o	o	o	o	o	—
Anti-SC35	PF	x	x	x	x	x	x	x	x	—
	IG	xx	xx	xx	xx	xx	xx	xx	xx	—
	IGAZ	o	o	o	o	o	o	o	o	—
Anti-Sm complex of snRNPs	PF	xx	xx	xx	xx	xx	xx	xx	xx	—
	IG	xx	xx	xx	xx	xx	xx	xx	xx	—
	IGAZ	x	x	x	x	x	x	x	xx	—
Anti-poly(A) polymerase	PF	xx	xx	xx	xx	xx	xx	xx	xx	—
	IG	o	o	o	o	o	o	o	o	—
	IGAZ	o	o	o	o	o	o	o	o	—
Anti-TFIID	PF	xx	xx	xx	xx	xx	xx	xx	xx	—
	IG	x	x	x	x	x	x	x	x	—
	IGAZ	x	x	x	x	x	x	x	x	—

o, x, xx, xxx, Estimation of intensity of labeling; PF, perichromatin fibrils; IG, interchromatin granules; IGAZ, IG-associated zones; —, not done.

chicken probe (EY Labs, San Mateo, CA), diluted 1:100 in PBS/Tween/BSA, was used as a bridge before gold-complex labeling. Finally, all grids were thoroughly rinsed with PBS and ultrapure water and air-dried. The preparations were stained by the regressive technique, which is preferential for nuclear ribonucleoproteins (Bernhard, 1969): 4.7% aqueous uranyl acetate for 45 sec, 0.02 M EDTA for 3 min, and lead citrate for 45 sec.

As controls, noninjected cells were processed as above. Moreover, the injected cells treated without primary antibodies or without rabbit anti-chicken bridge probe were used. To confirm the RNA nature of Br-labeling, some sections were also submitted to RNA digestion with 0.2% ribonuclease (type IA, Sigma) in 1 mM triethanolamine-acetic acid buffer, pH 7.3, for 18 h at 37°C.

The grids were examined with a Philips (Eindhoven, The Netherlands) CM 100 electron microscope at 80 kV using a 30–40 μ m objective aperture.

Evaluation of the Gold Grain Density

For semiquantitative evaluation, the estimation refers to the minimal and maximal presence of colloidal gold granules found on subnuclear compartments. Local density of gold grains was estimated separately for each antibody. The evaluation of the specific labeling was as follows: zero = very weak or no signal; single symbol = intensity of signal was moderate, but evidently above background level; double symbol = intensity of gold labeling is between minimal and maximal level; triple symbol = maximal labeling intensity. A similar method was also used for the estimation of colocalization between two antibodies reflected by codistribution of different size colloidal gold particles. Background staining was evaluated as a binding of antibodies to the resin over regions free of cells.

RESULTS

The results of this study show that microinjection of BrUTP into living cells is a very useful tool for *in vivo*

labeling of RNA and its subsequent *in situ* localization by electron microscopical methods. The ability to reveal the incorporated precursor directly on ultrathin resin sections allows one to avoid permeabilization of cells and therefore prevents deleterious effects of detergents and makes possible a more satisfactory level of preservation of ultrastructural details. In addition, sectioning makes accessible antigenic sites in compact nuclear domains and thus prevents antibody penetration problems encountered in fluorescence or preembedding immunocytochemistry. The use of gridded coverslips allowed us to visualize, with good precision, the individual microinjected cells. In the ultrathin sections of cells processed *in situ*, the fine structural morphology of the cell nucleus was well preserved so that all the nuclear constituents could be identified easily.

