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Localization of the glucocorticoid receptor in discrete clusters in the cell nucleus

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SUMMARY

The cell nucleus is highly organized. Many nuclear functions are localized in discrete domains, suggesting that compartmentalization is an important aspect of the regulation and coordination of nuclear functions. We investigated the subnuclear distribution of the glucocorticoid receptor, a hormone-dependent transcription factor. By immunofluorescent labeling and confocal microscopy we found that after stimulation with the agonist dexamethasone the glucocorticoid receptor is concentrated in 1,000-2,000 clusters in the nucleoplasm. This distribution was observed in several cell types and with three different antibodies against the glucocorticoid receptor. A similar subnuclear distribution of glucocorticoid receptors was found after treatment of cells with the antagonist RU486, suggesting that the association of the glucocorticoid receptor

in clusters does not require transformation of the receptor to a state that is able to activate transcription. By dual labeling we found that most dexamethasone-induced receptor clusters do not colocalize with sites of pre-mRNA synthesis. We also show that RNA polymerase II is localized in a large number of clusters in the nucleus. Glucocorticoid receptor clusters did not significantly colocalize with these RNA polymerase II clusters or with domains containing the splicing factor SC-35. Taken together, these results suggest that most clustered glucocorticoid receptor molecules are not directly involved in activation of transcription.

Key words: nuclear domain, matrix, steroid receptor, transcription, hormone response element, confocal microscopy

INTRODUCTION

The glucocorticoid receptor (GR) belongs to a large family of ligand-dependent transcription factors. Its molecular working mechanism is fairly well understood. After hormone binding the GR is localized in the nucleus and controls the expression of specific genes by binding to regulatory DNA sequences, named hormone response elements (HREs). Chromatin structure and interactions with other transcription factors are important additional elements that play a role in the regulation of gene expression by the GR (Adler et al., 1992; Hayes and Wolffe, 1992; Pearce and Yamamoto, 1993; Truss and Beato, 1993).

Many nuclear functions and components are concentrated in discrete domains (de Jong et al., 1990; van Driel et al., 1991; Spector, 1993). For example, splicing factors and poly(A)⁺ RNA are concentrated in 20-50 nucleoplasmic speckles (Fu and Maniatis, 1990; Spector, 1990; Carter et al., 1993). Also replication activity is localized in discrete nuclear domains (Berezney, 1991b). Synthesis of rRNA and assembly of ribosomes take place in the nucleolus (Scheer et al., 1993). In

addition, a number of nuclear domains with unknown function have been described, such as coiled bodies (Lamond and Carmo-Fonseca, 1993) and other nuclear bodies (Stuurman et al., 1992). Compartmentalization obviously is a general principle of nuclear organization and is probably a key mechanism in the regulation and coordination of nuclear functions.

Recently, a new technique was developed to visualize sites of synthesis of pre-mRNA in the nucleus (Jackson et al., 1993; Wansink et al., 1993). This technique is based on the incorporation of the nucleotide analogue 5-bromouridine 5'-triphosphate (BrUTP) into nascent RNA. Sites of incorporation are visualized by immunofluorescence microscopy using a monoclonal antibody that specifically recognizes bromouridine. Using this method a punctate nuclear staining is observed. This indicates that transcription by RNA polymerase II (RPII) is concentrated in specific domains in the nucleoplasm. However, little is known about the subnuclear location of components of the transcription machinery, such as RPII and transcription factors.

Many observations suggest that transcription is associated

with the nuclear matrix. The nuclear matrix is operationally defined as the residual nuclear structure that remains after extraction of more than 90% of the chromatin and all soluble and loosely-bound components (Cook, 1988; Verheijen et al., 1988; Berezney, 1991a; van Driel et al., 1991). It consists of a peripheral lamina and a fibrogranular internal network. The residual DNA present in the nuclear matrix is enriched in actively transcribed genes (Ciejek et al., 1983; Gerdes et al., 1994). Pulse-labelled, nascent RNA is associated with the nuclear matrix (Jackson et al., 1981; Ciejek et al., 1982; Jackson and Cook, 1985) and displays in nuclear matrices the same spatial distribution as in intact nuclei (Jackson et al., 1993; Wansink et al., 1993). RPII activity has been demonstrated in nuclear matrix preparations (Jackson and Cook, 1985; Razin and Yarovaya, 1985). Steroid and thyroid hormone receptors and a number of other transcription factors are associated with the nuclear matrix (Kaufmann et al., 1986; Barrack, 1987; Evan and Hancock, 1987; Klempnauer, 1988; Getzenberg and Coffey, 1990; Stein et al., 1991; van Steensel et al., 1991; Bidwell et al., 1993; van Wijnen et al., 1993). Taken together, these data suggest that the nuclear matrix plays a role in the regulation and spatial organization of transcription.

