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Nuclear distribution of transcription factors in relation to sites of transcription and RNA polymerase II

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SUMMARY

We have investigated the spatial relationship between sites containing newly synthesized RNA and domains containing proteins involved in transcription, such as RNA polymerase II and the transcription factors TFIID, Oct1, BRG1, E2F-1 and glucocorticoid receptors, using dual immunofluorescence labelling followed by confocal microscopy on cultured cells. As expected, a high degree of colocalisation between the RNA polymerase II and sites containing newly synthesised RNA was observed. Like the newly synthesised RNA and the RNA polymerase II, we found that all the transcription factors that we studied are distributed more or less homogeneously throughout the nucleoplasm, occupying numerous small domains. In addition to these small domains, TFIID was found concentrated in coiled bodies and Oct1 in a single large domain of

about 1.5 μm in 30% of the cells in an asynchronous HeLa cell culture. Remarkably, we found little or no relationship between the spatial distribution of the glucocorticoid receptor, Oct1 and E2F-1 on the one hand and RNA polymerase II and transcription sites on the other hand. In contrast, a significant but incomplete overlap was observed between the spatial distributions of transcription sites and BRG1 and TFIID. These results indicate that many of the transcription factor-rich nuclear domains are not actively involved in transcription. They may represent incomplete transcription initiation complexes, inhibitory complexes, or storage sites.

Key words: Transcription, Nuclear organization, Confocal microscopy, BRG1, Oct1, E2F-1, TFIID, TFIIF, RNA polymerase II

INTRODUCTION

In the last two decades we have witnessed an explosive growth in our understanding of the molecular mechanisms that underlie replication, transcription and RNA processing. In contrast, our knowledge about the structure and functional organization of the interphase nucleus in which all these processes occur is still quite limited. Recently, Wansink et al. (1993) and Jackson et al. (1993) showed that in cultured mammalian cells transcription by RNA polymerase II is concentrated in numerous domains, scattered throughout the nucleoplasm. Furthermore, Van Steensel et al. (1995a, 1996) demonstrated that two transcription factors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), occur in many small clusters in the nucleus. A similar distribution was found for RNA polymerase II (Bregman et al., 1995; Van Steensel et al., 1995a). Remarkably, the spatial distribution of the clusters of GRs was different from that of the sites of transcription and that of RNA polymerase II (Van Steensel et al., 1995a), whereas a partial, though significant colocalization was observed for the GR and MR clusters in rat hippocampus cells (Van Steensel et al., 1996).

In this paper we analyze the nuclear localization of several other transcription factors in relation to the spatial distribution of RNA polymerase II and of transcription sites. We focus on the basal transcription factors TFIID (p62) and TFIIF (RAP74)

and the specific transcription factors Oct1, E2F-1, the glucocorticoid receptor and BRG1.

The basal transcription factors TFIID and TFIIF are required for transcription by RNA polymerase II. Naively, one would expect a significant colocalization between these regulatory proteins and transcription sites. Oct1 is a transcription factor necessary for producing RNA from some highly active genes, like the snRNA genes (Murphy et al., 1989; Sive and Roeder, 1986; Tanaka et al., 1988) and histone H2B, which is highly transcribed only during S-phase (Fletcher et al., 1987; Heintz, 1991). E2F is a factor involved in the regulation of the cell-cycle (reviewed by Lathangue, 1994; Muller, 1995; Weinberg, 1995). Its transacting activity is inhibited by association with the hypophosphorylated form of the retinoblastoma tumour suppressor protein (pRb), or its relatives p107 and p130 (Cao et al., 1992; Cobrinik et al., 1993; Helin et al., 1993; Hiebert et al., 1992). Multiple E2F family members have been identified. Here we use an antibody against E2F-1 (Dyson et al., 1993). BRG1 is one of the two known human homologues of the yeast SNF2/SWI2 protein and the brahma protein in *Drosophila*, the other one being hbrm (Khavari et al., 1993; Muchardt and Yaniv, 1993). This protein is thought to activate transcription through remodelling chromatin structure (Hirschhorn et al., 1992; Kruger and Herskowitz, 1991; Kruger et al., 1995; Kwon et al., 1994; Prelich and Winston, 1993).

