

Fluorescent Labeling of Nascent RNA Reveals Transcription by RNA Polymerase II in Domains Scattered Throughout the Nucleus

Derick G. Wansink, Wouter Schul, Ineke van der Kraan, Bas van Steensel, Roel van Driel, and Luitzen de Jong

E. C. Slater Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands

Abstract. Several nuclear activities and components are concentrated in discrete nuclear compartments. To understand the functional significance of nuclear compartmentalization, knowledge on the spatial distribution of transcriptionally active chromatin is essential. We have examined the distribution of sites of transcription by RNA polymerase II (RPII) by labeling nascent RNA with 5-bromouridine 5'-triphosphate, *in vitro* and *in vivo*. Nascent RPII transcripts were found in over 100 defined areas, scattered throughout the nucleoplasm. No preferential localization was observed in either the nuclear interior or the periphery. Each transcription site may represent the activity of a single gene or, considering the number of active pre-mRNA genes in a cell, of a cluster of active genes. The relation between the distribution of nascent RPII transcripts and that of the essential splicing factor SC-35

was investigated in double labeling experiments. Antibodies against SC-35 recognize a number of well-defined, intensely labeled nuclear domains, in addition to labeling of more diffuse areas between these domains (Spector, D. L., X. -D. Fu, and T. Maniatis. 1991. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3467-3481). We observe no correlation between intensely labeled SC-35 domains and sites of pre-mRNA synthesis. However, many sites of RPII synthesis colocalize with weakly stained areas. This implies that cotranscriptional splicing takes place in these weakly stained areas. These areas may also be sites where splicing is completed posttranscriptionally. Intensely labeled SC-35 domains may function as sites for assembly, storage, or regeneration of splicing components, or as compartments for degradation of introns.

THE cell nucleus comprises all factors required for faithful replication of the genome and regulated synthesis, processing and transport of RNA. In recent years much information on nuclear organization has become available. It is clear now that the nucleus is highly organized (reviewed by de Jong et al., 1990; Jackson, 1991; van Driel et al., 1991). The most conspicuous subnuclear domain is the nucleolus in which ribosomal genes from different chromosomes are clustered and ribosome assembly takes place (reviewed by Scheer and Benavente, 1990; Hernandez-Verdun, 1991). Other examples of a domainlike organization in the nucleus are: replication clusters during S-phase (reviewed by Berezney, 1991), clustered splicing components (Spector, 1990; Fu and Maniatis, 1990; Carmo-Fonseca et al., 1992), hnRNP proteins (Piñol-Roma et al., 1989; Ghetti et al., 1992), and tracks and foci of specific RNAs (Lawrence and Singer, 1991; Huang and Spector, 1991). In addition, a number of structures have been visualized of which the function is still unknown (Ascoli and Maul, 1991; Saunders et al., 1991; Raška et al., 1991; Stuurman et

al., 1992). The structural basis of the occurrence of nuclear activities in domains and the functional significance of this organizing principle for the regulation of gene expression and DNA replication are not understood.

Essential for understanding nuclear organization is how the sites of transcription are spatially related to nuclear domains involved in RNA processing and replication. Three DNA-dependent RNA polymerases (RPs)¹ are responsible for nuclear transcription (Sentenac, 1985; Bautz and Petersen, 1989). RPI transcribes rRNA genes in the nucleolus. RPII is located in the nucleoplasm, synthesizing pre-mRNA and most snRNAs. RPIII is also located in the nucleoplasm and synthesizes small RNAs, like 5S rRNA, U6 snRNA and tRNAs. RPII transcription, pre-mRNA splicing and RNA transport appear to be closely linked activities (Chang and Sharp, 1989; Lawrence et al., 1989; Beyer and Osheim, 1991). Regulation at any of these processes may control the rate at which a specific transcript will be exported as a mature mRNA to the cytoplasm. Knowledge on the relationship between sites of RPII transcription and compartments en-

Address correspondence to Derick G. Wansink, E. C. Slater Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands.

1. *Abbreviations used in this paper:* BrUTP, 5-bromouridine 5'-triphosphate; Br(d)U, bromo(deoxy)uridine; RPI-II-III, RNA polymerase I-II-III; snRNP, small nuclear ribonucleoprotein particle.

gaged in RNA processing will lead to a better understanding of nuclear organization and regulation of gene expression.

Strikingly, the spatial distribution of transcriptionally active chromatin in the interphase nucleus is still unclear. Indirect immunofluorescence with antibodies against RPII (Bona et al., 1981), visualization of nuclease-sensitive sites (Hutchison and Weintraub, 1985; de Graaf et al., 1990), and electron microscopy combined with autoradiography on [³H]uridine-labeled cells (reviewed by Fakan, 1986) have not resulted in an unambiguous picture of the distribution of RPII transcription. In addition, localization of specific genes and transcripts by *in situ* hybridization has not resulted in conclusions with regard to the spatial organization of active genes (reviewed by Lawrence and Singer, 1991).

We have studied the spatial distribution of RPII transcription sites in the interphase nucleus by visualizing nascent (*i.e.*, attached to engaged RPII) RNA *in vitro* and *in vivo*. Our technique is based on the incorporation of the UTP-analogue-5-bromouridine 5'-triphosphate (BrUTP) into nascent RNA. Incorporated BrUTP is visualized by indirect immunofluorescence microscopy. A discrete, punctated labeling is observed, which strongly suggests that RPII transcription takes place in numerous small domains dispersed throughout the nucleus. We compare this labeling pattern with the distribution of the essential splicing component SC-35 (Spector et al., 1991).

Materials and Methods

Cell Culture

T24 (human bladder carcinoma) cells were grown at 37°C under a 10% CO₂ atmosphere in DME (Gibco, Paisly, UK) supplemented with 10% (vol/vol) heat-inactivated FCS (Boehringer, Mannheim, Germany), 2 mM L-glutamine (Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco). Human skin fibroblasts (Heng89AD or 85AD5035F) were grown under a 5% CO₂ atmosphere in a 1:1-mixture of Ham's F-10 medium (Gibco) and DME, and contained the supplements described above.