To obtain insight into the relationship between nuclear structure and the regulation of transcription by the GR, we investigated the subnuclear distribution of the GR by immunofluorescent labeling and confocal laser scanning microscopy (CSLM). We demonstrate that the hormone-activated GR is concentrated in clusters in the nucleus. These clusters of the GR are associated with the nuclear matrix. Surprisingly, GR clusters do not significantly colocalize with foci of newly synthesized pre-mRNA. We also report here that RPII is concentrated in clusters in the nucleus. Clusters of the GR do not show a significant overlap with these clusters of RPII. These data suggest that a large fraction of the GR clusters is not directly involved in the activation of transcription.

MATERIALS AND METHODS

Cell culture

T24 (human bladder carcinoma), HeLa (human cervix carcinoma) and NRK (newborn rat kidney) cells were grown at 37°C under a 10% CO₂ atmosphere in DMEM (Gibco, Paisly, UK) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) (Gibco), 2 mM L-glutamine (Gibco), 100 i.u./ml penicillin and 100 µg/ml streptomycin (Gibco). To prepare steroid-free medium, FCS was replaced with charcoal-treated FCS (Brink et al., 1992). For microinjection experiments T24 cells were cultured under 5% CO₂.

For immunofluorescence labeling, cells were grown at least 24 hours on Alcian Blue-treated coverslips (Brink et al., 1992) or gelatin-coated microinjection slides. In some experiments (Fig. 1) normal medium was replaced by steroid-free medium 18 hours before fixation. One hour before fixation cells were treated with 10⁻⁷ M dexamethasone (Sigma) or 10⁻⁷ M RU486 (Roussel-Uclaf, France).

Nuclear matrix preparation

All incubations were carried out on ice. HeLa cells grown at least one day on Alcian Blue-treated microscope coverslips were treated for one hour with 10⁻⁷ M dexamethasone and extracted *in situ* as follows. Cells were washed twice in CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5 mM benzamidine, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml aprotinin) (Fey

et al., 1986), incubated 3 minutes with 0.5% Nonidet P-40 and 0.5 mM sodium tetrathionate in CSK, then 5 minutes with CSK/0.5 mM sodium tetrathionate and washed twice briefly with CSK. Subsequently, cells were washed once with digestion buffer (CSK with 50 mM NaCl instead of 100 mM NaCl), followed by incubation for 1 hour with 250 µg/ml DNase I (type IV, Sigma) in digestion buffer. Then cells were extracted with 0.25 M (NH₄)₂SO₄ in digestion buffer for 10 minutes and washed twice with CSK. The thus obtained nuclear matrices were fixed and labeled as described below. Microscopic inspection of nuclear matrices labeled with Hoechst 33258 confirmed that most DNA was extracted.

Microinjection

Microinjection of BrUTP was performed as described previously (Wansink et al., 1993).

Immunofluorescent labeling of cultured cells and nuclear matrices

All incubations were carried out at room temperature, unless stated otherwise. Cells and nuclear matrices on coverslips or microinjection slides were fixed 10 minutes in freshly prepared 2% formaldehyde in PBS. Then they were permeabilized 10 minutes in 0.5% Nonidet P-40 in PBS, incubated 5 minutes in 100 mM glycine in PBS to inactivate free aldehyde groups and blocked 10 minutes in PBG (0.5% BSA, 0.1% gelatin in PBS).

