Journal of Cellular Biochemistry 57:465–478 (1995)

Domains of the Human Androgen Receptor and Glucocorticoid Receptor Involved in Binding to the Nuclear Matrix

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Abstract Steroid receptors have been reported to bind to the nuclear matrix. The nuclear matrix is operationally defined as the residual nuclear structure that remains after extraction of most of the chromatin and all soluble and loosely bound components. To obtain insight in the molecular mechanism of the interaction of steroid receptors with the nuclear matrix, we studied the binding of several deletion mutants of the human androgen receptor (hAR) and the human glucocorticoid receptor (hGR) to the nuclear matrix. Receptor binding was tested for two different nuclear matrix preparations: complete matrices, in which most matrix proteins are retained during the isolation procedure, and depleted matrices, which consist of only a subset of these proteins. The results show that the C-terminal domain of the hAR binds tightly to both depleted and complete matrices. In addition, at least one other domain of the hAR binds to complete matrices but not to depleted matrices. In contrast to the hAR, the hGR binds only to complete matrices. For this interaction both the DNA-binding domain and the C-terminal domain of the hGR are required, whereas the N-terminal domain is not. We conclude that specific protein domains of the hAR and the hGR are involved in binding to the nuclear matrix. In addition, our results indicate that the hAR and the hGR are attached to the nuclear matrix through different molecular interactions.

Key words: steroid receptors, C-terminal domain, DNA binding domain, transcription, nuclear localization, nuclear matrix, nuclear matrix acceptors, interaction, disulfide bridges

Steroid receptors are hormone-dependent transcription factors. They can regulate the expression of specific genes by binding to regulatory DNA sequences named hormone response elements (HREs). At least three functional domains can be distinguished in steroid receptors. These include a C-terminal steroid binding domain, a DNA-binding domain (DBD), and an N-terminal domain which is required for maximal transcriptional regulatory activity [Evans, 1988; Green and Chambon, 1988].

The DBDs of the glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, and androgen receptor are highly homologous. This is reflected by their ability to bind to the same HRE. However, in vivo these steroid receptors regulate gene expression differentially. Evidence is accumulating that this target gene specificity is determined by the interplay between steroid receptors, other transcription factors, and chromatin structure [Adler et al., 1992; Hayes and Wolffe, 1992; Pearce and Yamamoto, 1993; Truss and Beato, 1993].

Many members of the steroid and thyroid hormone receptor superfamily have been reported to be bound to the nuclear matrix [reviewed in Barrack, 1987; Getzenberg et al., 1990]. 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, 1991; van Driel et al., 1991]. It consists of a peripheral nuclear lamina and an internal fibrogranular network. Nuclear matrices are generally isolated by treatment of cell nuclei

Received July 6, 1994; accepted August 8, 1994.

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with nonionic detergents, digestion with nucleases, and extraction with a buffer of high ionic strength [Berezney and Coffey, 1974; Kaufmann et al., 1981; He et al., 1990].

Numerous data indicate that transcription is closely associated with the nuclear matrix. Active genes are enriched in the residual DNA that remains bound to the nuclear matrix [reviewed in Getzenberg et al., 1991]. Pulse-labeled, nascent RNA is associated with the nuclear matrix [Jackson et al., 1981, 1993; Ciejek et al., 1982; Wansink et al., 1993]. Transcriptional activity has been demonstrated in the nuclear matrix [Jackson and Cook, 1985; Razin and Yarovaya, 1985]. Steroid and thyroid hormone receptors [Barrack and Coffey, 1980; Barrack, 1983, 1987; Kaufmann et al., 1986; Kirsch and Miller-Diener, 1986; Kumara-Siri et al., 1986; Alexander et al., 1987; van Steensel et al., 1991] and a number of other transcription factors [Evan and Hancock, 1987; Klempnauer, 1988; Getzenberg and Coffey, 1990; Stein et al., 1991; Bidwell et al., 1993; van Wijnen et al., 1993] are associated with the nuclear matrix. These data indicate that the nuclear matrix may play an important role in the regulation of transcription.