BrUTP Incorporation in Permeabilized Cells (Run-on Transcription)

Cells in Suspension. T24 cells (~50% confluent) were collected by trypsinization and resuspended in TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂) containing 0.5% BSA (Sigma Chemical Co., St. Louis, MO). Cells were washed with TBS and glycerol buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 25% glycerol, 0.5 mM PMSF, 0.5 mM EGTA), respectively. Then the cells were permeabilized in glycerol buffer containing 0.05% Triton X-100 (Sigma Chemical Co.) for 3 min at room temperature (~2 × 10⁷ cells/ml). The permeabilized cells were washed once. Run-on transcription was performed in transcription buffer (100 mM KCl, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM EGTA, 25% glycerol, 25 µM S-adenosyl-L-methionine (Boehringer), 5 U/ml RNase inhibitor from human placenta (Boehringer), 1 mM PMSF, 0.5 mM ATP, 0.5 mM CTP, 40 µM [³H]GTP (500 dpm/pmol; NEN DuPont, 's Hertogenbosch, The Netherlands) and 0.2 mM BrUTP (Sigma Chemical Co.) or UTP; ~3 × 10⁶ permeabilized cells/100 µl of assay) at room temperature. The reaction was stopped by adding TCA to a final concentration of 5%, and 1.6 mg/ml BSA as carrier protein, and was kept on ice for 30 min. Precipitates were recovered by centrifugation and washed six times with 5% TCA. Finally, the precipitates were solubilized in Solvable (DuPont De Nemours, Bad Homburg, Germany) according to the manufacturer's instructions and counted in a LKB 1214 Rackbeta liquid scintillation counter. α -Amanitin (Sigma Chemical Co.) was included in some run-on transcription assays to discriminate between the different RPs (Roeder, 1976) as indicated in the text.

Cells on Coverslips. Cells were transferred onto gelatine-coated glass coverslips and allowed to grow for 40–45 h. Subsequently, the coverslips

were washed once with TBS and once with glycerol buffer. Then the cells were permeabilized with glycerol buffer containing 0.05% Triton X-100 for 3 min at room temperature. Identical results were obtained when 1- α -lysolecithin or digitonin were used as permeabilizing agents (data not shown). The detergent-containing buffer was removed and transcription buffer containing 0.5 mM of ATP, CTP, GTP and 0.2 mM BrUTP was added. In control experiments α -amanitin or actinomycin D was included. Run-on transcription was performed at room temperature for 10–30 min. Then the coverslips were washed once with TBS containing 0.5% Triton X-100 and 5 units/ml RNase inhibitor for 3 min, and once with TBS containing 5 U/ml RNase inhibitor. Cells were fixed immediately afterwards.

Fixation and Immunofluorescence Microscopy

Cells were fixed in either 2% (wt/vol) formaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), or in a mixture of 2% formaldehyde and 0.01% glutaraldehyde (grade 1, specially purified 25% aqueous solution; Sigma Chemical Co.) in PBS for 15 min at room temperature. More cells were retained on the coverslips when 0.01% glutaraldehyde was present while no effect on the labeling pattern was observed. The formaldehyde solution was freshly prepared from paraformaldehyde (Merck, Darmstadt, Germany) by depolymerization. Subsequently, the coverslips were incubated as follows: 2 × 5 min in PBS; 10 min in PBS containing 0.5% Triton X-100; 2 × 5 min in PBS; 10 min in PBS containing 0.4% NaBH₄ (Fluka Chemical Co., Buchs, Switzerland) (only when glutaraldehyde was present during fixation); 10 min in PBS containing 100 mM glycine; 2 × 5 min in PBS; 3 × 15 min in PBG (PBS containing 0.5% [wt/vol] BSA and 0.05% [wt/vol] gelatin [from cold water fish skin, Sigma Chemical Co.]); overnight at 4°C with a rat mAb raised against BrdU (Sera-Lab, Crawley Down, UK) diluted 1:500 in PBG; 4 × 5 min in PBG; 1.5 h with biotin-conjugated donkey anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:30 in PBG; 4 × 5 min in PBG; 30 min with either streptavidin-FITC conjugate (Gibco) diluted 1:500 in PBG or streptavidin-Texas Red conjugate (Amersham, Amersham, UK) diluted 1:250 in PBG; 2 × 5 min in PBG; 2 × 5 min in PBS; 3 min in PBS containing 0.4 µg/ml Hoechst 33258 (Sigma Chemical Co.) to stain DNA; 5 min in PBS. Coverslips were mounted in PBS containing 90% glycerol and 1 mg/ml p-phenylenediamine (Sigma Chemical Co.). Preparations were examined in a Leitz Aristoplan microscope equipped with epifluorescence optics, and photographed on Kodak Tri-X films at 400 ASA.

Microinjection

Cells were grown on marked glass coverslips (Cell-line Associates, Newfield, NJ) as described above. They were injected into the cytoplasm by glass micropipettes essentially as described by Capecchi (1980) and Graessmann and Graessmann (1983). The injection buffer contained 140 mM KCl, 100 mM BrUTP, 2 mM Pipes, pH 7.4. To inhibit RPII α -amanitin (20 µg/ml) was included in the injection buffer in control experiments. About 5% cell volume was injected. After microinjection cells were either fixed directly or were returned to normal cell culturing conditions for up to 60 min and then processed for immunofluorescence as described above.

Double Immunolabeling, Confocal Laser Scanning Microscopy, and Image Processing

MAb anti-SC-35, recognizing a 35-kD non-snRNP splicing factor (Fu and Maniatis, 1990) was purified from culture supernatant on a protein A-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). The mAb was eluted with 100 mM glycine-HCl, pH 2.5 (Goding, 1983).

For double-immunolabeling experiments, preparations were processed exactly as described above. Coverslips were incubated overnight at 4°C with anti-BrdU and anti-SC-35 simultaneously. After washing the coverslips, anti-BrdU was detected with biotin-conjugated donkey anti-rat IgG(H+L) (Jackson ImmunoResearch Laboratories) diluted 1:30, whereas anti-SC-35 was detected with TRITC-conjugated goat anti-mouse Ig (Nordic Immunological Lab., Tilburg, The Netherlands), diluted 1:100 in PBG. Subsequently, the coverslips were incubated with streptavidin-FITC conjugate (Gibco) diluted 1:500 in PBG. When anti-SC-35 and anti-BrdU were incubated separately the same localization results were obtained. The specificity of each of the secondary antibodies was tested by omitting one of the primary antibodies. No cross reaction was observed.

Optical sections of doubly stained cells were recorded with a Leica confocal laser scanning microscope equipped with a 63×/1.4 N.A. oil immer-

sion lens. A dual-wavelength laser was used to excite FITC and TRITC simultaneously at 488 nm and 514 nm, respectively. A 580-nm longpass dichroic beamsplitter separated red and green fluorescence. A 590 nm longpass filter and a 530–555 nm bandpass filter in front of two detectors were used. The z-resolution was about 0.9 μm . No crosstalk was observed. The fluorescence signals from both fluorochromes were recorded simultaneously in one scan. Photographs were taken directly from screen, using Kodak Tri-X 400 ASA black and white film.

Results

BrUTP Is a Substrate for Mammalian RPII

To study the spatial distribution of RPII transcription sites we have developed an immunocytochemical technique to visualize nascent RNA. A method to label DNA with bromodeoxyuridine (BrdU) has been used successfully already (Gratzner et al., 1975; Gratzner, 1982; Wilson et al., 1985; Nakamura et al., 1986; Nakayasu and Berezney, 1989; Mazotti et al., 1990). Antibodies raised against BrdU also recognize bromouridine (BrU) with high specificity and affinity (Vanderlaan and Thomas, 1985; Schutte et al., 1987). If cells are exposed to BrU in their growth medium, however, BrU will most likely be identified as a thymidine analogue and incorporated into DNA, rather than RNA (Eidinoff et al., 1959; Brockman and Anderson, 1963). Therefore, we have used BrUTP instead of BrU to label nascent RNA in living cells after microinjection, and in run-on experiments in permeabilized cells.