Figure 1 shows a confocal optical section of a cell nucleus visualizing BrUTP-labeled RNA after 10 min incubation and the distribution of anti-PANA labeling. Anti-Br-RNA signal exhibits the typical distribution pattern demonstrating diffuse labeling with a number of small spot-like areas, which represents transcriptionally active regions on the periphery of condensed chromatin clumps (Figure 1A). The nucleoli (large dark areas) remain virtually unlabeled because of an insufficient antibody penetration (Wansink *et al.*, 1993). An immunofluorescence assay with the anti-PANA probe recognizing a marker protein of IG (Clevenger and Epstein, 1984) reveals several speckled

Table 3. Summary of immunoelectron microscopic colocalization of Br-RNA and of transcription and processing factors

Nuclear area	Antibody	4-5 min	6-7 min	8-9 min	10-11 min	12-13 min	14-15 min	20-25 min	30-35 min	90 min
PF	Anti-pol II	xxx	xxx	xxx	xx	xx	xx	xx	xx	xx
	Anti-hnRNP	x	x	xx	xx	xx	xx	xxx	xxx	—
	Anti-SC35	x	xx	xx	xx	xx	xx	xx	xx	—
	Anti-Sm complex	x	xx	xx	xx	xx	xx	xx	xxx	—
	Anti-poly(A)pol	x	x	x	x	x	x	x	x	—
	Anti-TFIIH	xx	xx	xx	xx	xx	xx	xx	xx	—
IG	Anti-pol II	o	o	o	o	o	o	o	o	—
	Anti-hnRNP	o	o	o	o	o	o	o	o	—
	Anti-SC35	o	o	o	o	o	x	x	xx	—
	Anti-Sm complex	o	o	o	o	o	x	x	xx	—
	Anti-poly(A)pol	o	o	o	o	o	o	o	o	—
	Anti-TFIIH	o	o	o	o	o	x	xx	xx	—
IGAZ	Anti-pol II	o	o	o	o	x	x	x	x	—
	Anti-hnRNP	o	o	o	o	o	o	o	o	—
	Anti-SC35	o	o	o	o	o	o	o	o	—
	Anti-Sm complex	o	o	o	o	x	x	x	xx	—
	Anti-poly(A)pol	o	o	o	o	o	o	o	o	—
	Anti-TFIIH	o	o	o	o	x	x	x	x	—

o, x, xx, xxx, Estimation of intensity of colocalization with anti-BrdU antibodies; PF, perichromatin fibrils; IG, interchromatin granules; IGAZ, IG-associated zones; —, not done.

regions corresponding to IG clusters (Figure 1B). When incubation with the anti-PANA antibody is carried out in a double-labeling experiment, the PANA-positive speckles occur in nucleoplasmic areas that do not exhibit significant anti-BrUTP labeling (Figure 1C).

Tables 2 and 3 summarize results of ultrastructural immunolabeling of Br-RNA and of the distribution of antibodies recognizing different transcription and processing factors and the estimation of the extent of their colocalization, respectively.

The anti-BrdU antibodies used in our experiments showed the strongest signal of several probes of different commercial origins; the antibody from Partec exhibits especially high affinity for Br-RNA. As to the specificity of anti-RNA polymerase II antibodies used in our experiments, the rabbit affinity-purified antibody (Kim and Dahmus, 1986) reacts predominantly with the phosphorylated C-terminal domain of the RNA polymerase II α subunit (Cadena and Dahmus, 1987), whereas the chicken antibody (Carroll and Stollar, 1983) obviously recognizes several forms of the enzyme.

The distribution pattern of Br-RNA and of the different factors involved in pre-mRNA formation investigated in the present work were evaluated with regard to different nucleoplasmic domains previously reported as being involved in pre-mRNA metabolism. In the tables describing the degree of labeling in such nucleoplasmic domains, we analyzed PF as the major RNP constituent of the perichromatin region, whereas IG and IGAZ-associated zones obviously represent the most important and remarkable domains in the interchromatin nucleoplasmic space.