Antibody incubations were done in PBG. Primary antibodies against the GR were mouse monoclonal antibody mAb7 (Okret et al., 1984), ascites fluid, diluted 1:2,000; mouse monoclonal antibody GR778-795 (Flach et al., 1992), culture supernatant, diluted 1:2; rabbit polyclonal antipeptide antiserum 57 (Affinity BioReagents, Neshanic Station, NJ) (Cidowski et al., 1990), diluted 1:250. Purified mouse monoclonal antibody 8WG16 against RNA polymerase II (Thompson et al., 1989) was diluted to 0.1 µg/ml. Anti-SC35 mouse monoclonal antibody (Fu and Maniatis, 1990) culture supernatant was diluted 1:10. Rat monoclonal antibody against bromodeoxyuridine (Sera-Lab, Crawley Down, UK) was diluted 1:500. All primary antibodies were incubated overnight at 4°C. After 4× 5 minutes washing in PBG, coverslips were incubated 1–2 hours at room temperature with secondary antibodies. Depending on the experiment, these were biotinylated sheep anti-mouse (Amersham, Amersham, UK), TRITC-conjugated donkey anti-mouse, FITC- or TRITC-conjugated donkey anti-rabbit or biotinylated donkey anti-rat antibodies (all from Jackson ImmunoResearch Laboratories, West Grove, PA). If a biotinylated secondary antibody was used, then coverslips were washed 4× 5 minutes in PBG, followed by 1 hour FITC-conjugated streptavidin (Jackson ImmunoResearch Laboratories or Gibco). Next, coverslips were washed 4× 5 minutes in PBG, 10 minutes in PBS containing 0.4 µg/ml Hoechst 33258 and 10 minutes in PBS. Coverslips were mounted in embedding medium (78% glycerol, 1 mg/ml *p*-phenylene diamine in PBS, pH 8.0).

Immunofluorescent labeling of rat hippocampus

Male Wistar rats weighing ~250 gram were adrenalectomized and kept one week on standard food and water containing 0.9% NaCl. One hour before brain fixation rats were injected subcutaneously with 300 µg/100 g bodyweight corticosterone dissolved in polyethylene glycol. Control rats received vehicle only. Rat brain fixation and labeling was carried out as described previously (van Steensel et al., 1994a). Briefly, rats were anaesthetized with Nembutal. Brain was fixed by intracardiac perfusion with 0.9% NaCl for 2 minutes, followed by fixative solution (4% formaldehyde in PBS, pH 7.4) for 15 minutes. The brain was removed from the skull and stored overnight in fixative, followed by 24 hours in PBS. Brain sections (30 µm) were cut with a vibratome and stored at -20°C in 78% glycerol in PBS, pH 7.4. For immunolabeling, hippocampus was dissected from the brain sections and washed in excess PBS. Hippocampus sections were incubated 1 hour in 5% normal donkey serum and 0.1% (w/v) Nonidet P-40 in

PBS, pH 7.4, followed by overnight incubation at 4°C with mouse monoclonal antibody mAb7 (Okret et al., 1984) diluted in PBGN (0.5% BSA, 0.1% gelatin and 0.1% (w/v) Nonidet P-40 in PBS). Next, sections were washed 4× 10 minutes in PBGN and subsequently incubated 1 hour with TRITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories) diluted in PBGN. Finally sections were washed 10 minutes in PBGN and 2× 10 minutes in PBS and mounted in embedding medium.

Confocal scanning laser microscopy

CSLM images were collected with a Leica confocal microscope, equipped with a 488/514 dual band argon ion laser. An oil-immersion objective (×100; NA = 1.32) was used. Emitted fluorescence was detected using a 525DF10 bandpass filter for FITC and a 550 nm longpass filter for TRITC. Pairs of images were collected simultaneously in the green and red channels. Generally, serial optical sections of labeled nuclei were made. Voxel dimensions were in most images 49 nm lateral and 208 nm axial.

Specificity of labeling

Controls demonstrated that labeling was specific and that there was no cross-reactivity of any of the antibodies that were used. Omission of any of the primary antibodies resulted in complete loss of the corresponding nuclear signal. Labeling with anti-BrU antibody was only found in cells that were microinjected with BrUTP. Sensitivity of BrU-RNA labeling to α -amanitin and RNase has been demonstrated previously (Wansink et al., 1993).

Image processing and analysis

Images were processed and analyzed using SCIL-IMAGE software (developed for 3-D image analysis at the University of Amsterdam). Optical cross-talk between the red and green channel was quantified and subtracted as described previously (Manders et al., 1992). Typically, cross-talk of FITC fluorescence into the red channel was 20–30% and cross-talk of TRITC fluorescence into the green channel was below detection level. After removal of cross-talk, noise was reduced by a 3×3×1 uniform filter. Image intensities were stretched to fill the 256 steps of the grey scale.