We have investigated the efficiency of incorporation of BrUTP into RNA by mammalian RPII to establish the usefulness of BrUTP for our purposes. With purified calf thymus RPII RNA synthesis followed simple Michaelis-Menten steady state kinetics for UTP and BrUTP (data not shown). The K_m of calf thymus RPII was 17 μM for BrUTP, and 22 μM for UTP. The V_{max} of [^3H]GTP incorporation into RNA in the presence of BrUTP was $\sim 40\%$ lower than with UTP. Comparable results on BrUTP incorporation into RNA have been obtained with cherry salmon RPII (Nakayama and Saneyoshi, 1984). The rate of transcription was essentially constant in time up to at least 60 min. This indicates that RPII was not inactivated by BrUTP.

The incorporation rate of BrUTP into RNA was also determined in a run-on transcription assay using permeabilized human bladder carcinoma cells. Because very little, if any, initiation of transcription occurs under run-on conditions, mainly elongation of in vivo-initiated transcription is measured (Weber et al., 1977). Run-on transcription took place at progressively decreasing rates (Fig. 1; See also Weber et al., 1977). α -Amanitin was used to discriminate between the three different nuclear RPs (Roeder, 1976). The difference between the values measured in the presence of 1 $\mu\text{g}/\text{ml}$ α -amanitin and those determined in the absence of α -amanitin represents RNA synthesis by RPII (inset, Fig. 1). RPII transcription accounted for $\sim 75\%$ of total RNA synthesis under our conditions. At any timepoint the amount of [^3H]GTP incorporation by RPII in the presence of BrUTP was $\sim 20\%$ lower than in the presence of UTP. This shows that the decrease of the rate of synthesis by RPII during run-on transcription with BrUTP is the same as with UTP. Therefore, the incorporation of BrUTP did not result in an increase of premature termination of transcription.

From the inset of Fig. 1 it can be calculated that after 30

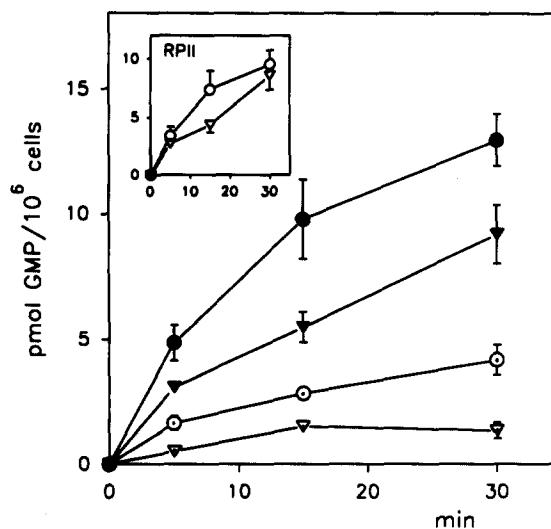


Figure 1. Time course of incorporation of BrUTP during run-on transcription in permeabilized human bladder carcinoma cells. Human bladder carcinoma cells were permeabilized with Triton X-100 and incubated with ATP, CTP, [^3H]GTP, and either UTP (circles) or BrUTP (triangles) (See Materials and Methods). Reaction mixtures were incubated for 5, 15, or 30 min at room temperature. Reactions were stopped with TCA, and radioactivity in TCA-precipitable material was measured. Reactions were done in the presence (dotted symbols) or absence (filled symbols) of 1 $\mu\text{g}/\text{ml}$ α -amanitin, which blocks RNA polymerase II activity. The radioactivity which was measured when UTP as well as BrUTP were omitted from the reaction mixture was subtracted. The difference between transcription in the absence and presence of 1 $\mu\text{g}/\text{ml}$ α -amanitin represents transcription by RNA polymerase II and is depicted in the inset. Shown are the results of an experiment in triplicate. Error bars denote standard errors.

min of run-on transcription 2×10^7 nucleotides per cell had been incorporated into RNA by RPII, in the presence of BrUTP, assuming equimolar use of all four ribonucleotides. The number of engaged RPII molecules in a growing cell is $\sim 20,000$ per cell (Cox, 1976; and references therein), so that on the average, $\sim 1,000$ nucleotides have been incorporated in each run-on transcript. Because full-length RPII transcripts contain on the average 10,000 nucleotides (Levin, 1980), only a small fraction of the engaged RPII molecules can have terminated transcription properly under our experimental conditions. This implies that BrUTP-labeled run-on transcripts represent predominantly nascent RNA, i.e. RNA bound to engaged RPII.

BrUTP was also incorporated by RPI (not sensitive to α -amanitin), albeit less efficiently. The rate of incorporation of BrUTP by RPI was about 50% of the rate in the presence of UTP. Finally, only very little RPIII (activity blocked by 100 $\mu\text{g}/\text{ml}$ α -amanitin) was detected in the presence of either UTP or BrUTP (data not shown). This is in agreement with reported low activities of RPIII in nuclei of cultured cells (Marzluff and Huang, 1984).

We conclude that BrUTP substitutes for UTP in RNA under these conditions, thereby validating our use of this nucleotide analogue for visualizing sites of RNA synthesis.

Run-on RPII Transcripts Are Concentrated in Subnuclear Domains

Having demonstrated that BrUTP is efficiently incorporated

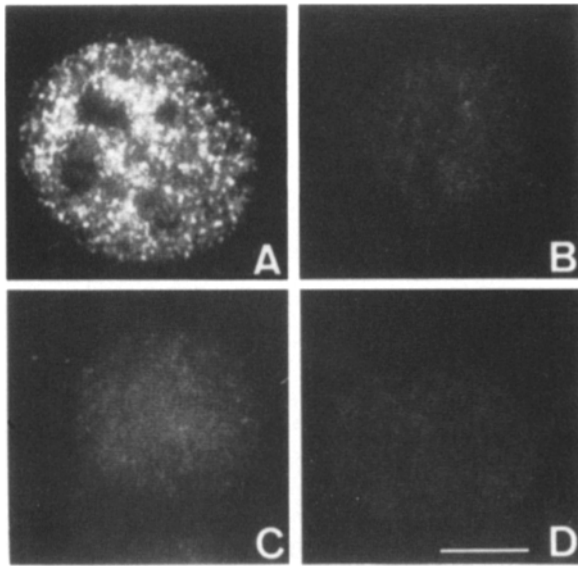


Figure 2. Localization of BrUTP-labeled RNA in vitro. Run-on transcription was carried out in Triton X-100 permeabilized human bladder carcinoma cells for 30 min at room temperature. Subsequently, cells were fixed in 2% formaldehyde. Coverslips were incubated with an anti-BrdU mAb from rat, followed by biotin-conjugated donkey anti-rat Ig and streptavidin-FITC conjugate. (A, C, and D) Run-on transcription was carried out in the presence of BrUTP. (B) BrUTP was substituted by UTP to examine the specificity of the mAb. (C) To inhibit RPII transcription 1 $\mu\text{g}/\text{ml}$ α -amanitin was included during run-on transcription. (D) Before fixation cells were incubated with 50 $\mu\text{g}/\text{ml}$ RNAase A for 10 min at room temperature. Bar, 5 μm .

