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Ultrastructural Localization of Active Genes in Nuclei of A431 Cells

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Abstract We have studied the ultrastructural localization of active genes in nuclei of the human epidermoid carcinoma cell line A431. Nascent RNA was labeled by incorporation of 5-bromouridine 5'-triphosphate, followed by pre-embedment or postembedment immunogold labeling and electron microscopy using ultrasmall gold–conjugated antibodies and silver enhancement. This combination of techniques allowed a sensitive and high resolution visualization of RNA synthesis in the nucleus. Transcription sites were identified as clusters of 3–20 gold particles and were found throughout the nucleoplasm. The clusters had a diameter of less than 200 nm. The distribution of clusters of gold particles in nuclei is preserved in nuclear matrix preparations. Nascent RNA is associated with fibrillar as well as with granular structures in the matrix. A431 nuclei contained on average about 10,000 clusters of gold particles. This means that each cluster represents transcription of probably one active gene or, at most, a few genes. Our study does not provide evidence for aggregation of active genes. We found transcription sites distributed predominantly on the surface of electron-dense nuclear material, probably lumps of chromatin. This supports a model of transcription activation preferentially on the boundary between a chromosome domain and the interchromatin space.

Key words: 5-bromouridine 5'-triphosphate, electron microscopy, domain, nuclear matrix, RNA polymerase II, transcription, ultrasmall gold

For a thorough understanding of the regulation of gene expression the spatial distribution of active genes in relation to nuclear ultrastructure is essential. An important contribution to this field was made in the 1970s by electron microscopic autoradiography (EMARG) on 3H-uridine-labeled cells. By using the regressive EDTA staining, evidence has been presented that extranucleolar RNA synthesis is associated with ultrastructural entities called perichromatin fibrils [Fakan and Puvion, 1980; Fakan, 1986]. These fibrillar structures are distributed throughout the nucleoplasm on the surface of chromatin domains and probably correspond to newly synthesized hnRNA, associated with hnRNP core proteins and splicing factors [Fakan, 1994]. Unfortunately, the strength of the EMARG technique is limited by its low resolution.

A nonradioactive technique to visualize sites of transcription has recently been developed by us [Wansink et al., 1993]. It is based on the incorporation of the UTP analog 5-bromouridine 5'-triphosphate (BrUTP) into nascent RNA [Jackson et al., 1993; Wansink et al., 1993]. Using immunofluorescence microscopy, a punctate pattern consisting of several hundreds of fluorescent spots of nascent RNA synthesized by RNA polymerase II (RPII) was observed throughout the nucleoplasm. The number of active RPII molecules [Cochet-Meibach et al., 1974; Cox, 1976; Crerar and Pearson, 1977] is one to two orders of magnitude larger than the number of fluorescent spots. Most likely, the number of transcribed genes is also much higher than the number of fluorescent spots, since many active genes probably contain a single elongating RPII molecule and only a few, highly active genes contain more than one elongating RPII.
molecule at any moment. Two possibilities can be considered to relate the number of elongating RPII molecules and the number of fluorescent transcription domains. (1) Fluorescent spots of nascent RNA reflect the existence of discrete compartments in which several RPII molecules, transcribing several active genes, are concentrated in a higher order structure. (2) Active genes are not organized in higher order structures; light microscopic resolution is too low to visualize individual sites of transcription: one fluorescent spot reflects the activity of a number of genes that happen to be close together, or, in some cases, the activity of one gene that happens to be at a relative large distance from its nearest neighbors.

In order to discriminate between these possibilities, we have combined the BrUTP technique with the high resolving power of electron microscopy to study the ultrastructural localization of transcription in nuclei and nuclear matrix preparations of A431 cells.