Segmentation of the image to determine the number of GR clusters in 3-D images of labeled nuclei was done either by a simple thresholding procedure or by detection of local maxima (see also Results). For thresholding the effect of various threshold levels was compared. The number of clusters (a cluster is defined as a group of more than 10 contiguous voxels in the resulting binary images) was counted automatically and plotted against the threshold level (see Fig. 3b). Local maxima were identified using a 9×9×5 maximum filter. The size of this filter is slightly larger than the size of a typical GR cluster (about 7×7×4 voxels). This local maximum filter detected not only clearly visible GR clusters in the nucleus, but also a large number of very small fluctuations in the weak cytoplasmic signal. For this reason local maxima corresponding to very low voxel values in the original image were removed by a threshold filter. This thresholding resulted in removal of the local maxima in the weakly labeled cytoplasmic compartment, but did not significantly affect the local maxima in the much stronger nuclear signal.

Fig. 1. Distribution of the GR in nuclei of cultured cell lines. T24 (a–c) or NRK cells (d–i) were grown on cover slips in steroid-free medium. One hour before fixation cells were incubated with 10^{-7} M Dex (b, c, e–h), 10^{-7} M RU486 (i), or without added hormone (a, d). After fixation with 2% formaldehyde cells were labeled with antiserum 57 (a–f, i), GR_{788–795} (g), or mAb7 (h) followed by a TRITC-conjugated secondary antibody. Single CSLM optical sections through the middle of cell nuclei are shown. Bars, 5 μ m.

RESULTS

The GR is concentrated in clusters in the cell nucleus

The subnuclear localization of the hormone-stimulated GR was visualized by indirect immunofluorescence labeling and confocal laser scanning microscopy (CSLM). Fig. 1a-f shows CSLM optical sections of the human bladder carcinoma cell line T24 and the rat kidney cell line NRK after labeling with antiserum 57. This antiserum specifically recognizes the human and rat GR (Cidlowski et al., 1990). In the absence of hormone a weak cytoplasmic and nuclear labeling was obtained (Fig. 1a and d). After treatment with the GR agonist dexamethasone (Dex) all cells displayed a strong nuclear labeling (Fig. 1b and e). At higher magnification it is clearly visible that this nuclear GR-immunoreactivity is concentrated in numerous domains scattered throughout the nucleoplasm (Fig. 1c and f). No strong labeling was found in nucleoli. Similar clustered patterns of GR-labeling were observed in nuclei of human fibroblasts, rat hepatoma cells (data not shown) and HeLa cells (cf. Fig. 4). This distribution was also found after labeling of Dex-treated cells with monoclonal anti-GR antibodies GR₇₈₈₋₇₉₅ (Flach et al., 1992) (Fig. 1g) and mAb7 (Okret et al., 1984) (Fig. 1h). We found similar labeling patterns with different fixation methods. Fixation of cells in a mixture of 0.01% glutaraldehyde, 2% formaldehyde and 0.1% Nonidet P-40 resulted in the same distribution as fixation in 2% formaldehyde followed by permeabilization with 0.5% Nonidet P-40 (data not shown). We conclude that the GR is concentrated in clusters in the cell nucleus. This nuclear distribution is present in many cell types from rat and human origin, and is not dependent on the antibody that is used.

In Dex-treated HeLa and T24 cells some weakly labeled GR clusters were also found in the cytoplasm (Fig. 1b and c). This suggests that the ability of the GR to form clusters is not restricted to nuclear forms of the receptor. However, the majority of GR-immunoreactivity is present in the nucleus. It is not clear whether cytoplasmic clusters are biochemically identical to nuclear clusters.

Activation of the GR by Dex and other agonists involves a multi-step transformation of the receptor to a form that becomes associated with DNA and activates the transcription

machinery (Beato, 1991). The GR antagonist RU486 binds with high affinity to the GR but fails to induce gene expression (Philibert, 1984; Beck et al., 1993). We investigated whether the formation of nuclear GR clusters could also be induced by treatment of cells with RU486. Fig. 1i shows that NRK cells treated with RU486 displayed the same clustered nuclear GR distribution as Dex-treated cells. Thus, formation of GR clusters in the cell nucleus does not require activation of the GR to a state that is able to activate gene expression.