into RNA by mammalian RPII, we developed an immunocytochemical method to visualize the sites of incorporation in the cell nucleus. Run-on transcription in permeabilized cells was used to incorporate BrUTP into nascent RNA. After run-on transcription for a defined period of time, nonincorporated BrUTP was removed. Then cells were fixed with formaldehyde, and incorporated BrUTP was detected with a mAb raised against BrdU, which also recognizes BrU. A clear nuclear staining was first observed after 10 min of incubation. Longer labeling (up to 30 min) resulted in a similar, more intense punctated pattern (Fig. 2 A). The anti-BrdU mAb was specific for BrUTP, because no labeling was seen when UTP was used instead of BrUTP (Fig. 2 B). The staining was fully obliterated by incubation with RNAse A, or by carrying out the run-on reaction in the presence of 1 $\mu\text{g}/\text{ml}$ α -amanitin (Fig. 2, C and D). This indicates that the punctated pattern represents RNA synthesized by RPII.

The question arises whether the punctated immunofluorescence pattern reflects the actual sites of RPII transcription, or other sites where newly synthesized RNA may have accumulated. We have already argued above that the majority of engaged RPII molecules can not have terminated transcription properly after 30 min of run-on transcription. This implies that BrUTP-labeled transcripts represent predominantly nascent RNA. Premature termination or cleavage of run-on transcripts cannot be excluded, however. Resulting fragmented transcripts may have accumulated in places spatially separated from the sites of RNA synthesis. To test this possibility we examined whether chasing with

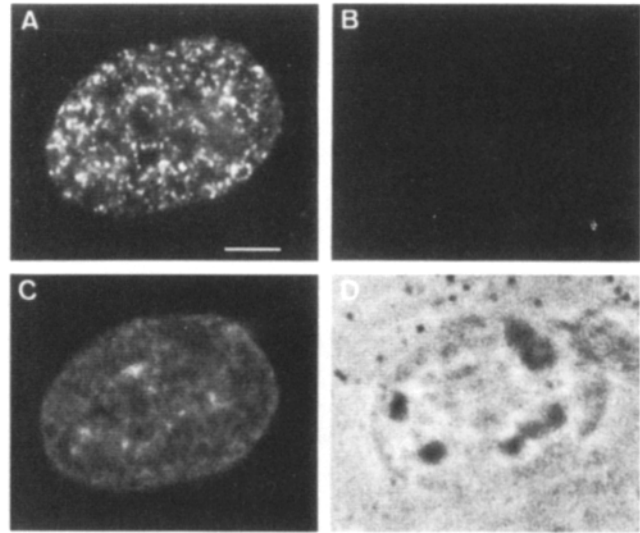


Figure 3. Localization of BrUTP-labeled RNA in DNase-digested and salt-extracted nuclei. Run-on transcription was carried out in Triton X-100 permeabilized human skin fibroblasts in the presence of BrUTP for 30 min at room temperature. Subsequently, labeled cells were treated with sodium tetrathionate, digested with RNase-free DNase and extracted with 0.25 M ammonium sulfate. Then the extracted nuclei (nuclear matrices) were fixed and immunolabeled as described in Fig. 2. (A) Nascent RNA, (B) DNA stained with Hoechst 33258, 0.25-s exposure, under these conditions nuclei give a bright image, (C) as in B but 10 s exposure, (D) phase contrast. Note that most DNA has been extracted. In these extracted preparations nucleolar RNA is stained (compare D with A), whereas the nucleoplasmic staining has not altered, as compared to labeling in unextracted preparations. Bar, 5 μm .

UTP after a short BrUTP labeling would affect the labeling pattern. We found that a chase with UTP after a 10-min BrUTP pulse neither affected the intensity of the spots, nor the overall labeling pattern, whereas longer labeling times with BrUTP only resulted in a pattern of spots of higher intensity (data not shown). Therefore, it is unlikely that the fluorescent spots correspond to sites containing prematurely terminated or artifactually cleaved RNA, that was synthesized somewhere else in the nucleus. In conclusion, the punctated pattern represents the sites of RNA synthesis by RPII.

In most experiments we did not observe nucleolar labeling. This observation was unexpected, because RPI, which is localized in the nucleolus and responsible for 20–30% of total nuclear transcription under our conditions, did incorporate BrUTP (see above). However, nucleolar labeling became visible when BrUTP-labeled, permeabilized cells were incubated with DNase I and extracted with buffer containing 0.25 M ammonium sulfate (Fig. 3 A). By this procedure, $\sim 90\%$ of the DNA and protein material in the nucleus was removed (Fig. 3, B and C), whereas most BrUTP-labeled RNA remained associated with the residual structure (the nuclear matrix; van Driel et al., 1991; Jack and Eggert, 1992). Nucleolar remnants could still be recognized (Fig. 3 D). Under these conditions BrUTP-labeled nucleolar RNA became detectable. This indicates that the absence of fluorescent labeling in nucleoli of permeabilized cells is due to inaccessibility of the nucleoli to the mAb against BrU. The question arises then whether also some RPII transcripts

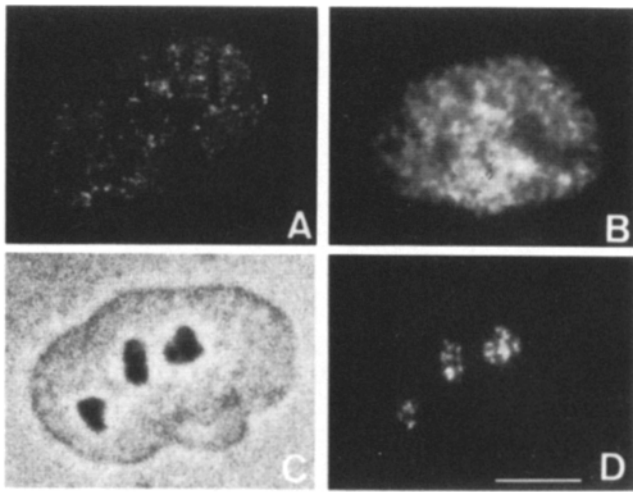


Figure 4. Localization of BrUTP-labeled RNA in vivo. Human skin fibroblasts were microinjected with BrUTP to investigate transcription in vivo. After microinjection, cells were cultivated for 15 min (A, C, and D) or 1 h (B) at 37°C. To inhibit RPII α -amanitin was co-injected (C and D). Cells were processed as described in Fig. 2. (A, B, and D) Newly synthesized RNA. (C) Phase contrast of nucleus shown in panel (D). Nucleolar labeling became apparent after RPII inhibition. Bar, 5 μ m.

might escape detection because of antibody inaccessibility. Removal of 90% of the DNA and protein did not alter the nucleoplasmic staining, neither its punctated pattern, nor its overall intensity. Therefore, we conclude that nascent RPII transcripts in permeabilized cells are accessible to the anti-BrU mAb, so that the fluorescent pattern represents the sites of RPII transcription.