MATERIALS AND METHODS

Cell Culture

A431 (ATCC CRL 1555, human epidermoid carcinoma) cells, free of mycoplasm, were grown at 37°C under a 7.5% CO2 atmosphere in DMEM (Gibco, Paisley, UK) supplemented with 10% (v/v) heat-inactivated FCS (Gibco), 10 IU/ml penicillin, and 10 μg/ml streptomycin (Gibco). For a transcription labeling, A431 cells were grown for about 24 hr on coverslips to less than 50% confluency.

BrUTP Incorporation

Cells were permeabilized in 0.05% Triton X-100, and BrUTP was incorporated into nascent RNA as described [Wansink et al., 1993, 1994a], except that the MgCl2 concentration was lowered to 0.5 mM during permeabilization and 2.5 mM during transcription (giving a free Mg2+ concentration of about 0.5 M). After run-on transcription for 30 min, coverslips were placed on ice and rinsed in ice-cold TBS (10 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.5 mM MgCl2, and 1 mM PMSF) containing 0.05% Triton X-100 and 5 U/ml RNasin (Promega Co., Madison, WI) for 3 min. Then the coverslips were rinsed in ice-cold TBS containing 5 U/ml RNasin for 5 min. Subsequently, cells were either fixed immediately in 2% formaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) for 15 min at room temperature, or used for in situ nuclear matrix preparation.

In Situ Nuclear Matrix Preparation

BrUTP-labeled cells were incubated as follows (all steps at room temperature): 5 min in TBS containing 5 U/ml RNasin, and 2 mM sodium tetrathionate (Sigma Chemical Co., St. Louis, MO) to stabilize nuclear matrix structure [Kaufmann and Shaper, 1984; Stuurman et al., 1992]; 2 × briefly in TM50 (25 mM Tris.HCl, pH 7.4, 5 mM MgCl2, 50 mM KCl, 1 mM EGTA, and 1 mM PMSF) containing 5 U/ml RNasin; 30–45 min in TM50 containing 750 U/ml RNase-free DNase I (Boehringer, Mannheim, Germany) and 30 U/ml RNasin; 2 × 10 min in 25 mM Tris.HCl, pH 7.4, 5 mM MgCl2, 100 mM KCl, 1 mM EGTA, 1 mM PMSF, 0.25 M (NH4)2SO4, and 5 U/ml RNasin; 2 × briefly in TBS containing 5 U/ml RNasin. Subsequently, nuclear matrices were fixed in 2% formaldehyde in PBS for 15 min.

Immunofluorescence Microscopy

After fixation, coverslips with either BrUTP-labeled nuclei or nuclear matrices were processed, immunolabeled, and embedded as described earlier [Wansink et al., 1993, 1994a]. Before embedding, DNA was stained with Hoechst 33258. BrUTP-labeled RNA was detected with a rat monoclonal antibody against IgG(H + L) (Jackson Immunoresearch Laboratories, West Grove, PA), and FITC-conjugated streptavidin (Jackson) as described [Wansink et al., 1993, 1994a]. Preparations were examined in a Leitz Aristoplan microscope equipped with epifluorescence optics using a 63×/1.4 N.A. oil-immersion lens, and photographed on Kodak Tri-X films at 400 ASA.

BrUTP Incorporation and Electron Microscopy

For electron microscopy, A431 cells were grown on Thermoplan coverslips (LUX, Miles Lab, Naperville, IL). Cells were permeabilized and labeled with BrUTP for 30 min as described above. BrUTP-labeled nuclei were either fixed directly in PBS containing 2% formaldehyde and 0.01% glutaraldehyde for 15 min at room temperature, or used for in situ nuclear matrix preparation as described above, and then fixed in the same fixative. In control experiments
BrU TP was substituted for UTP during run-on transcription.

After fixation coverslips were incubated as follows (at room temperature): 5 min in PBS containing 0.5% Triton X-100; 2 × 5 min in PBS; 10 min in PBS containing 100 mM glycine; 5 min in PBS. Then these EM samples were processed for either pre-embedment or post-embedment immunogold labeling. Both techniques are described separately below.