The clustered subnuclear distribution of the GR may be a specific property of cultured tumor cell lines. To test this, we labeled the GR in sections of rat hippocampus tissue. Pyramidal neurons in the hippocampus are terminally differentiated cells that express the GR at high levels. CSLM images shown in Fig. 2a and b demonstrate that the GR-immunoreactivity in these neurons depends on the presence of glucocorticoids. In adrenalectomized rats, which are depleted of circulating corticosteroid, a weak cytoplasmic and no nuclear labeling was observed (Fig. 2a). After treatment of these rats with corticosterone a strong nuclear signal was observed (Fig. 2b). The GR in nuclei of hippocampal neurons showed a clustered distribution (Fig. 2c). This indicates that the clustered subnuclear distribution of the GR is a property of nonproliferating cells in tissue as well as of cultured cell lines.

The number of GR clusters per cell nucleus was estimated by computer analysis of three-dimensional CSLM images of Dex-treated NRK cell nuclei that were labeled with antiserum 57. Two approaches were followed. In one approach, images of nuclei labeled with antiserum 57 (Fig. 3a) were thresholded to obtain binary images (Fig. 3c). In such a binary image a cluster was defined as a group of more than 10 contiguous voxels having value 1. The number of clusters according to this definition depended on the threshold level that was applied (Fig. 3b). When a high threshold level was selected, weakly labeled clusters were not detected. When the threshold was low, adjacent clusters became fused and thus were counted as one single cluster. The number of clusters that corresponded to the maximum of the curve depicted in Fig. 3b was taken as an estimate of the number of GR clusters in the nucleus. In this way about 800 clusters per nucleus were counted. However, this method probably results in an underestimation, because weakly labeled clusters are not detected at the selected threshold level.

Fig. 2. Subnuclear distribution of the GR in rat hippocampus pyramidal neurons. Rats were adrenalectomized to deplete endogenous corticosteroids. Seven days after adrenalectomy rats were treated 1 hour with 300 µg corticosterone per 100 gram body weight (b,c) or with vehicle only (a). Brains were fixed by intracardiac perfusion with 4% formaldehyde. Brain slices of 30 µm were cut on a vibratome and labeled with antibody mAb7 followed by TRITC-conjugated secondary antibody. Single CSLM optical sections of pyramidal neurons in the hippocampal CA1 area are shown. Bar, 5 µm.

Fig. 3. Estimation of the number of GR clusters per cell nucleus. An unprocessed 3-D CSLM image of an NRK cell nucleus labeled with antiserum 57 (a) was thresholded and the clusters in the resulting binary image were counted after removal of clusters smaller than 10 voxels. The number of clusters was plotted against the threshold level (b). Threshold level 60 resulted in binary image (c). Detection of clusters by a 9×9×5 local maximum filter, after removal of the cytoplasmic signal, resulted in binary image (d). Corresponding single sections of a 3-D confocal image are shown in a, b and d.

In the second approach, GR clusters were identified by application of a filter that detects local maxima (Fig. 3d). The number of clusters found by this approach was about 2,300 per nucleus. This method is relatively sensitive to noise and may detect small local variations in the background signal, resulting in an overestimation of the number of GR clusters. Based on these two different methods we conclude that an NRK cell nucleus contains between one and two thousand GR clusters.

GR clusters are retained in the nuclear matrix

Biochemical studies have demonstrated that the GR is bound to the nuclear matrix (Kaufmann et al., 1986; van Steensel et al., 1994b). This binding could play a role in the spatial organization of the GR in the nucleus. Therefore, we studied the distribution of the GR in nuclear matrix preparations. Nuclear matrices were prepared from Dex-treated HeLa cells, fixed with 2% formaldehyde and labeled for immunofluorescence microscopy with monoclonal antibody mAb7 against the GR. CSLM images show that the distribution of the GR in nuclear matrices (Fig. 4c and d) was similar to the distribution in nuclei of directly fixed cells (Fig. 4a and b). The labeling intensity of the GR in nuclear matrices was comparable to that in directly fixed cells. These data demonstrate that clustered GR molecules are tightly bound to the nuclear matrix.