Perichromatin Region

Intensive labeling of newly synthesized RNA, mainly occurring on the periphery of condensed chromatin areas where PFs were the major labeled nucleoplasmic constituents, was observed 20–35 min after BrUTP microinjection (Figures 2 and 3); however, evident signal was already identified at much shorter periods, from 4 min onward, after microinjection of cells. In this case, mainly individual gold particles were observed associated predominantly with PFs (Figures 4 and 5). No striking local accumulation of gold particles was observed, regardless of the duration of the labeling period. The labeling intensity within the perichromatin regions was increasing with the increasing labeling time. Some aggregation of labeled PFs that had accumulated within these regions was observed after prolonged incubation periods (20–90 min); however, the general distribution pattern of Br-RNA within this region remained comparable for all labeling periods. Anti-RNA polymerase II antibodies colocalized frequently with Br-RNA, especially after shorter incubation periods (Figure 6). This frequency of colocalization, however, decreased with the increasing duration of BrUTP labeling.

The other specific probes colocalized to different extents during all BrUTP incubation periods (Figures 7 and 8 and Table 3). With increasing BrU incubation time, the frequency of BrU colocalization with anti-poly(A) polymerase and with anti-TFIIH remained equal while the colocalization with anti-hnRNP and anti-SC35 was increasing.

Interchromatin Region

We have not observed major variations in the overall BrUTP labeling of the interchromatin space, which remained relatively low at all incubation periods. After longer BrUTP labeling (12 min onward), the signal was associated with recently described IG-associated zones (Visa *et al.*, 1993a). A rather frequent colocalization with anti-RNA polymerase II was found here (Figure 9). In these areas, TFIIF and Sm complex of snRNPs were also accumulated and colocalized to some extent with anti-BrdU (Figure 10); however, anti-p80-coilin antibody was not associated with such zones (our unpublished observation).

Anti-BrdU antibody recognized some BrU label that was also in association with clusters of IG, but only after longer incubation times (14–90 min); however, the centers of these clusters remained devoid of label (Figures 7 and 11). Incorporated BrUTP was partly colocalized with SC-35 and Sm complex of snRNPs (Figure 10). No significant signal for anti-RNA polymerase II (Figure 11) and antipoly(A) polymerase was revealed in IG clusters. A low signal for TFIIF was identified in both IG clusters and IG-associated zones.

Nucleolus

The nucleolus exhibited detectable labeling after all BrUTP incubation periods. Although short pulses gave rise to signal limited to the dense fibrillar component (Figure 2), the granular component became labeled only after prolonged incubation (90 min) with the precursor (our unpublished results). No detectable signal was observed in fibrillar centers, regardless of the labeling period duration.

Cytoplasm

After paraformaldehyde fixation, ultrastructural morphology of the cytoplasmic constituents is not very well preserved. Nevertheless, the occurrence of brominated RNA in the cytoplasm, mainly after 90 min incubation, was detected repeatedly. During such incubation periods, label was also observed close to some nuclear pores. No detectable signal was identified after short labeling pulses, showing at the same time that the intracellular pool of unincorporated BrUTP was removed during washing of cells and subsequent dehydration.

Controls

The results of control experiments, in which the primary or the bridge antibody was omitted, were negative. Also the background on the resin areas outside the cells was negligible. Moreover, noninjected cells observed in the sections were devoid of label.

Ribonuclease digestion of sections labeled with brominated precursor leads to a complete removal of labeled RNA and gives rise to a signal corresponding to background level. This assay therefore confirms that the observed labeling represents RNA. The overall contrast of RNA-containing nuclear constituents, in particular of PFs usually visualized after the EDTA staining, was also reduced after ribonuclease treatment (our unpublished observations).

DISCUSSION

Our immunoelectron microscopic observations of cells microinjected with BrUTP reveal the following points. 1) During all different incubation periods with BrUTP (4–90 min), PFs occurring mainly on the periphery of condensed chromatin regions represent the major labeled nucleoplasmic ribonucleoprotein constituents. All transcription or processing factors, which we probed for, colocalized to different extents on PFs. 2) After longer BrUTP incubation periods, interchromatin granule clusters become occasionally labeled only on their periphery, whereas they remain unlabeled after short pulses. They do not contain detectable amounts of hnRNP core proteins, RNA polymerase II, and poly(A) polymerase, but they accumulate splicing factors. 3) Interchromatin granule-associated zones contain significant amounts of Br-RNA after longer labeling periods. They also accumulate RNA polymerase II and transcription factor TFIIF as well as the Sm complex of snRNPs. 4) After short incubation periods, BrUTP is incorporated only into the dense fibrillar component of the nucleoli, whereas after 90 min the signal also occurs in the granular component. 5) After 90 min incubation with the brominated precursor, label can be observed in the proximity of nuclear pores as well as in the cytoplasm.