**Pre-embedment Immunogold Labeling**

Coverslips were incubated as follows (at room temperature unless specified otherwise): 5 min in PBG [PBS containing 0.5% (w/v) BSA and 0.05% (w/v) gelatin (from cold water fish skin, Sigma)]; 10 min in PBS containing 10% (w/v) BSA; overnight at 4°C with anti-BrUTP diluted 1:1,500 in PBG; 6 × 5 min in PBG; 10 min in 25% normal donkey serum (Jackson) in PBS; 2 hr in biotin-conjugated donkey anti-rat IgG(H + L), diluted 1:600 in PBG (note that until this step the preparation method was identical to the immunofluorescent labeling procedure); 6 × 5 min in PBG-extra-gelatin [PBS containing 0.5% (w/v) BSA and 0.25% (w/v) gelatin]; overnight at 4°C in goat anti-biotin antibodies coupled to ultrasmall gold with a diameter smaller than 0.8 nm (Aurion, Wageningen, The Netherlands), diluted 1:100 in PBG-extra-gelatin; 8 × 15 min in PBG-extra-gelatin; 3 × 10 min in PBS. Samples were postfixed in 1% glutaraldehyde in PBS for 10 min. The ultrasmall gold particles were enlarged by silver enhancement (Danscher, 1981). These postembedding-labeled sections were stained with uranyl acetate and lead citrate and then examined in a Philips EM 420 electron microscope operated at 80 kV.

**RESULTS**

**Ultrastructural Localization of Active Genes**

Nascent RNA, synthesized by RPII in the presence of BrUTP, can be visualized by immunofluorescence microscopy (Wansink et al., 1993; Jackson et al., 1993). The fluorescence pattern in all cell types studied thus far consists of several hundreds of spots scattered throughout the nucleoplasm. A431 cells showed a similar punctate fluorescence pattern of BrU-labeled nascent RNA (results not shown). To investigate whether this punctate fluorescence pattern reflects a higher organization of active genes, we have studied the spatial distribution of transcription sites at the ultrastructural level by adapting the technique of labeling nascent RNA with BrUTP in permeabilized cells to immunogold electron microscopy. Incorporated BrU was detected by antibodies coupled to ultrasmall gold particles, followed by silver enhancement. We first employed pre-embedment immunogold labeling to ensure an optimal labeling efficiency. A labeling pattern consisting mainly of clusters of gold particles, scattered through the entire nucleoplasm except nucleoli, was observed (Fig. 1A; Fig. 1C shows a higher magnification of part of another nucleus). Only a few solitary gold particles were seen. Clusters of gold particles had an apparent diameter of about 50-200 nm and consisted of three to up to 20 gold particles. In many cases neighboring clusters were located less than about 0.25 μm apart, which means that they cannot be resolved by light microscopy. However, no obvious higher order organization of clusters, i.e., groups of clusters, was observed.

As a control, run-on transcription was done in the presence of UTP instead of BrUTP. In that case only a few gold particles were observed in the nucleus, whereas clusters of gold particles...
Fig. 1. Visualization of nascent RNA in nuclei of A431 cells by electron microscopy. A: Nascent RNA was labeled with BrUTP for 30 min during run-on transcription in permeabilized A431 cells. Sites of BrUTP incorporation were labeled with antibodies conjugated to ultrasmall colloidal gold in a pre-embedding immunogold labeling procedure. Samples were cut into 0.25 μm sections and inspected by electron microscopy as described in Materials and Methods. Mainly clusters of gold particles are present throughout the nucleus, predominantly on the surface of electron-dense material. Overall picture of nucleus is shown in the inset. B: Control experiment to demonstrate the specificity of the BrUTP labeling. UTP was used instead of BrUTP during run-on transcription. Labeling with anti-BrUTP antibodies was carried out as described in A. Only a few gold particles are observed, which indicates that the anti-BrUTP staining is specific for BrUTP-containing RNA. C: Transcription sites are localized mainly on the surface of electron-dense nuclear domains that contain short fibers with an apparent width of 20–30 nm (arrows). Cy, cytoplasm; No, nucleolus; N, nucleus. Bars: A, 1 μm; B, 0.5 μm; C, 0.2 μm.
were seen only rarely (Fig. 1B). Gold particles were sometimes found attached to cytoplasmic remnants after BrUTP labeling, as well as after UTP labeling. This was probably the result of aspecific binding of the gold-conjugated antibody.