We show that the transcription factors BRG1, Oct1, E2F-1,

TFIIH and also TFIIF are distributed in a similar punctate pattern in nuclei of HeLa cells, similar to what is observed for the GR and the MR (Van Steensel et al., 1995a, 1996). In addition, Oct1 occurs in about 30% of the cells in high local concentration in a 1 to 1.5 μm diameter nuclear domain of unknown function. Similarly, TFIH and TFIIF are concentrated in nuclear foci that we identified as coiled bodies. Remarkably, we find little or no relationship between the spatial distribution of the GR, Oct1 and E2F-1 on the one hand and RNA polymerase II and transcription sites on the other hand. In contrast, a significant but incomplete overlap was observed between the spatial distributions of transcription sites and BRG1 and TFIH, as well as between RNA polymerase II and TFIH.

MATERIALS AND METHODS

Cell culture

HeLa (human cervix carcinoma, ATCC) cells were grown at 37°C in a 10% CO₂ atmosphere in DMEM (Gibco, Breda, the Netherlands), supplemented with 10% (v/v) heat-inactivated FCS (Boehringer, Almere, the Netherlands), 2 mM L-glutamine (Gibco), 100 i.u./ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco). Fibroblasts (F3035) were grown at 37°C under a 5% atmosphere in DMEM/HAMF10 (Gibco), supplemented with 10% heat-inactivated FCS and the additions mentioned above. Where indicated, the following transcriptional inhibitors were added to the culture media at the indicated final concentrations and times: α -amanitin (50 $\mu\text{g}/\text{ml}$, 3 hours), 5,6-dichloro-1 β -D-ribofuranosyl benzimidazole (DRB) (50 μM , 2 hours) or actinomycin D (10 $\mu\text{g}/\text{ml}$, 1 hour) (all from Sigma, Bornem, Belgium).

Fixation and immunocytochemistry

Cells were grown on coverslips for 48 hours and fixed, directly or after *in vivo* incorporation of microinjected BrUTP, in 2% formaldehyde in PBS for 15 minutes at room temperature. Then they were permeabilised for 10 minutes in 0.5% Triton X-100 in PBS, incubated for 10 minutes in 100 mM glycine in PBS to inactivate free aldehyde groups and aspecific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 10 minutes, followed by 5 minutes in PBG: PBS containing 0.5% (w/v) BSA and 0.1% (w/v) gelatin from cold water fish skin (Sigma, Bornem, Belgium). Incubations with the primary antibody were done in PBG overnight at 4°C. The coverslips were subsequently washed four times for 5 minutes in PBG and incubated with the corresponding secondary antibodies for 1.5 hours in PBG at room temperature. Secondary antibodies were TRITC-conjugated donkey anti-rat, FITC- or cy3-conjugated donkey anti-rabbit, biotinylated donkey anti-rabbit, DTAF- or TRITC-conjugated donkey anti-mouse (all from Jackson ImmunoResearch Laboratories) or biotinylated sheep anti-mouse (Amersham, Amersham, UK). If a biotinylated secondary antibody was used, coverslips were washed four times 5 minutes in PBG, followed by 30 minutes FITC- or cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories). Next, coverslips were washed two times 5 minutes in PBG, two times 5 minutes in PBS, 10 minutes in PBS containing 0.4 $\mu\text{g}/\text{ml}$ Hoechst 33258, or 1 $\mu\text{g}/\text{ml}$ propidium iodide and 10 minutes in PBS. Coverslips were mounted in Vectashield (Vector Labs). Primary antibodies and sera were: rabbit anti-Oct1 (Zwilling et al., 1994), rabbit anti-BRG1 (Khavari et al., 1993), rabbit anti-TFIIF(RAP74) (Yonaha et al., 1993), rabbit pab57 against the glucocorticoid receptor (Cidlowski et al., 1990), rabbit anti-p80-coilin (Bohmann et al., 1995b), monoclonal KH20 anti-E2F-1 (Dyson et al., 1993), a monoclonal antibody against the p62 subunit of TFIH (Schaeffer et al.,

1994), monoclonal H5 against hyperphosphorylated RNA polymerase II, monoclonal antibody H14 against hypo-, intermediate- and hyper phosphorylated RNA polymerase II (Bregman et al., 1995), and monoclonal mouse anti-NuMa (Compton et al., 1992). Rat anti-BrdU (Sera-lab, Crawley Down, UK) was used for detection of BrdU incorporated in DNA and BrU in nascent RNA.