Most GR clusters do not colocalize with newly synthesized RNA, RNA polymerase II or splicing factor SC-35

The clustered distribution of the GR closely resembles the distribution of nascent pre-mRNA that was reported previously (Jackson et al., 1993; Wansink et al., 1993). Therefore, we

Fig. 4. Distribution of the GR in HeLa nuclei and nuclear matrices. Nuclear matrices were prepared from Dex-treated cells by in situ extractions as described in Materials and Methods. Intact cells (a,b) and nuclear matrices (c,d) were fixed and labeled with antibody mAb7, followed by biotinylated secondary antibody and streptavidin-FITC. Single confocal sections of different cells and nuclear matrices are shown.

compared the spatial distribution of the GR and that of newly synthesized pre-mRNA by dual labeling. Dex-treated T24 cells were microinjected with BrUTP and cultured 20 minutes to allow in vivo incorporation of BrUTP into nascent pre-mRNA. After fixation of the cells the GR and incorporated BrU were visualized by dual immunofluorescent labeling and CSLM. Representative CSLM optical sections of a cell nucleus are shown in Fig. 5a and d. Both the GR (green) and newly synthesized RNA (red) are localized in discrete domains. However, there is no obvious correlation between the two patterns. Although some colocalization (yellow) can be observed, a large fraction of the GR clusters shows little or no BrU labeling, and vice versa. We analysed the degree of colocalization in a more quantitative way by plotting the signal intensities of the red and green signals along a single line drawn through the image of a doubly labeled nucleus. Plots of two arbitrarily chosen lines, as indicated by arrowheads in Fig. 5a, are shown in Fig. 6a. These plots show that there is no clear correlation between the spatial distributions of the GR and newly synthesized RNA. Almost never does a local maximum in the green (GR) signal coincide with a peak in the red (BrU-RNA) signal.

It is unlikely that the low BrU-immunoreactivity in GR clusters is caused by steric hindrance of the anti-BrU antibody by the anti-GR antibody, considering the large number of BrU residues that is incorporated into nascent RNA (Wansink et al., 1993). Moreover, after single BrU labeling we found about the same number of BrU clusters per nucleus as after dual GR-BrU labeling, indicating that competition between the antibodies does not occur (data not shown).

We also studied the degree of colocalization between the GR and RNA polymerase II (RPII). Dex-treated T24 cells were fixed and labeled with mouse monoclonal antibody 8WG16 against RPII (Thompson et al., 1989) and rabbit antiserum 57 against

the GR. CSLM optical sections (Fig. 5b and e) demonstrate that RPII is also concentrated in numerous small clusters in the nucleoplasm. Preliminary results indicate that similar distributions of RPII are present in nuclei of other cell types (D. G. Wansink, unpublished results). Combination of GR labeling and RPII labeling demonstrates that there is no clear correlation between the localization of GR clusters and that of RPII clusters. This is confirmed by plots of the red and green pixel values along single lines through doubly labeled images (Fig. 6b).

Most factors that are involved in pre-mRNA splicing are known to be concentrated in specific nuclear domains (Fu and Maniatis, 1990; Spector, 1990). A marker for these domains is the essential splicing factor SC-35 (Fu and Maniatis, 1990). Dual labeling of antiserum 57 against the GR with an antibody against SC-35 shows that most GR clusters do not colocalize with SC-35 domains (Fig. 5c and 5f). This is confirmed by single line intensity plots (Fig. 6c). Although a few GR clusters were found in or near an SC-35 domain, we conclude that most GR clusters do not colocalize with SC-35 domains.

DISCUSSION

By immunofluorescent labeling and CSLM we studied the

spatial distribution of the GR in the cell nucleus. We found that the liganded GR is concentrated in one to two thousand clusters in the nucleus. This was observed in a variety of human and rat cell lines and in rat hippocampus neurons, indicating that the clustered distribution of the GR is not restricted to specific cell types. We found that the distribution of GR clusters in the nucleoplasm shows no obvious relationship with the distribution of newly synthesized pre-mRNA, clusters of RPII, or domains that contain the essential splicing factor SC-35.

How many GR molecules are present in one cluster? Radioligand binding assays indicate that a HeLa cell contains approximately 1×10^5 GR molecules (S. Deurloo and B. van Steensel, unpublished results). Similar numbers have been reported for other cell types (Akner et al., 1990). Quantification of CSLM images of whole cells shows that after DEX treatment at least 80% of the GR-immunoreactivity is located in the nucleus (data not shown). We observed approximately 1,000-2,000 GR clusters per nucleus. Thus, on the average one cluster may contain 40-80 GR molecules.