Transcription Sites Are Located Preferentially on the Surface of Chromatin Domains

Clusters of gold particles were found throughout the nucleus, predominantly on the surface of domains of electron-dense material (Fig. 1A,C). In this dense material short 20–30 nm thick fibers were present (arrows in Fig. 2). Fibers of comparable size have been observed in similarly permeabilized CHO cells [Belmont and Bruce, 1994]. The electron-dense material probably represents tightly packed chromatin. This suggests that active loci are predominantly located at the surface of compact chromatin domains, i.e., on the border with the interchromatin space.

To exclude the possibility that the lack of labeling inside the electron-dense domains is the result of inaccessibility to antibodies, BrUTP-labeled nuclei were also subjected to post-embedding immunogold labeling. This labeling technique allows detection of antigens on the surface of a thin section, and should, therefore, be able to detect BrUTP also inside the dense nuclear structures corresponding to chromatin domains. Inspection of postembedding-labeled sections showed that transcription sites were indeed localized predominantly on the border of dense material (Fig. 2). The preferential localization of active genes on the surface of chromatin domains in direct contact with the interchromatin space has important implications for our understanding of the regulation of gene expression.

Fig. 2. Localization of nascent RNA in nuclei of A431 cells using a postembedding labeling procedure. Nascent RNA was labeled with BrUTP for 30 min during run-on transcription in permeabilized A431 cells. Cells were embedded in Epon, 100 nm sections were made, and sites of BrUTP incorporation were stained with antibodies conjugated to ultrasmall gold particles as described in Materials and Methods. The distribution of gold clusters is similar to that observed in pre-embedding-labeled sections: transcription sites are predominantly situated at the surface of electron-dense nuclear material, probably representing chromatin. Bar, 0.5 µm.
How Many Transcription Sites Are Present in A431 Nuclei?

The results presented thus far indicate that sites of transcription by RPII can be identified as clusters of gold particles. About 20 pre-embedment-labeled sections of different nuclei were analyzed to determine the average number of gold clusters per nucleus. A cluster was defined as containing ≥3 gold particles closely together, with a maximum distance between nearest neighbors of 50 nm. Data were corrected for background labeling determined in control nuclei that had been incubated under run-on conditions in the presence of UTP instead of BrUTP (Fig. 1B). After subtraction of background labeling, the number of clusters of gold particles varied considerably between nuclei, which is probably the result of differences in transcriptional activity and differences in labeling efficiency. We found on average five clusters of gold particles/µm² of nucleoplasm (range from two to seven). Since a section had a thickness of about 0.25 µm, an A431 nucleus contained on average about 20 (range 8–28) clusters/µm³. Given a total nuclear volume of about 500 (range 200–800) µm³, this implies that an average BrUTP-labeled A431 nucleus contained about 10,000 (range 2,000–22,000) gold particle clusters.

How does the number of clusters of gold particles relate to the number of active RPII molecules? On the assumption that there are about 20,000 active RPII molecules per cell [Cochet-Meilhac et al., 1974; Cox, 1976; Crerar and Pearson, 1977], each gold cluster represents the activity of, on average, about two RPII molecules. The number of gold particles in a cluster ranges from three to up to 20. If each actively transcribing RPII molecule has the same turnover number, the number of clustered gold particles decorating the nascent RNA at a transcription site is probably related to the number of elongating RPII molecules at that site. Given the broad range of number of gold particles in a cluster, some clusters may contain more than two active RPII molecules, transcribing either the same or different genes, and many clusters probably reflect the activity of not more than a single RPII molecule, i.e., only one gene. We did not observe local concentrations of clusters of gold particles. So, our results do not provide evidence for the concentration of several active genes in a specific higher order structure.