Several cell lines from human, mouse, rat and chicken origin were tested to establish whether the observed punctated pattern represents a common distribution of nascent RPII transcripts in the interphase nucleus. All cell lines investigated so far displayed similar punctated patterns. In all cases no labeling was observed if 1 μ g/ml α -amanitin was present during RNA synthesis, or after incubation with RNase A (data not shown). This shows that the pattern represents transcription by RPII. The number of spots per nucleus differed between cell lines, ranging from \sim 100 to several hundreds. We conclude that the observed punctated pattern represents a common distribution of RPII transcription sites in interphase nuclei.

Visualization of Newly Synthesized RNA In Vivo

To analyze the distribution of nascent RNA in vivo, we have studied RNA synthesis after microinjection of BrUTP into living cells followed by immunofluorescence. Incubation with BrUTP for 15 min resulted in a clear punctated nucleoplasmic pattern (Fig. 4 A). Labeling was sensitive to RNase A as well as to α -amanitin (1 μ g/ml) (data not shown). This shows that RPII incorporates BrUTP into RNA in vivo, similarly as observed in vitro. A similar, but weak punctated nuclear staining was already observed after 4 min of incubation with microinjected BrUTP (data not shown). Given a mean length of \sim 10,000 nucleotides for primary RPII transcripts and an in vivo incorporation rate of 20 nucleotides/s, \sim 70% of the incorporated BrUTP is in na-

nascent RNA after 4 min of incubation with BrUTP. This indicates that the punctated pattern represents sites of RNA synthesis.

Permeabilization with Triton X-100 before fixation with formaldehyde did not change the spatial distribution of BrUTP-labeled RNA. This excludes the possibility that the punctated pattern results from a reorganization of newly synthesized transcripts after permeabilization. In vivo labeling after microinjection resulted in the same punctated pattern as observed after run-on transcription in permeabilized cells. This indicates that our in vitro labeling procedure did not cause any major distortion of nuclear structure.

As in permeabilized cells, nucleoli in most microinjection experiments were not labeled. However, a bright nucleolar labeling was frequently observed in vivo when RPII transcription was inhibited by co-injecting α -amanitin with BrUTP (Fig. 4 D). This labeling must be the result of RPI transcription, because RPII was inhibited by α -amanitin and the contribution of RPIII transcription was too small to be detected (compare Fig. 2 C). Therefore, the observed nucleolar staining can not result from a reorganization of non-nucleolar transcripts with the nucleolus. The α -amanitin-induced nucleolar staining can be explained by a change in antibody accessibility, related to an indirect effect of RPII inhibition on nucleolar structure. It has been shown that inhibition of RPII in vivo (e.g., by α -amanitin or 5,6-dichloro-1- β -D-ribofuranosyl benzimidazole) results in disaggregation of nucleoli (reviewed by Brasch, 1990). This disaggregation may loosen the dense nucleolar ultrastructure and enable the antibodies to penetrate. The observed nucleolar staining in the presence of α -amanitin consisted of some tens of dots inside nucleoli, whereas the nucleoplasm remained unlabeled. Nucleolar rRNA dots seemed to be larger than nucleoplasmic spots containing RPII transcripts. The nuclear RNA labeling pattern was reminiscent of the localization of RPI (Scheer and Rose, 1984; Scheer and Raška, 1987). These observations are in agreement with the notion that RPI transcription takes place in nucleolar fibrillar centers (Scheer and Benavente, 1990; Thiry and Goessens, 1992).

Unlike labeling in vitro, the in vivo labeling pattern changed as microinjected cells were incubated for a longer time. After 1 h, the discrete labeling pattern had changed to a strong, diffuse labeling throughout the nucleoplasm (Fig. 4 B). This diffuse labeling may reflect intranuclear transport of BrUTP-labeled RNA. RNA tracks, as have been reported after in situ hybridization using a probe for a specific RNA species (Lawrence et al., 1989), were never observed. Only a weak cytoplasmic staining was detectable after 1 h of incubation with BrUTP (data not shown). This suggests that BrUTP-labeled RNAs are processed and transported to the cytoplasm. It is known, however, that in a normal cell only part of the total transcript population ever leaves the nucleus (Salditt-Georgieff et al., 1981). So, even if BrUTP-labeled transcripts are transported out of the nucleus, the concentration of newly synthesized transcripts in the cytoplasm is likely to be low compared to that in the nucleus after 1 h of labeling (assuming that the volume of the nucleoplasm and the volume of the cytoplasm are about the same).

Summarizing, we conclude that labeling of living cells with BrUTP is a rapid, simple, and sensitive technique to visualize newly synthesized RNA. The fluorescent domains of incorporated BrUTP observed after labeling for a short

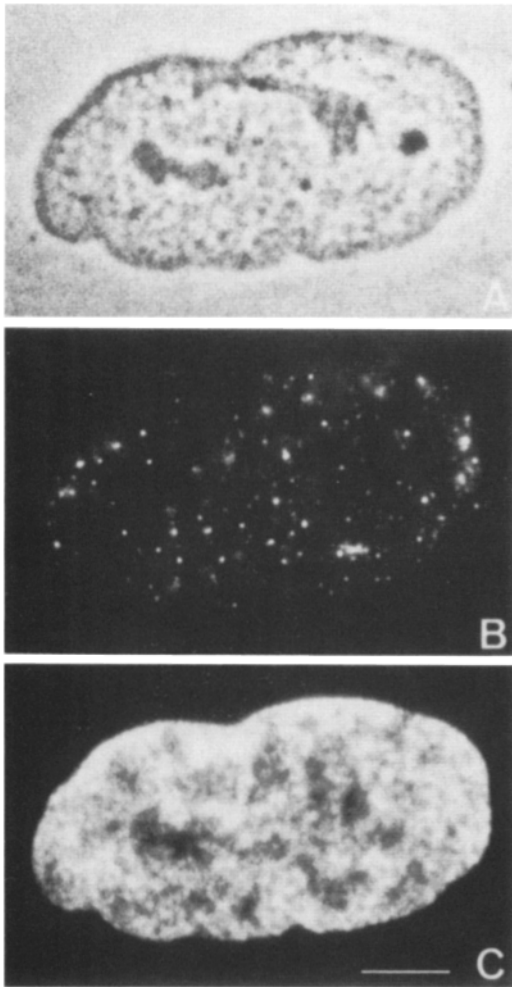


Figure 5. Distribution of nascent RPII transcripts in the nucleus. Triton X-100 permeabilized human skin fibroblasts were labeled with BrUTP for 15 min at room temperature. After fixation with 2% formaldehyde, incorporated BrUTP was detected by incubating coverslips with anti-BrdU, biotin-conjugated donkey anti-rat Ig, and streptavidin-Texas Red conjugate. The use of streptavidin-Texas Red conjugate resulted in a somewhat less diffuse background staining compared to streptavidin-FITC. The nucleus of a single cell is shown comparing phase contrast (A), nascent RNA (B), and DNA stained by Hoechst 33258 (C). Note that nascent-RPII transcripts are present near the nuclear envelope as well as in the nuclear interior. Nascent RNA is present in areas of low and of high DNA concentration. No nucleolar labeling is observed. Bar, 5 μm .

period of time *in vivo* obviously correspond to genuine sites of RPII transcription.