Specificity of antibodies

Immunoblots of whole cell extract were used to confirm the specificity of the antibodies against the transcription factors we used. To prepare whole cell extract, HeLa cells were grown in a culture flask, harvested with trypsin, washed with PBS containing 5% BSA to block the trypsin, washed twice with PSB and subsequently dissolved in boiling SDS-PAGE sample buffer to which the following proteinase inhibitors were added: 5 $\mu\text{g}/\text{ml}$ trypsin inhibitor, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM PMSF and 1 mM EGTA. Proteins were then separated with SDS-PAGE and transferred to nitrocellulose. Blots were immunostained as described by van Steensel et al. (1995b). For E2F-1, Oct1, TFIH and TFIIF no cross-reactivity of any of the antibodies was observed. The anti-BRG1 antibody did show crossreactivity in whole cell extract. To prove that the cross-reacting proteins are located in the cytoplasm, a cell extract was prepared from cells which had been permeabilised with 40 $\mu\text{g}/\text{ml}$ digitonin in the presence of the proteinase inhibitors mentioned above, prior to the addition of the SDS-PAGE sample buffer. Omission of any of the primary antibodies resulted in complete loss of the fluorescent signal. Nuclear labelling with anti-BrU antibody was observed only in cells that had been micro-injected with BrUTP; no signal was detectable in control cells. Sensitivity of BrU labelling of nascent RNA to α -amanitin and to RNase has been demonstrated earlier (Wansink et al., 1993).

Microinjection

Over a period of 10 minutes, 100 mM BrUTP was injected into approximately 100 non-confluent HeLa or fibroblasts cells grown on Cellocate coverslips (Eppendorf, Hamburg, Germany). Cells were cultured for 10 minutes at 37°C to allow incorporation of the BrUTP in the RNA, fixed and labelled as described above.

Confocal laser scanning microscopy

Images were collected with a Leica confocal laser scanning microscope, equipped with a 488/514 dual-band argon-ion laser and an oil-immersion objective ($\times 100$, NA=1.32). Fluorescence was detected using a 525DF10 bandpass filter for FITC or DTAF and a 550 nm long pass filter for TRITC or cy3. Pairs of images were collected simultaneously in the green and red channels. Images were collected as single optical sections or as 512 $\times$ 512 $\times$ 32 voxel images (sampling distance 49 nm lateral and 208 nm axial).

Image processing and image analysis

For image processing and analysis the software package SCILIMAGE was used (Van Balen et al., 1994). Optical crosstalk was quantified and subtracted as described previously (Manders et al., 1992). To enhance the effective resolution of the confocal images, we applied a novel restoration technique, which corrects for diffraction-induced distortions in the image (Van der Voort and Strasters, 1995). To quantitatively analyze the relationship between the spatial distribution of two complex nuclear 3-D distributions, we used a cross-correlation method, recently developed by Van Steensel et al. (1996). Briefly, in this method the two 3-D images are shifted with respect to each other over a distance of n voxels along the x -axis. After each shift ($n=1,2,3$ etc) the overlap between the two images is calculated, expressed as Pearson's coefficient r_p (Gonzalez and Wintz, 1987). When r_p is plotted versus the shift n , a correlation function (CCF) is generated. As demonstrated earlier (Van Steensel et al., 1996), r_p is independent of the shift n if the two distribution are unrelated. In contrast, if r_p shows a maximum at $n=0$ the overlap between the distributions is more than expected for unrelated distribution. Likewise, if the CCF

shows a minimum around $n=0$, the two distributions (partially) exclude each other.

RESULTS

Specificity of antibody labelling

The conclusions that we draw from this work about the spatial distribution of different components of the transcription machinery, using immunolabelling, critically depend on the specificity of the antibodies used. Fig. 1 shows immunoblots of the antibodies tested on whole cell extracts: anti-BRG1 (lane 1), anti-E2F-1 (lane 3), anti-Oct1 (lane 4), monoclonal antibody against the p62 subunit of TFIIF (lane 5) and antibodies against the 74 kDa subunit of TFIIF (lane 6). Blots showing the specificity of the RNA polymerase II antibodies and the glucocorticoid receptor are not shown, since their monospecificity has already been shown elsewhere (Cidlowski et al., 1990; Bregman et al., 1995). Results show that the antibodies against E2F-1, TFIIF, and TFIIF are monospecific. The double band shown for anti-Oct1 in lane 4 is most likely due to different phosphorylation states, as was shown by Roberts et al. (1991). Lane 1 shows that in addition to the BRG1 protein of 205 kDa (Khavari, 1993) some other protein bands are detected by the BRG1 antibody. These cross reacting bands most likely represent soluble cytoplasmic proteins, since (i) immunofluorescent labelling with the anti-BRG1 antibody shows nuclear staining as well as some cytoplasmic labelling (see Figs 2A and 3C), (ii) if cells are permeabilised with 40 µg/ml digitonin prior to fixation, the cytoplasmic labelling disappears, while the nuclear labelling appears unaltered, (iii) an immunoblot prepared from digitonin-permeabilised cells shows that under these conditions only the 205 kDa protein remains, while all cross reacting protein bands disappear (Fig. 1, lane 2), and (iv) the antibody recognizes only the 205 kDa band in immunoblots prepared from nuclear extracts of several tissues and primary cultures (Khavari et al., 1993). We concluded that nuclear labelling by the anti-BRG1 antibody is entirely due to recognition of the 205 kDa BRG1 protein.