Promoter and enhancer regions of glucocorticoid-controlled genes typically contain only one or two HREs to which the GR can bind as a dimer. Thus, the number of receptor molecules in one GR cluster is more than tenfold larger than the number of receptor molecules that are bound to the flanking regions of

Fig. 5. Subnuclear localization of the GR relative to newly synthesized RNA (a,d), RNA polymerase II (b,e) or essential splicing factor SC-35 (c,f) in T24 cells. To all cells 10^{-7} M Dex was added 1 hour before fixation. About 20 minutes before fixation cells were microinjected with BrUTP (a,d). Fixed cells were double-labeled with antiserum 57 against the GR and anti-BrU antibody (a,d), antibody 8WG16 against RPII (b,e) or anti-SC-35 antibody (c,f), followed by appropriate secondary antibodies. The localization of the GR is shown in green; BrU-RNA, RPII and SC-35 are shown in red. Top row shows representative cell nuclei; bottom row shows enlargements of areas indicated by dotted squares. Bars, 1 μ m.

one typical glucocorticoid-responsive gene. A model has been proposed in which active genes and components of the transcription machinery are clustered in transcription 'domains' or 'factories' (Jackson et al., 1993; Wansink et al., 1993). If GR clusters represent such transcription domains, then one would expect to find considerable transcriptional activity in these GR clusters. However, we found no correlation between the distribution of GR clusters and the distribution of BrU-labeled pre-mRNA. It is therefore unlikely that GR clusters represent such 'transcription factories'. Rather, our results suggest that most GR clusters contain predominantly GR molecules that are not directly involved in transcriptional activation.

To obtain a sufficiently strong BrU labeling in the GR-BrU dual labeling experiments the time between microinjection of BrUTP and fixation of the cells had to be approximately 20 minutes. Assuming an average transcript size of ~10 kb and a transcription rate of ~20 nucleotides per second, synthesis of a typical pre-mRNA transcript takes approximately 8 minutes. Thus, after 20 minutes a considerable fraction of the BrU-labeled transcripts is terminated. We cannot exclude the possibility that these terminated transcripts move away from their sites of synthesis. Nevertheless, part (~30%) of the BrU labeling represents RNA that is still being transcribed. These two populations of RNA (i.e. terminated and nascent RNA) cannot be distinguished by our labeling technique. However, we found that most GR clusters are not enriched in BrU labeling at all. This suggests that most GR clusters represent sites in the nucleus where little or no transcription takes place.

Fig. 6. Intensities of the red and green signal along single lines taken from Fig. 5. Values were taken from two horizontal lines between the blue arrowheads in Fig. 5a (a), 5b (b) and 5c (c). Solid lines: red signal, corresponding to BrU-RNA (a), RPII (b) and SC-35 (c); dotted lines: green signal, corresponding to GR. Pixel values are in arbitrary units.

More insight into the relationship between GR clusters and sites of transcription may be obtained in future experiments in which GR-immunolabeling is combined with fluorescent *in situ* hybridization with probes against glucocorticoid-responsive genes or their transcripts.

By immunofluorescent labeling and confocal microscopy we found RPII also to be localized in numerous clusters in the nucleus. Previously, a diffuse distribution of RPII was reported (Jimenez-Garcia and Spector, 1993). However, these results were obtained by conventional immunofluorescence microscopy, which has a lower resolution than CSLM. Preliminary dual labeling experiments suggest partial colocalization of RPII clusters and BrU-labeling (B.v.S., K.v.d.M. and D.G.W., unpublished results). Dual labeling of the GR and RPII in T24 cells revealed that the majority of GR clusters does not colocalize with RPII clusters. Although it is not yet clear whether all RPII clusters are actively engaged in transcription, the lack of correlation between the distributions of the GR and RPII is consistent with the notion that most clustered GR molecules are not directly involved in the activation of gene expression. GR clusters also did not significantly colocalize with SC-35 domains. This is not surprising, as there is no biochemical evidence that the GR interacts with components of the splicing machinery.

Nuclear GR clusters were also induced by treatment of NRK cells with RU486. This GR antagonist may exert its antiglucocorticoid activity at two stages of receptor action: (i) prevention of transformation of the GR to a DNA-binding state, and (ii) alteration of a step subsequent to DNA-binding (Mao et al., 1992). Recently, it was reported that RU486 fails to induce DNA-binding of the GR (Heck et al., 1994). However, other data indicate that DNA-binding of the GR is only partially impaired in the presence of RU486 (Beck et al., 1993). Therefore, we cannot exclude the possibility that RU486-induced GR clusters contain DNA-associated receptor molecules. Nevertheless, formation of GR clusters obviously does not require complete activation of the GR to a state that is able to activate transcription.