Distribution of Nascent RPII Transcripts in the Nucleus

The distribution of transcription by RPII was investigated in nuclei of human skin fibroblasts in more detail. As in all other cell types investigated so far, nascent RPII transcripts were found in well-defined nuclear domains, rather than being distributed homogeneously throughout the nucleus. Some hundred nascent-RNA spots per nucleus were observed (Fig. 5 B). The spots had different intensities and were scattered seemingly at random throughout the nucleoplasm. Spots were present in the periphery as well as in the nuclear interior and near the nucleolus. The nucleolus itself

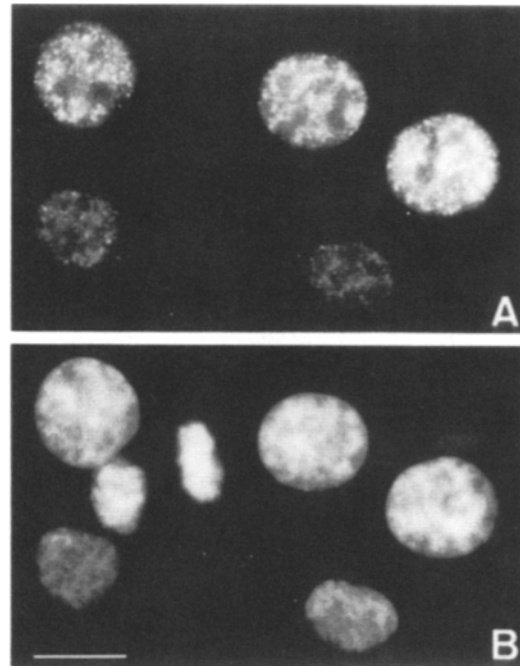


Figure 6. The intensity of the RPII transcription pattern differs from cell to cell. Human bladder carcinoma cells were permeabilized with Triton X-100 and incubated with BrUTP for 15 min at room temperature. Coverslips were processed as indicated in Fig. 2. (A) Nascent RNA. (B) DNA stained by Hoechst 33258. A typical group of cells exhibiting patterns of different intensities is shown. Note cell in anaphase. Bar, 10 μm .

was not labeled. The nascent-RNA pattern was compared with the distribution of DNA, visualized by the DNA-specific fluorochrome Hoechst 33258 (Fig. 5 C). Nascent transcripts were observed in both low and high DNA concentration areas.

The localization of nascent RNA during different stages of the cell cycle was investigated in asynchronous cultures. We did not observe RNA labeling in any stage of mitosis (Fig. 6 A, cell in anaphase). In interphase cells the intensity of the spots, but not their overall distribution, differed considerably from cell to cell (Fig. 6 A). These differences probably reflect fluctuations in transcriptional activity related to different stages in the cell cycle, as was reported earlier by Fakan and Nobis (1978). Pfeiffer and Tolmach (1968) determined that the overall rate of RNA synthesis is constant during G1, doubles during the first half of S-phase, and then remains constant during the second half of S-phase up to mitosis. Probably, the doubling in transcription rate is due to a doubling of the number of active genes, which are replicated predominantly in early S phase (see Goldman, 1988).

Summarizing, we find that RPII transcription domains are not restricted to any specific region of the nucleoplasm, but are scattered throughout the nucleus, with exception of the nucleolus.

Localization of Nascent RNA with Respect to Domains Enriched in Splicing Components

Several co- and posttranscriptional processes like capping, cleavage, polyadenylation, methylation, splicing, and intranuclear transport occur between pre-mRNA synthesis and translocation of mature mRNA through the nuclear pores.

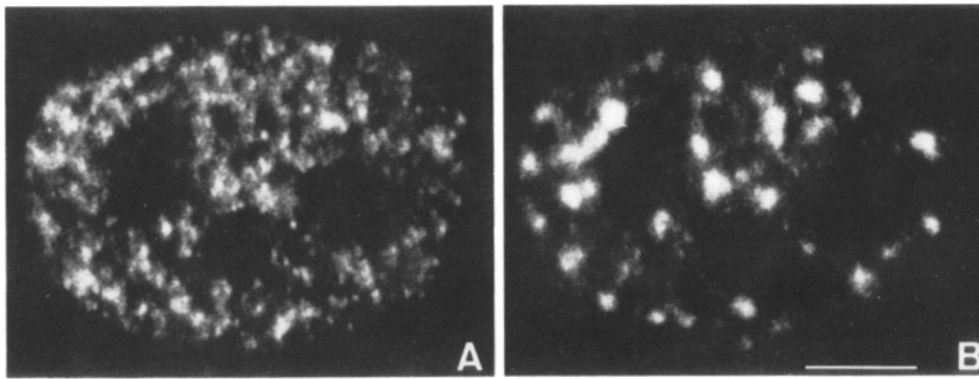


Figure 7. Double labeling of nascent RNA and SC-35 domains enriched in splicing components. Triton X-100 permeabilized human skin fibroblasts were labeled with BrUTP for 15 min at room temperature. Cells were fixed in 2% formaldehyde. Incorporated BrUTP was visualized as described in Fig. 2. In the same cell domains enriched in snRNPs were visualized with anti-SC-35, a mAb against an essential splicing component.

Anti-SC-35 was detected by TRITC-conjugated goat anti-mouse Ig. Preparations were examined in a confocal laser scanning microscope. (A) Nascent RNA labeled with BrUTP. (B) Domains enriched in splicing components stained by anti-SC-35 (same optical section as in A). Essentially no colocalization was observed between sites containing nascent RNA and intensely labeled SC-35 domains. Some colocalization was observed between sites containing nascent RNA and areas showing weak SC-35 staining. Bar, 5 μ m.

Thus far, the nuclear location of most of the above mentioned RNA processing events has not been identified yet. Only the distribution of splicing components has been extensively studied (Lerner et al., 1981; Nyman et al., 1986; Verheijen et al., 1986; Fu and Maniatis 1990; Carmo-Fonseca et al., 1991, 1992; Zhang et al., 1992). Pre-mRNA splicing takes place in spliceosomes, which are composed of small nuclear ribonucleoprotein particles (snRNPs) associated with non-snRNP proteins (reviewed by Lührmann et al., 1990; Green, 1991).