Many transcription factors are located in numerous small domains throughout the nucleoplasm

We have compared the spatial distribution of several transcription factors, RNA polymerase II and nascent RNA by confocal microscopy after dual indirect immunofluorescent labelling. Before analysing the relationship between these complex 3-D distributions it was necessary to carry out image restoration in order to correct for diffraction-induced and other distortions due to microscope hardware (Van der Voort and Strasters, 1995). Subsequently, the relationship between two distributions was analyzed by a cross correlation method developed recently by Van Steensel et al. (1996).

Fig. 2 shows the distribution of BRG1 (A), Oct1 (B), TFIIF(p62) (C), glucocorticoid receptor (GR) (D), E2F-1 (E) and TFIIF(RAP74) (F). All transcription factors occupied numerous, often interconnected, small domains, throughout the nucleoplasm and were excluded by the nucleoli. In addition to these small domains, TFIIF and TFIIF labelling was found in several foci (usually about 3) per cell in almost all HeLa cells. The Oct1 antibody showed labelling of a large (1.5 µm) domain, which was often located close to a nucleolus, in about

Fig. 1. Immunoblots prepared from whole cell extract were used to determine the monospecificity of the antibodies used in this paper: anti-BRG1 (lane 1), anti-E2F-1 (lane 3), anti-Oct1 (lane 4), an antibody against the p62 subunit of TFIIF (lane 5) and antibodies against the 74 kDa subunit of TFIIF (lane 6). For anti-BRG1, an additional immunoblot was prepared from digitonin permeabilised cells (lane 2).

30% of the cells. E2F-1, BRG1 and Oct1 also showed some cytoplasmic labelling.

To check whether the observed distributions were dependent on the fixation method, we repeated all immunolabellings after fixation in acetone, instead of formaldehyde. For TFIIF, TFIIF, GR, Oct1 and BRG1 indistinguishable distributions were observed after the two fixation methods (data not shown). For E2F-1, acetone fixation destroyed nuclear labelling. To analyze if any of the observed distributions was specific for HeLa cells (a tumour cell line), we also investigated the distribution of these transcription factors in human skin fibroblasts (a non-tumour cell line). The distribution of the factors was very similar for both cell lines (data not shown). The only difference was that in fibroblasts TFIIF and TFIIF did not show the two to five foci observed in HeLa cells and the Oct1 antibody labelled several smaller nuclear domains, i.e. four to six of 0.5–1 µm diameter, rather than one domain of 1.5 µm in HeLa cells.

We conclude that all transcription factors investigated here are localised in numerous, often interconnected small domains throughout the nucleoplasm, excluding the nucleoli. In addition to these small domains TFIIF and TFIIF are concentrated in one to three additional foci and Oct1 is present also in a single large domain.

Fig. 2. Distribution of (A) BRG1, (B) Oct1, (C) TFIID(p62), (D) glucocorticoid receptors, (E) E2F-1 and (F) TFIID(RAP74) in HeLa cells. Single confocal sections are shown. Bar, 2 μ m.

Dual labelling of sites of transcription and the transcription factors BRG1, E2F-1, TFIID and Oct1

The punctate distributions of Oct1, E2F-1, TFIID and BRG1 resemble that of nascent RNA (Jackson et al., 1993; Wansink et al., 1993). Therefore, we compared the spatial distribution of nascent RNA with that of these transcription factors by dual-labelling immunocytochemistry and confocal microscopy. Sites of active transcription were labelled by microinjection of the UTP-analogue BrUTP, after which the cells were cultured for ten minutes to allow incorporation of the BrUTP into the nascent RNA. Fig. 3A-D shows single confocal sections of HeLa nuclei that had been double labelled with BrUTP (red) and (in green) BRG1 (A), Oct1 (B), TFIID (C) and E2F-1 (D). These images show that, although there is some overlap between the sites of transcription and the domains containing the different transcription factors, many

of transcription factor domains show little or no BrUTP labelling and vice versa. In fibroblasts very similar results were obtained (data not shown). In all nuclei some BrUTP labelling can be observed inside the nucleoli. This represents transcription by RNA polymerase I.