Localization of Active Genes in Nuclear Matrices

The nuclear matrix is the structure that remains after extracting most of the chromatin from the nucleus, and is thought to be involved in the functional organization of the interphase nucleus [Cook, 1988; Verheijen et al., 1988; Berezney, 1991; van Driel et al., 1991]. Depending on the isolation procedure that is used, other terms for this chromatin-depleted nuclear structure are nuclear scaffold [Mirkovitch et al., 1984] and nucleoskeleton [Jackson and Cook, 1985]. Actively transcribed genes are associated with the nuclear matrix [Ciejek et al., 1983; Jackson and Cook, 1985]. Moreover, at the light microscopical level, the spatial distribution of BrUTP-labeled transcription domains is retained in the nuclear matrix [Wansink et al., 1993]. The same results were obtained for A431 cells (results not shown). This indicates that the nuclear matrix plays a role in the spatial organization of transcription and RNA processing. It is not known how nascent RNA is localized with respect to the fibrogranular ultrastructure of the nuclear matrix.

To address this question, nascent RNA was labeled with BrUTP during run-on transcription in A431 cells, as described before. Subsequently, nuclear matrices were prepared for electron microscopy. As in intact nuclei, a labeling pattern consisting of clusters of gold particles was observed (Fig. 3). The diameter of the individual gold particles in nuclear matrices was often larger than in whole nuclei. This was probably caused by a more efficient penetration of the viscous silver enhancement buffer into the open structure of the nuclear matrix. In agreement with the data obtained at the light microscopical level [Wansink et al., 1993], the distribution of transcription sites in nuclear matrices at the ultrastructural level resembled the distribution of transcription sites in intact nuclei. In this case too no local concentrations of clusters of gold particles were observed. Clusters of gold particles were found about uniformly on the surface of granular structures and also on fibrous structures of the nuclear matrix (Fig. 3). At present, the nature of these structures is unknown, but our observations show that both contain nascent RNA.

Remnants of nucleoli in nuclear matrices also contained gold particles (Fig. 3), in spite of a lack of nucleolar labeling in intact nuclei (Fig. 1), which is probably caused by inaccessibility of
Fig. 3. Visualization of transcription sites in nuclear matrices by electron microscopy. Nascent RNA was labeled with BrUTP for 30 min during run-on transcription in permeabilized A431 cells. Nuclear matrices were prepared in situ, labeled with ultrasmall gold-conjugated antibodies, and then embedded as described in Materials and Methods. Shown is a detail of a nuclear matrix; an overall picture of this matrix is shown in the inset. The individual gold particles are relatively large in this preparation due to a more efficient silver enhancement in matrices compared to in whole nuclei. A dense labeling of predominantly clusters of gold particles was found throughout the nuclear matrix. These clusters were located on the surface of granular components (black arrows) as well as on fibrous components of the nuclear matrix (arrowheads). Also, transcription in nucleolar remnants (white arrow) is observed. L, nuclear lamina; No, nucleolar remnants. Bar, 0.5 μm.

DISCUSSION

We have investigated the spatial distribution of newly synthesized RNA at the ultrastructural level. RNA was labeled by incorporating BrUTP under run-on conditions in permeabilized cells and visualized with gold-conjugated antibodies. Sites of newly synthesized RNA appeared as clusters of gold particles, scattered throughout the nucleoplasm, except nucleoli. Several lines of evidence indicate that clusters of gold particles represent sites of nascent RNA synthesized by RPII, i.e., predominantly pre-mRNA.

1. Incorporation of BrUTP in nucleoplasmic RNA is completely prevented by 1 μg/ml α-amanitin [Wansink et al., 1993; Roeder, 1976].