We have examined the distribution of nascent RPII transcripts with respect to the localization of the essential splicing component SC-35 (Fu and Maniatis, 1990). SC-35 is a non-snRNP protein involved in spliceosome assembly, that is essential for the first step of the splicing reaction (Fu and Maniatis, 1990, 1992*a,b*). We carried out double immunofluorescence labeling experiments by combining anti-SC-35 staining with BrUTP labeling of nascent RNA. Fig. 7 A shows BrUTP labeling in permeabilized human skin fibroblasts, detected by confocal laser scanning microscopy. Fig. 7 B shows the same optical section labeled with anti-SC-35. In the SC-35 pattern intensely labeled structures and weakly labeled, smaller structures can be recognized. The majority of nascent-RNA sites did not colocalize with the intensely labeled domains (compare Fig. 7, A and B). Although nascent transcripts sometimes were found close to these domains, they only rarely coincided with them. Many nascent-RNA domains were not situated near a SC-35 cluster. Therefore, there seems to be no relationship between the distribution of RNA synthesis and the intensely labeled, large SC-35 domains. Careful visual inspection of confocal images of nuclei double labeled for nascent RNA and SC-35 showed some colocalization between sites containing nascent RPII transcripts and areas showing weak SC-35 staining. The above described double labeling was also carried out for human bladder carcinoma cells, leading to identical conclusions (data not shown).

Discussion

Visualization of Nascent RPII Transcripts In Situ

We have examined the distribution of RPII transcription sites in the interphase nucleus of a number of cell types. To this

end, we developed a technique based on the incorporation of BrUTP into nascent RNA. BrUTP-labeled RNA is visualized by immunofluorescence microscopy using a specific anti-Br(d)U antibody. The immunofluorescence pattern obtained after short time *in vivo* transcription or after run-on transcription consists of hundreds of spots scattered throughout the nucleoplasm.

Several lines of evidence indicate that this staining represents sites of synthesis of authentic RPII transcripts. (a) BrUTP is efficiently incorporated by RPII. The time courses of run-on transcription are similar for BrUTP and UTP, except that at any time point the rate of transcription in the presence of BrUTP is \sim 20% lower than in the presence of UTP. This shows that BrUTP does not induce premature termination of transcription. We conclude that BrUTP simply substitutes for UTP in RNA, so that the same transcripts are synthesized in the presence of either BrUTP or UTP. (b) Labeled RNA is already detectable after 4 min of *in vivo* labeling, or after 10 min of run-on transcription. In both cases, most BrUTP-labeled RNA is still nascent. (c) The same punctuated pattern is observed in living cells, after microinjection with BrUTP, and in permeabilized cells, under run-on transcription conditions. It is known that during run-on transcription the same RNAs, in the same relative amounts, are made as *in vivo* (McKnight et al., 1979; Darnell, 1982). In addition, short term *in vivo* labeling is independent of permeabilization before fixation. So, the BrUTP-labeled domains are not a result of reorganization of newly synthesized RNA after permeabilization of labeled cells. (d) Extraction of \sim 90% of the DNA and protein from nuclei of permeabilized cells did neither alter the spatial distribution, nor the number and intensity of nascent-RNA domains in the nucleoplasm. This shows that there are no "hidden" RPII transcription sites that might have escaped detection. Moreover, these results are in agreement with the notion that nascent RNA is attached to an underlying organizing structure (Ciejek et al., 1982; Xing and Lawrence, 1991).

Summarizing, labeling nascent RNA with BrUTP is to our knowledge the first technique to *in situ* visualize sites of RNA synthesis by immunofluorescence microscopy. The technique is simple, rapid, and sensitive, and provides interesting possibilities for studying the localization of active chromatin in relation to other nuclear structures and functions. Independently, Jackson et al. (1993) used the same

technique to visualize newly synthesized RNA in permeabilized cells.

The Spatial Distribution of RPII Transcription Sites in the Nucleus

Our results and those of Jackson et al. (1993) show that nascent RPII transcripts are concentrated in a few hundred domains that are scattered throughout the nucleoplasm. RPII transcripts were found near the nuclear envelope as well as in the nuclear interior and near the nucleolus. This observation has important consequences for our understanding of the relation between RPII transcription and RNA processing, which will be discussed below.

The distribution of newly synthesized RNA in interphase nuclei has been investigated by labeling transcripts with [³H]uridine, followed by autoradiography (Fakan and Bernhard, 1971; Fakan et al., 1976; reviewed by Fakan, 1986). A strong labeling of the nucleoli was observed, obviously representing transcripts of RPI. The nucleoplasm was labeled only weakly; no specific pattern or clusters could be discerned. Autoradiography in combination with immunogold labeling has shown [³H]uridine at perichromatin fibrils where also snRNPs and hnRNPs were localized (Fakan et al., 1976; Bachellerie et al., 1975). A cluster of perichromatin fibrils may correspond to one BrUTP-labeled transcription domain. In that case, results obtained with our BrUTP labeling technique would be compatible with earlier autoradiographic studies.

Because, under our conditions, we visualize nascent RPII transcripts, we label at the same time the sites where active genes are localized in the nucleus. Other techniques have been used to investigate the localization of active chromatin in interphase nuclei. Actively transcribed chromatin is characterized by an increased sensitivity to nucleases (Weintraub and Groudine, 1976). Because nuclease-sensitive DNA was found predominantly at the nuclear periphery (Hutchison and Weintraub, 1985; de Graaf et al., 1990; Krystosek and Puck, 1990), this was interpreted to indicate that most active genes are located in that specific region of the interphase nucleus. Our results clearly show that RNA synthesis by RPII is not confined to the nuclear periphery but takes place throughout the nucleoplasm. Therefore, nuclease sensitivity may not be a useful in situ parameter for the localization of active chromatin.

The localization of a few individual genes has been examined by in situ hybridization. Integrated, actively transcribed Epstein-Barr virus genes in a human lymphoma cell are only present in the nuclear interior (Lawrence et al., 1988, 1989). The dystrophin gene, on the other hand, was found near the nuclear envelope, irrespective whether the gene was active or inactive (Lawrence et al., 1990; Lawrence and Singer, 1991). Although the evidence is still limited, it seems that each gene, active or inactive, is confined to its own region in the interphase nucleus. The lack of correlation between a geometric nuclear region and gene activity is in agreement with our results that RPII transcription domains are localized throughout the entire nucleoplasm. In situ hybridization has also been used to localize RNA. Specific transcripts have been found to be concentrated in foci and tracks in interphase nuclei (Lawrence et al., 1989; Spector et al., 1990). It is not clear how these tracks are spatially related to the actual sites of transcription. Therefore, it would be interesting

to investigate the spatial distribution of specific transcripts visualized by in situ hybridization in relation to nascent RNA domains.