To analyze the dual labelled images we used two tools: (i) cross correlation analysis between the two 3-D distributions (Van Steensel et al., 1996); and (ii) linescans. In a linescan the intensity values along a straight line through the dual image (in the x,y plane) is plotted. Such analysis shows whether or not areas of high local signal intensity in one image coincide with those in the other image. The cross correlation function (CCF) is used to establish whether or not a relationship exists between the two complex 3-D distributions in a dual labelled image, i.e. whether two images overlap more, or less than expected for two non-related images (Van Steensel et al., 1996).

BRG1 and sites of transcription. (G,H) CCF and linescan of Oct1 and sites of transcription. Asterisk indicates the position of the large Oct1-containing domain. (I,J) CCF and linescan of TFIID and sites of transcription. (K,L) CCF and linescan of E2F-1 and sites of transcription.

Fig. 3E,G,I and K show CCFs that were obtained by comparing the 3-D distribution of the sites of transcription with that of BRG1 (E), Oct1 (G), TFIID (I), E2F-1 (K), respectively. The maximum in the CCFs at zero shift in Fig. 3E and I shows that the distributions of BRG1 and TFIID have more overlap with that of nascent RNA than expected for non-related distributions of the same complexity. This conclusion is supported by the linescans Fig. 3F and J, which show that in some cases local maxima in the dual labelled images coincide. However, it is evident that this coincidence is far from complete. Fig. 3G and K show that the spatial distributions of Oct1 and E2F-1 do not show a clear relationship with the distribution of nascent RNA. These conclusions are confirmed by the line scans in Fig. 3H and L, which show that local maxima in the distribution of nascent RNA almost never coincide with local maxima in concentration of E2F-1 or Oct1. The position of the linescans has been marked in Fig. 3A-D by white arrows. The linescan in Fig. 3H shows that the large domain with local high concentration of Oct1 in Fig. 3B (position in Fig. 3H marked with an asterisk) does not correspond to a region with a high rate of transcription. The dis-

tributions of the four transcription factors do not change significantly if RNA synthesis is inhibited by α -amanitin (50 μ g/ml, 3 hours), DRB (50 μ M, 2 hours) or actinomycin D (10 μ g/ml, 1 hour) except that TFIID disappeared from its nuclear foci (data not shown). From these experiments we conclude that a large fraction of the transcription factors analysed here is not involved in transcription activation.

RNA polymerase II and sites of transcription

Most of the nascent RNA that is labelled with BrUTP is the product of RNA polymerase II (Wansink et al., 1993). Therefore, we investigated the relationship between the localization of nascent RNA and RNA polymerase II. Two major forms of RNA polymerase II can be distinguished: (i) PolII₀, which is hyperphosphorylated predominantly on the ser and thr residues of the C-terminal domain and (ii) PolII_A, the hypophosphorylated form (Dahmus, 1981; Zhang and Corden, 1991). PolII₀ is considered to be the active form of RNA polymerase II (Cadena and Dahmus, 1987; Laybourn and Dahmus, 1990; Payne et al., 1989). We used two antibodies, i.e. H5 recognizing PolII₀, and H14 recognizing

Fig. 4. (A,B) Dual labelling in HeLa cells of sites of transcription (red) with (A) RNA polymerase II (green) and (B) hyperphosphorylated RNA polymerase II (PolIII0 in green) in HeLa cells. (C-F) Dual labelling of hyperphosphorylated RNA polymerase II (PolIII0 in red) and in green: (C) BRG1, (D) Oct1, (E) TFIH and (F) glucocorticoid receptors. Areas marked by a rectangle are shown four times enlarged as insets. Left inset is red signal only, middle inset is green signal only, right inset is overlay. Arrowhead in left (red) inset of panel B indicates a

domain that contains newly synthesised RNA only, arrowhead in middle (green) inset of B indicates a domain that contains both PolIII0 and newly synthesised RNA. Bars: 2 μ m in all main panels, and 0.3 μ m in all insets. (G-H) CCF and linescan of RNA polymerase II and sites of transcription. (I,J) CCF and linescan of hyperphosphorylated RNA polymerase II (PolIII0) and sites of transcription. (K,L) CCF and linescan of PolIII0 and BRG1. (M,N) CCF and linescan of PolIII0 and Oct1. (O,P) CCF and linescan of PolIII0 and TFIH. (Q,R) CCF and linescan of PolIII0 and the glucocorticoid receptor.