To avoid deleterious effects of cell permeabilization on nuclear fine structure, as well as a possible protein extraction and/or displacement attributable to detergent treatment, we have microinjected BrUTP into the cytoplasm of living cells. Visualization of the incorpo-

Figure 2 (facing page). Immunoelectron microscopic localization of BrU (mouse antibody) in BrUTP-microinjected T24 cells after 20-min incubation. Gold marker labels the perichromatin region in the nucleoplasm and the dense fibrillar component in the nucleolus (N). Condensed chromatin areas (C) and the interchromatin region (contrasted areas) are mostly devoid of label. Bar, 1.0 μ m.

Figure 3 (facing page). Thirty minutes after microinjection of BrUTP, two regions located on condensed chromatin periphery, looking like transcriptional complexes with multiple transcripts, exhibit strongly anti-BrdU-labeled (mouse anti-BrdU antibody) PFs (arrows). Bar, 0.2 μ m.

Figure 4 (facing page). A high-power micrograph shows, after pulse of 7 min 40 sec, anti-BrdU label (mouse anti-BrdU antibody) on PFs arranged in an array resembling a transcription complex. Bar, 0.1 μ m.

Figures 2, 3, and 4.

Figure 5. Localization of brominated RNA (mouse anti-BrdU antibody) after incubation of 4 min 20 sec. Mostly individual 5-nm gold particles are localized on PFs (arrows). Bar, 0.2 μ m.

Figure 6. Double-labeling with mouse anti-BrdU antibody (small grains, 6 nm) and chicken anti-RNA polymerase II antibody (large grains, 15 nm) after incubation of 7 min 20 sec. Colocalization is visible on PFs (arrows). Bar, 0.2 μ m.

Figure 7. Mouse anti-BrdU antibody (6-nm grains) and hnRNP (15-nm grains) colocalization on PFs after 8-min incubation. Note unlabeled IGs (arrow). Bar, 0.2 μ m.

Figure 8. Colocalization of Br-RNA (mouse anti-BrdU antibody; 6-nm particles) and poly(A)-polymerase (15-nm particles) on PFs after 5 min incubation. Bar, 0.2 μ m.

Figure 9. Colocalization of brominated RNA (mouse anti-BrdU antibody; 6-nm grains) and RNA polymerase II (chicken anti-pol II antibody; 15-nm grains) in the IG-associated zone (asterisk) after 30-min incubation. Bar, 0.2 μ m.

rated brominated nucleotide by means of postembedding immunoelectron microscopy on sections of cells cut in situ allowed us to follow, at high resolution, sites of transcription and the association of newly synthesized RNA with nuclear structural constituents. Moreover, the use of gridded coverslips as cell supports allows for identification of individual cells and recording of a precise labeling period after microinjection of RNA precursor. This labeling system was previously used in fluorescence digital imaging analyses of the intranuclear spatial distribution of newly synthesized RNA (Wansink *et al.*, 1993; Fay *et al.*, 1997). In our laboratories, we have not been able to obtain detectable labeling, at either light or electron microscopic level, using direct incorporation of Br-uridine by intact cells in culture. Because previous studies of Br-labeled RNA processing indicated alteration of RNA processing events attributable to the presence of bromine atoms within the pre-mRNA molecule (Wansink *et al.*, 1994b; Fay *et al.*, 1997), our analyses were

mainly concentrated on the investigation of transcription sites and on topological relationships between localization of newly synthesized RNA and constituents of nuclear architecture.