2. No splicing of BrUTP-labeled RNA occurs in vitro [Wansink et al., 1994b], so the labeled sites cannot represent accumulated introns that had been spliced elsewhere in the nucleus.

3. Labeled transcripts are elongated by about 10% on average of their full length during our run-on
transcription conditions [Wansink et al., 1993], so gold-conjugated antibodies label predominantly sites of nascent RNA, rather than sites where RNA accumulates after transcription termination.

In the study presented here we have used permeabilized cells, incubated for 30 min under run-on transcription conditions. Upon permeabilization some nuclear components are extracted and structural changes of the nuclei may occur. This raises the question whether a spatial redistribution of transcription sites has occurred under our experimental conditions. Although we cannot fully exclude this possibility, we did not observe such a reorganization at the level of the light microscope [Wansink et al., 1993; Schul et al., unpublished observations] when we compared the spatial distribution of nascent RNA labeled with BrU in vitro, i.e., in permeabilized cells, and in vivo, i.e., after microinjection of BrUTP in intact cells.

Visualization of Transcription in the Nucleus on a Single Gene Level

The average number of gold particle clusters found per nucleus was 10,000. The number of clusters of gold particles is in the same order of magnitude as the number of active RPII molecules. Therefore, one gold particle cluster probably labels the nascent RNA synthesized by a single RPII molecule, or by only a few RPII molecules, transcribing the same or different genes. Hence, each cluster of gold particle labels nascent RNA transcribed from a single gene or at most a few genes. Detection of nascent RNA at a single gene level offers new possibilities to study many aspects of the synthesis, processing, and transport of pre-mRNA at the ultrastructural level.

By immunofluorescence microscopy, a punctate pattern of BrU-labeled nascent RNA has been observed in different cell types, consisting of several hundreds of fluorescent spots [Jackson et al., 1993; Wansink et al., 1993]. This pattern can be explained in two ways. The first possibility is that several RPII molecules, transcribing different active genes, are concentrated in a higher order structure, each corresponding to a fluorescent spot. In the second possibility, active genes are not organized in higher order structures. In that case one fluorescent spot probably reflects the activity of several genes of which the position of the corresponding nascent RNAs cannot be resolved by light microscopy, or the activity of a single gene that happens to be at a relatively large distance from its nearest neighbors. The study presented here supports the second possibility. The number of clusters of gold particles is one order of magnitude larger than the number of fluorescent transcription sites. Many clusters of gold particles are separated from neighboring clusters by less than about 0.25 μm, i.e., they are not resolved by light microscopy. No concentration of clusters of gold particles in domains was observed under the experimental conditions, neither in intact nuclei, nor in nuclear matrix preparations. In conclusion, the data presented here do not provide evidence for a higher organization of many active genes grouped together in discrete transcription domains.

Localization of Active Class II Genes in the Nucleus

Active genes are located more or less uniformly throughout the nucleoplasm, i.e., in the nuclear periphery as well as in the nuclear interior. However, we found that active genes are not distributed at random throughout the nucleus. They were predominantly located on the surface of electron-dense domains. These electron-dense areas probably correspond to domains of relatively condensed chromatin. We have shown by postembedding labeling that this specific localization was not due to inaccessibility of these structures to antibodies. A similar localization of transcription on the border of condensed chromatin was found after labeling cells in vivo with 3H-uridine followed by EMARG and regressive EDTA staining [Fakan and Bernhard, 1971; Fakan et al., 1976]. Together, these findings support a model in which transcription takes place on the surface of chromatin domains. The interior of these domains apparently corresponds to transcriptionally silent chromatin. Other experimental approaches have resulted in similar models with respect to the higher order organization of the cell nucleus [Cremer et al., 1993; Kramer et al., 1994]. It would be interesting to investigate by in situ hybridization the position of silent and active genes in chromatin domains. Transcription-dependent positioning of genes relative to chromatin domains and to the interchromatin space may represent a level of regulation of gene expression that has hitherto received little attention.
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