PolIII0, PolIIIA and intermediate phosphorylation states of RNA polymerase II (Bregman et al., 1995). Fig. 4 shows nuclei labelled with BrUTP (A,B, red) and H14 (A, green) and H5 (B, green). The distributions of the two forms of RNA

polymerase II do not differ significantly, although PolIII0 labelling appears to be more discrete than PolIIIA labelling and the local maxima in the PolIIIA distribution seem more interconnected. The overall intensity of both labellings varies

Fig. 5. Nuclei of HeLa cells dual labelled for (A) TFIIF (red) and TFIIF (green) and for (B) E2F-1 (red) and the glucocorticoid receptor (green). Cells labelled for the GR were pre-treated with 100 nM dexamethasone for one hour to induce nuclear localisation of the receptor. Insets are four times enlargements of the indicated boxes. Left inset is red image only, middle inset is green image only, right inset is overlay image. In A a second inset (also four times enlargement) shows an overlay dual image of a coiled body labelled for the two transcription factors. Bars: 2 μ m in all main panels and 0.3 μ m in all insets. Linescans in E and F were taken at the position indicated by the arrowheads in images A and B, respectively. (C,E) CCF and linescan of TFIIF and TFIIF dual labelled image. (D,F) CCF and linescan of E2F-1 and the GR dual labelled image.

from cell to cell. Contrary to the findings of Bregman et al. (1995) we found no labelling of speckles in HeLa cells with either of the two anti-RNA polymerase II antibodies. With each of the antibodies we found that RNA polymerase II-containing domains colocalise with sites of transcription and vice versa (e.g. see arrow in enlarged overlay insert in Fig. 4B). However, there are also many domains that are strongly labelled by anti-RNA polymerase II and contain little or no BrUTP label, and vice versa (see enlarged insets in Fig. 4A,B). The linescans presented in Fig. 4H and J confirm that that local maxima of RNA polymerase II often, but not always, coincide with maxima in nascent RNA. Especially for hyperphosphorylated RNA polymerase II (PolIII0) we observed many domains that contain nascent RNA and little or no PolIII0 (arrow in the red insert of Fig. 4B) and domains that contain PolIII0 and no nascent RNA (arrow in the green insert of Fig. 4B). Cross correlation analysis (CCFs in Fig. 4G and I) strongly supports the notion that the spatial distribution of RNA polymerase II (as detected by each of the two antibodies) is strongly and positively related to the spatial distribution of nascent RNA.

Dual labelling of BRG1, TFIIF, Oct1 and the GR with RNA polymerase II

We investigated the spatial relationship between the nuclear distribution of BRG1, Oct1, TFIIF and the GR with RNA polymerase II, using antibody H5 that detects only the hyperphosphorylated form of the enzyme. Results in Fig. 4C-F show that there is no clear relationship between at the one hand the distributions of BRG1 (C, green), Oct1 (D, green) and the GR (F, green) and at the other hand that of PolIII0 (red in Fig. 4C,D,E,F). This conclusion is confirmed by cross correlation analysis (CCFs in Fig. 4K,M,Q) and the line scans (Fig. 4L,N,R). In contrast, the spatial distribution of the general transcription factor TFIIF (Fig. 4E) overlaps significantly with that of RNA polymerase II. Inspection of the line scan (Fig. 4P) and the CCF (Fig. 4O) confirms this. Note that the large Oct1-containing domain visible in Fig. 4D does not coincide with a domain containing PolIII0. The same results were obtained with the antibody H14, which recognizes the hypophosphorylated and all other phosphorylated states of the enzyme (not shown).

Thus, most of the specific transcription factors GR, BRG1

Fig. 6. TFIIF is concentrated in coiled bodies. HeLa cells were dual labelled with an antibody against p80-coilin, a protein specific for coiled bodies (A) and an antibody against TFIIF (B). Bar, 2 μ m.

and Oct1 do not appear to be part of an active or inactive transcription complex that contains RNA polymerase II. In contrast, the basal transcription factor TFIIF shows significant colocalization with the RNA polymerase.