The results of our experiments confirm earlier ultrastructural observations carried out by means of high-resolution autoradiography of ^3H -uridine in vivo labeled cells (e.g., Bachellerie *et al.*, 1975; Fakan *et al.*, 1976) and clearly identify PF as the in situ forms of nascent transcripts. It is interesting to note that after short incubation periods following BrUTP microinjection (Figure 5), label associated with PFs containing Br-RNA is mostly represented by individual gold grains, thus suggesting that transcription takes place in individual transcription sites rather than within "transcription factories" (Iborra *et al.*, 1996; Jackson *et al.*, 1998). Moreover, similar to autoradiographic observations (for review, see Fakan, 1978), our present findings show that most transcription sites occur on the periphery of condensed chromatin areas.

Figure 10. Double-labeling with rat anti-BrdU antibody (6-nm particles) and mouse anti-Sm antibody (15-nm particles) after pulse of 8 min 40 sec. Sm complex occurs in both the IGs and the IG-associated zone (asterisk), whereas Br-RNA is not detected in these areas. Bar, 0.2 μ m.

Figure 11. BrUTP (mouse anti-BrdU antibody; 6-nm grains) and RNA polymerase II (rabbit anti-pol II antibody; 15-nm grains) double-labeling after 90-min incubation. Even after a prolonged incubation time, the center of the IG cluster remains devoid of label. Some BrU signal can be observed on the periphery of the cluster, whereas no RNA polymerase II is associated with the IG cluster. Bar, 0.2 μ m.

In agreement with previous reports, antibodies recognizing all transcription and splicing factors probed so far associate with PFs (e.g., Fakan *et al.*, 1984; Pu-vion *et al.*, 1984; Spector *et al.*, 1991; Spector *et al.*, 1993). A high degree of colocalization between Br-RNA 10 min after BrUTP microinjection and RNA polymerase II was reported using confocal immunofluorescence microscopy (Grande *et al.*, 1997), coinciding with our observations.

The decreasing colocalization of Br-RNA with RNA polymerase II with increasing BrUTP-labeling time further suggests that a portion of labeled RNA is separated from the polymerase complex after longer labeling periods. After longer BrUTP incubation periods, some label occurs on the periphery of clusters of interchromatin granules, in agreement with previous autoradiographic observations (e.g., Fakan and Bernhard, 1973), whereas virtually no label is identified after short labeling pulses. The data therefore strongly support the conclusion that significant transcription does not take place on the IG clusters and does not

favor the idea that these nuclear domains represent "transcript domains" in the nucleus (Carter *et al.*, 1993). Similarly, no accumulation of transcription sites on the periphery of IG clusters, suggesting a concentration of active genes within this region (Moen *et al.*, 1995), was noticed. With regard to RNA polymerase II distribution in the nucleoplasm, our findings demonstrate most anti-RNA polymerase II labeling associated with PFs within perichromatin regions, whereas only a very weak signal is revealed in interchromatin granule clusters. This is in agreement with immunofluorescence microscopic observation showing that in cells exhibiting abundant BrUTP incorporation, Br-RNA, the large RNA polymerase II subunit, and splicing and polyadenylation factors are located mostly within the nucleoplasm and do not concentrate in speckles (Zeng *et al.*, 1997).

The high-resolution observations of the present work are in agreement with findings of fluorescent microscopic experiments indicating a large number of BrUTP signal sites diffusely distributed in the nucleo-

plasm of various types of cells (Jackson *et al.*, 1993; Wansink *et al.*, 1993; Fay *et al.*, 1997). Moreover, an elegant fluorescence microscopic quantitative digital analysis of nascent Br-RNA, SC-35, and poly(A) distribution demonstrated that for all probes examined, most of the signal was diffusely distributed in the nucleoplasm (Fay *et al.*, 1997).