Summarizing, RPII transcription takes place in many small domains that are dispersed throughout the nucleus. No distinct, large nuclear regions exist that contain only active or only inactive chromatin.

Subnuclear Localization of Pre-mRNA Synthesis in Relation to Splicing

We have compared the localization of nascent RPII transcripts (i.e., predominantly pre-mRNA) with the distribution of the splicing machinery in the interphase nucleus. Conclusions are important for our understanding of the functional organization of the nucleus. As a marker for the localization of splicing components we have used the protein SC-35, which is essential for splicing of pre-mRNA (Fu and Maniatis, 1990, 1992a,b). In immunofluorescence, anti-SC-35 antibodies intensely label 20 to 50 domains in interphase nuclei. These domains colocalize with nuclear regions labeled by antibodies against snRNPs (Spector et al., 1991). In addition, anti-SC-35 antibodies and anti-snRNP antibodies weakly label diffuse areas between the sharply defined, strongly labeled domains (Spector et al., 1991). Another essential splicing factor, U2AF, shows a more widespread nucleoplasmic distribution (Zhang et al., 1992).

Our results show that there is no relationship between the distribution of clusters of nascent RPII transcripts and the subnuclear localization of domains containing a high concentration of SC-35 (Fig. 7). It is thought that splicing already starts before the transcript is completed, and may be continued post-transcriptionally (reviewed by Beyer and Osheim, 1991). This implies that cotranscriptional splicing takes place outside strongly labeled SC-35 domains. Scrutinizing confocal microscopy images after double labeling (like Fig. 7) shows that many sites of transcription coincide with weakly labeled SC-35-containing areas. So, these areas are most likely sites of cotranscriptional splicing. Evidently, if the strongly labeled SC-35 domains would be sites of post-transcriptional splicing, pre-mRNA has to be transported from the site of synthesis to these domains. However, assuming that BrUTP-labeled pre-mRNA is processed normally, we have no evidence that rapid post-transcriptional translocation of pre-mRNA to SC-35 domains occurs. This is based on the similarity of the spatial distribution of BrUTP-labeled nascent RNA in vitro and the distribution in vivo 15 minutes after microinjection with BrUTP, suggesting that also RNA labeled in vivo is located predominantly outside SC-35 domains. Therefore, it seems that no transport of BrUTP-labeled RNA takes place between sites of RNA synthesis and SC-35 domains, although many BrUTP-labeled transcripts are not nascent any more after 15 min and, therefore, available for translocation. We conclude that cotranscriptional splicing must occur outside the strongly labeled SC-35 domains and that post-transcriptional splicing may also occur at sites spatially separated from these domains.

Several observations relate to this problem. First, Spector et al. (1991) showed evidence that the weakly labeled SC-35-containing areas in the nucleus correspond to perichromatin fibrils. These fibrils become rapidly labeled after exposing a cell to [³H]uridine (Fakan, 1986), and contain also snRNPs (Fakan et al., 1984), indicating that cotranscrip-

tional splicing may occur at these structures. This is in agreement with our observations. Second, Wang et al. (1991) have presented evidence that microinjected pre-mRNA accumulates in the snRNP and SC-35-containing nuclear domains. Furthermore, in situ hybridization experiments with a biotinylated oligo-dT probe suggest that poly(A)-containing transcripts are localized in the same domains (Carter et al., 1991, 1993). These two observations suggest that RNA processing does take place in the SC-35 domains. However, accumulation of microinjected intron-containing pre-mRNA in these domains does not necessarily reflect the physiological pathway of endogenous pre-mRNA. It is also not clear whether the distribution of the oligo-dT probe after in-situ hybridization reflects the in vivo distribution of poly(A) RNA. Third, Spector et al. (1991) have presented evidence that the strongly labeled SC-35 domains, observed after immunofluorescent labeling, correspond to interchromatin granules seen by electron microscopy. Also snRNPs have been localized in interchromatin granules (Fakan et al., 1984). Fakan (1986) showed by electron microscopy, combined with autoradiography, that these structures become only slowly labeled after exposing living cells to [³H]uridine. These observations can best be explained by assuming that SC-35 domains are not major sites of RNA processing. Fourth, in two cases specific transcripts have been found close to both the respective genes (Xing et al., 1993) and domains enriched in splicing components (Huang and Spector, 1991; Xing et al., 1993). We also observed sites containing nascent RNA in close association with strongly labeled SC-35 domains. In addition, many transcription sites were found at some distance from those domains. Therefore, the localization of c-fos and fibronectin transcripts may not reflect a general organization principle of active chromatin. Fifth, very recently Jackson et al. (1993) reported colocalization of snRNPs with transcription sites. Unfortunately, they did not discriminate between strongly and weakly stained snRNP domains.

Taking these observations together the following picture emerges. Pre-mRNA synthesis takes place at numerous sites scattered throughout the nucleus. At many, if not all, of these sites splicing machinery is present (weakly labeled SC-35 areas), allowing cotranscriptional splicing. Subsequently, splicing is completed at the site of transcription. Presumably, as has also been suggested by others, strongly labeled SC-35 domains have other functions than splicing pre-mRNA, like storage of RNA processing components (Fakan et al., 1984; Wang et al., 1990), assembly of spliceosomes (Fu and Maniatis, 1990), regeneration of spliceosomes, or digestion of introns (Wang et al., 1990). It would be interesting to study the kinetics of recycling of splicing components between the weakly and strongly labeled domains.

Is Transcription by RPII Organized in Clusters of Active Genes?

Nascent RPII transcripts are localized in one hundred to several hundreds of nucleoplasmic domains per nucleus, depending on the cell type. The number of active pre-mRNA genes, however, in a typical eukaryotic cell is estimated to be 10,000 to 30,000 (Davidson and Hough, 1971; Galau et al., 1974; Bishop et al., 1984; Lewin, 1975). Only few genes are transcribed at a high rate. The majority of active genes

shows a relatively low transcription rate. In addition, there may be a limited set of active genes at distinct phases of the cell cycle. The discrepancy between the number of nascent-RNA domains and the number of active genes can be explained in two ways: (a) one RPII transcription domain corresponds to the position of one highly active gene; a large number of genes do not give rise to detectable accumulation of nascent RPII transcripts, or (b) one RPII transcription domain corresponds to the transcription of a cluster of a number of active genes. Our experiments do not discriminate between these two possibilities. However, like transcription, early replicating DNA is localized in a few hundred nuclear domains (Nakamura, 1986). Each replication domain contains 20–100 replicons and therefore must comprise on average several genes (Hand, 1978). Early replicating DNA is enriched in active genes (reviewed by Goldman, 1988). It has been claimed that the localization of early replicating DNA in discrete regions exists throughout the cell cycle and is passed on to daughter cells (Meng and Berezney, 1991). These findings support the idea that active genes are organized in clusters throughout interphase. Double-labeling experiments that combine visualization of replication and transcription should result in a better understanding of the higher organization of active chromatin.

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