Different transcription factors generally occupy separate sites

The punctate distributions of the transcription factors are very similar. We wondered how these proteins are localized with respect to each other. Therefore, we dual-labelled HeLa cells for: (i) TFIIF and TFIIF, two basal transcription factors (Fig. 5A); and (ii) the GR and E2F-1 (Fig. 5B). Surprisingly, the punctate distributions of TFIIF and TFIIF hardly showed any relationship, as can be appreciated from the cross correlation analysis and line scans (Fig. 5C and D, respectively). Only in the foci both transcription factors were present in high concentration. The punctate distributions of the GR and E2F-1 also showed little or no relationship, as can be concluded from the CCF and the line scans (Fig. 5E and F, respectively).

TFIIF and TFIIF are concentrated in coiled bodies and the large Oct1-containing domain is not specific for cells in S-phase

We have investigated the foci that are strongly labelled by anti-TFIIF and anti-TFIIF and the large domain that is enriched in Oct1 in some more detail. Using an antibody against p80 coilin, a protein that is exclusively localized in coiled bodies (Bohmann et al., 1995a), we were able to show that the TFIIF/TFIIF foci correspond to coiled bodies (Fig. 6A,B).

Identification of the structure labelled by Oct1 (see Figs 2B, 3B and 4D) is more difficult. The Oct1 domain was present in about 30% of the HeLa cells in an asynchronous cell culture. Since this represents approximately the percentage of cells in S-phase, and Oct1 is involved in transcription of histon H2B, a gene which is highly expressed in S-phase, we investigated whether the presence of this domain is typical for S-phase cells. To this end we labelled a HeLa cell culture using the thymidine analogue bromodeoxyuridine (BrdU), combined with an antibody recognizing incorporated BrdU. The cells were dual labelled with anti-Oct1. We did not find any relationship between cells in S-phase (recognized by strong BrdU labelling) and the presence of the Oct1 domain. Oct1-rich domains could be detected in both replicating and non-replicating cells (data not shown). The frequency of occurrence of the Oct-1 domain

(30% of the cells), its position (often close to the nucleolus) and its size do resemble nuclear domains labelled by anti-hnRNP-L (Pinol-Roma et al., 1989) and anti-hnRNP-I (Ghetti et al., 1992). To investigate whether the Oct1 domain is enriched in hnRNP-L or hnRNP-I, dual-labelling experiments with anti-Oct1 and anti-hnRNPL and anti-hnRNPI were carried out. The results showed that the antibodies label different nuclear structures (data not shown). Thus we conclude that in HeLa cells TFIIF and TFIIF are concentrated in coiled bodies and the Oct1 antibody labels a novel nuclear domain.

DISCUSSION

Immunofluorescent labelling combined with 3-D confocal microscopy, shows that in nuclei of HeLa cells RNA polymerase II and the transcription factors BRG1, Oct1, E2F-1, GR, TFIIF and TFIIF are distributed in punctate patterns throughout the nucleoplasm excluding the nucleoli. These distributions are similar to those observed for the glucocorticoid receptor (GR) and the mineralocorticoid receptor in other cell types and in hippocampus neurons (Van Steensel et al., 1994, 1995a, 1996) and that of nascent RNA (Jackson et al., 1993; Wansink et al., 1993). However, careful analysis of images of dual labelled cells showed consistently that there is remarkably little relationship between the spatial distributions of the transcription factors on the one hand and RNA polymerase II and nascent RNA on the other hand. This conclusion is based on: (i) visual inspection of the dual labelled 3-D images; (ii) cross correlation analysis of the two images in a dual labelled image pair; and (iii) line scans in which density profiles along a straight line in the dual image are compared. In contrast, a significant but incomplete colocalization was found: (i) between sites of transcription (marked by incorporation of Br-UTP into nascent RNA) and RNA polymerase II (using two different antibodies); (ii) between the basal transcription factor TFIIF and nascent RNA and RNA polymerase II; and (iii) between the transcription factor BRG1 and sites of transcription. The spatial distributions of the other transcription factors did not colocalize more with sites of transcription and with sites containing RNA polymerase II, than is expected for non-related, random spatial distributions of the same complexity. A similar observation was made by Van Steensel et al. (1995a) for the GR and nascent RNA in T24 (human bladder carcinoma) cells.