Anti-poly(A) polymerase labeling observed in our specimens occurred rather frequently on PFs, although it moderately colocalized with newly synthesized RNA; however, no signal was observed on IG clusters, contrary to reports for poly(A)⁺ RNA (Visa *et al.*, 1993b) and poly(A) binding protein II (Krause *et al.*, 1994), which both were partly localized in this domain, in addition to a frequent occurrence on PFs. The absence of the polyadenylation enzyme in IG aggregates further indicates that polyadenylation of pre-mRNA does not take place within this nucleoplasmic domain. Moreover, in the absence of significant amounts of hnRNP core proteins within IG clusters, the nature of poly(A)⁺ RNA previously detected in this nucleoplasmic constituent remains to be elucidated.

IG-associated zones, a novel nucleoplasmic domain recently described in HeLa cells (Visa *et al.*, 1993a), were also frequently observed in T24 cells. Originally reported as containing U1 but not U2 snRNP (Visa *et al.*, 1993a), this domain begins to be labeled 10–15 min after BrUTP microinjection and is strongly labeled later. Although we also found some RNA polymerase II, Sm complex of snRNP, and TFIIH in the IG-associated zones, we have not been able to identify p80-coilin within this domain (Puvion-Dutilleul *et al.*, 1995), although coiled bodies were labeled with anti-p80-coilin antibodies. Because IG-associated zones do not contain poly(A) polymerase, they do not seem to correspond to dots containing this enzyme and other pre-mRNA 3' processing factors observed close to the periphery of "speckles" by laser confocal microscopy (Schul *et al.*, 1998). The functional significance of this nucleoplasmic compartment, which has not been shown so far as a ubiquitous nuclear domain, remains to be elucidated.

Previous analysis of in vitro splicing of an adenovirus major late II pre-mRNA construct labeled with BrUTP has shown that splicing is strongly inhibited if all uridines were substituted for BrU, whereas it was restored to some extent at 50% substitution ratio and returned to an almost normal level when every tenth uridine was substituted (Wansink *et al.*, 1994b). Although we cannot estimate the percentage of BrU substitution in our experiments, the fact that some label, especially after long periods after microinjection, is found in the vicinity of nuclear pores and in the cytoplasm is in favor of some migration of Br-RNA. Whether the molecules reaching the cytoplasm have been properly processed remains unclear; however,

our observations show at the same time that there is much less intranuclear migration and export to the cytoplasm of Br-RNA compared with ³H-uridine-labeled RNA in cultured cells (e.g., Fakan and Bernhard, 1971; Fakan and Nobis, 1978). Whether accumulation of some Br-labeled PFs in small aggregates observed within perichromatin regions after longer incubation periods can account for "transcription factories" reported after comparable labeling times with Br-RNA precursors (e.g., Iborra *et al.*, 1996; Jackson *et al.*, 1998) remains to be elucidated.

As for the distribution of label in the nucleolus, it is interesting to note that during 4–30 min post-injection periods, the signal is observed exclusively on the dense fibrillar component and reflects pre-rRNA transcription sites. This confirms some previously reported data (e.g., Granboulan and Granboulan, 1965; Fakan and Bernhard, 1971; Hozak, 1995). After a 90 min incubation, Br-RNA occurs also on the nucleolar granular component. Even if this RNA represents correctly processed molecules, its translocation from the fibrillar component is considerably slower than after radioactive labeling (Granboulan and Granboulan, 1965).

Together, these observations suggest that some RNA molecules might, with a considerable delay, follow their intracellular pathways, although the number of such molecules would be much lower than that observed for tritium-labeled RNA.

In conclusion, our high-resolution observations demonstrate perichromatin regions as being the major sites of nucleoplasmic RNA transcription. PFs, which represent the in situ forms of nascent transcripts, are also the nucleoplasmic structural constituents that bring together all transcription and processing factors analyzed so far. These in situ findings therefore support a previous suggestion that splicing could take place cotranscriptionally on PFs. Furthermore, most transcripts appear more or less individually without any striking accumulation of label even after longer incorporation periods. Finally, no accumulation of transcripts occurs within the regions of IG aggregates. IG clusters contain limited amounts of rather slowly labeled RNA. Further studies that are now in progress aim at defining labeling conditions allowing for high-resolution analyses of RNA kinetics and intranuclear trafficking under conditions of unaltered pre-mRNA processing.

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