A clustered distribution of the GR was also found in nuclear matrices prepared from Dex-treated cells. This indicates that the nuclear matrix contains binding sites for the clustered GR population. Other transcription factors, including many members of the steroid receptor superfamily, have been reported to bind to the nuclear matrix (Feldman and Nevins, 1983; Kumara-Siri et al., 1986; Barrack, 1987; Bidwell et al., 1993; van Wijnen et al., 1993; Vassetzky et al., 1993). The role of the nuclear matrix in the working mechanism of transcription factors is unknown. It has been proposed that binding of transcription factors to the nuclear matrix serves to increase the local concentration of transcription factors in order to achieve a more efficient control of gene expression (Bidwell et al., 1993). Obviously, in matrix-associated GR clusters the local receptor concentration is high. However, because little newly synthesized RNA or RPII was found in most GR clusters in intact nuclei it is probable that most clustered matrix-bound GR molecules are not directly interacting with the transcription machinery.

Some earlier reports have described the subnuclear distribution of steroid receptors. Martins et al. (1991) reported a mottled distribution of the GR that had been overexpressed in COS cells. However, interpretation of these data was difficult

because the distribution of the GR may have been affected by overexpression. In the present paper we demonstrate clustered subnuclear localization of endogenous receptors in a variety of cell types. Akner et al. (1994) have reported a speckled distribution of the GR in human fibroblast cell nuclei after labeling with a monoclonal anti-GR antibody named MAb1. We found that the nuclear structures that are recognized by MAb1 correspond to domains enriched in splicing components (B.v.S., unpublished data). By western blotting it was shown that MAb1 not only recognizes the ~94 kDa GR but also strongly reacts with an unidentified protein of ~40 kDa (Akner et al., 1994, and B.v.S., unpublished results). For this reason we believe that immunofluorescence labeling data obtained with MAb1 are difficult to interpret.

The subnuclear localization of steroid receptors has also been the subject of immuno-electron microscopy studies. Using immunoperoxidase labeling, Press et al. (1985) found the estrogen receptor to be localized in euchromatin areas in the nucleus. A detailed immunogold labeling study by Vazquez-Nin et al. (1991) showed that the nuclear estrogen receptor is associated with fibrillar ribonucleoprotein aggregates. It is possible that the GR clusters that we report here represent similar structures. In contrast, progesterone receptor-immunoreactivity was reported to be predominantly present at the border regions between condensed chromatin and nucleoplasm (Perrot-Appianat et al., 1986; Isola, 1987). No significant clustering was found in these studies. These differences in subnuclear distribution may be attributed to differences in fixation and labeling techniques, or may reflect intrinsic differences between steroid receptor species. We are currently conducting immunogold electron microscopy experiments to study the subnuclear localization of the GR in more detail.

Evidence has been presented that hormone-stimulated steroid receptors shuttle continuously between cytoplasm and nucleus (Guiochon-Mantel et al., 1991; Guiochon-Mantel and Milgrom, 1993). This suggests that the nucleus contains a dynamic pool of receptor molecules. It is therefore likely that GR clusters are dynamic rather than static structures.

The physiological function of GR clustering remains unclear. Clustered GR molecules may be involved in suppression of specific genes (Cairns et al., 1993; Drouin et al., 1993). GR clusters may also represent sites of receptor storage. Sequestration of receptors in these clusters may be a mechanism to control the concentration of freely diffusing receptor in the nucleus. A similar model has been proposed to explain the accumulation of splicing factors in specific nuclear domains where virtually no transcription takes place (Jimenez-Garcia and Spector, 1993; Wansink et al., 1993).

Interestingly, a speckled nuclear distribution was recently reported for two other transcription factors: the retinoblastoma gene product (Mancini et al., 1994) and the tumour suppressor protein p53 (Jackson et al., 1994). Furthermore, we have found that the mineralocorticoid receptor displays a clustered subnuclear distribution (B.v.S., manuscript in preparation). These data indicate that concentration of transcription factors in specific nuclear domains may be a general phenomenon.

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