From these observations we conclude that in HeLa cells a large fraction of the transcription factor molecules in the nucleus is neither part of an active transcription complex, nor of an inactive complex containing RNA polymerase II. Only a relatively small fraction of the transcription factor molecules that we analysed here seems to be located at transcription sites. It is unclear what the function is of the numerous transcription factor-rich domains in the nucleus that do not contain RNA polymerase II or nascent RNA. Some of the domains that contain TFIIF may play a role in DNA repair, since TFIIF is an essential component of the nucleotide excision repair machinery (reviewed by Lehmann, 1995 and Maldonado et al., 1996). Alternatively, the domains may represent incomplete transcription initiation complexes or inhibitory complexes, most of them evidently lacking RNA polymerase II. Alternatively, transcription factor-rich domains may be storage sites from which proteins can be recruited if necessary. If so, we have to assume that such storage sites are, at least to some extent, transcription factor-specific, since no significant colocalization was found between TFIIF and TFIIF and between the GR and E2F-1. For that same reason it is unlikely that a large fraction of the transcription factor molecules diffuses freely and intermingles in the interchromatin space. Interestingly, Van Steensel et al. (1996) recently showed that in hippocampus neurons, GR-rich and mineralocorticoid-rich domains partially colocalized. It was speculated that this is related to the fact that in those cells these two transcription factors cooperate in the regulation of gene expression. For at least one type of nuclear component evidence has been presented that a considerable fraction of the molecules accumulates in specific storage compartments, i.e. the splicing machinery. Many splicing components are concentrated in so called speckle domains from which they can be recruited when necessary (Huang and Spector, 1996a,b).

RNA polymerase II was detectable at essentially all sites of transcription. The local ratio of nascent RNA and RNA polymerase II signals varied considerably from site to site in a nucleus. This can be due to several factors: (i) different rates of elongation; several factors are known to influence this rate (Bentley, 1995; Jones and Peterlin, 1994; Krumm and Groudine, 1995; Yankulov et al., 1994). (ii) Size of genes; short genes may re-initiate faster and thus have more associated RNA polymerase II. (iii) Pausing of RNA polymerase II complexes. Recent findings suggest that promoter-proximal pausing of RNA polymerase II defines a rate-limiting step after transcription initiation (Krumm and Groudine, 1995). Furthermore, RNA polymerase II in *Drosophila* has been found associated with many non-transcribed genes (Weeks et al., 1993). (iv) Initiation of transcription during the RNA labelling period, resulting in domains containing little RNA, but a relatively high concentration of RNA polymerase II. During our 10- to 20-minute RNA labelling period we also expect transcription termination to occur. We cannot exclude the possibility that during this period some newly synthesized RNA is transported away from the site of synthesis. However, considering the average time for synthesis of a pre-mRNA molecule (about 8 minutes, based on average transcription rate of 20 nt/second and an average transcript size of 10 kb), this is unlikely to be a quantitatively important effect. An interesting additional explanation for the variation of local RNA/RNA polymerase II ratios, is the idea that transcription of genes is discontinuous,

as has been proposed by Ross et al. (1994). In this hypothesis transcription in eukaryotic cells is proposed to occur in short bursts, interspersed with periods of inactivity. According to this hypothesis sites containing newly synthesised RNA represent sites where one or more short bursts occurred in the RNA labelling period after microinjection of BrUTP. If this model is correct, the local relative amounts of RNA polymerase and nascent RNA will strongly depend on the frequency of local transcriptional bursts.

Remarkably, the general transcription factor TFIIF significantly codistributes with RNA polymerase II and with sites containing nascent RNA, whereas BRG1, which is probably also part of a large fraction of the transcription complexes, colocalizes significantly with only transcription sites and not with RNA polymerase II. This suggests that BRG1 is selectively present at sites of transcription that contain a relatively small number of RNA polymerase II molecules.

Unexpectedly, we found that in HeLa cells Oct1 is present in high concentration in a 1 to 1.5 µm diameter subnuclear domain and that TFIIF and TFIIF are present in high concentration in foci that we identified as coiled bodies. The function of coiled bodies (for a review see Lamond and Carmo-Fonseca, 1993) is still unknown. TFIIF and TFIIF add to the growing list of proteins that are found to be present in these subnuclear structures (Bohmann et al., 1995a). The Oct1-rich domain is observed in about 30% of the cells in unsynchronized HeLa cell cultures, and differs from the large domains labelled by hnRNP-L and hnRNP-I, and thus probably represents a novel nuclear domain. We showed that its occurrence is not related to S-phase. In fibroblasts several smaller Oct1-rich domains are observed, rather than one large one.

Summarizing, our results shed new light on the spatial distribution of nuclear components involved in transcription by RNA polymerase II. RNA polymerase II and TFIIF show significant colocalisation with sites of transcription, whereas only a small fraction of the four transcription factors examined appear to be directly involved in transcription. The majority of these protein molecules seem to be idling either in compartments that have a storage function or are bound specifically or non-specifically at sites of which the function is presently unknown.

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