The interaction of steroid receptors with the nuclear matrix is dependent on the presence of hormone [Barrack, 1987]. By in vitro reconstitution experiments, binding of the rat androgen receptor [Barrack, 1983; Colvard and Wilson, 1984], the mouse estrogen receptor [Belisle et al., 1989; Metzger and Korach, 1990], and the chicken progesterone receptor [Schuchard et al., 1991] to the nuclear matrix was demonstrated to be saturable and of high affinity. This indicates that the nuclear matrix contains specific binding sites for steroid receptors. Little is known about the nature of these binding sites. Chick oviduct nuclear matrix contains a 10 kDa protein that has been shown to bind specifically and with high affinity to the progesterone receptor [Schuchard et al., 1991]. This suggests that binding of steroid receptors to the nuclear matrix is mediated by specific acceptor proteins. On the other hand, residual nuclear matrix DNA may be involved in steroid receptor binding.

The function of the interaction of steroid receptors with the nuclear matrix is presently unknown. To obtain more insight in this interaction, we set out to identify protein domains of the human androgen receptor (hAR) and the human glucocorticoid receptor (hGR) that are required for binding to the nuclear matrix.

MATERIALS AND METHODS

hAR and hGR Expression Plasmids

The hGR [Hollenberg et al., 1987; Hollenberg and Evans, 1988; Rupprecht et al., 1993] and hAR [Jenster et al., 1991, 1993] expression plasmids have been published previously. Plasmid pAR67 was constructed from pAR8 [Jenster et al., 1993] in which via site-directed mutagenesis a NarI site was introduced in codon 625 using primer pAR28.1 [Jenster et al., 1993]. NarI digestion resulted in the in-frame deletion of a cDNA fragment encoding residues 51–624.

Cell Culture and Transfections

For hGR studies, COS-1 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. Cells were transfected by electroporation with 5 μg receptor expression vector and 10 μg carrier DNA (pGEM4; Promega Corp., Madison, WI) as described [Rupprecht et al., 1993]. For nuclear binding studies, electroporated cells were replated in 6 cm petri dishes in Dulbecco's Modified Eagle's Medium supplemented with 10% charcoal-stripped steroid-free fetal calf serum and cultured 24 h in the presence or absence of 10^{-8} M dexamethasone (Sigma, St. Louis, MO). For immunofluorescence labeling, transfected cells were replated on 16 mm glass coverslips that were pretreated with alcian blue [Brink et al., 1992] and cultured as for nuclear binding studies.

For hAR and βgalNLS studies, cells were grown on alcian blue–treated coverslips or in 6 cm petridishes, transfected, and cultured as described previously [Jenster et al., 1993]. When indicated, cells were treated with 10^{-8} M R1881 (17α-methyltrienolone; NEN, Boston, MA) for 24 h.

Immunofluorescence Labeling and Microscopy

All incubations were carried out at room temperature. After hormone treatment for 24 h, hAR-transfected cells grown on coverslips were either directly fixed or first extracted in situ to obtain nuclear or nuclear matrix fractions, which were subsequently fixed. Fixation occurred by washing twice in PBS, fixing with 3.7% formaldehyde 10 min in PBS, washing twice in PBS, and permeabilization with 0.5% Nonidet P-40 in PBS for 10 min. After two wash steps in PBS, fixed and permeabilized specimens were incubated 20 min with 10% FCS in PBS, followed by incubation with the monoclonal antibody F39.4.1

[Zegers et al., 1991] (ascites fluid, diluted 1:1,000) or the polyclonal antibody SP066 [Kuiper et al., 1993] for 1 h, followed by 6 × 5 min washing in PBS and subsequent incubation with FITC-conjugated goat-antimouse antibody (Sigma) or FITC-conjugated goat-antirabbit antibody (Sigma) for 1 h. After six washing steps in PBS and 10 min incubation with 1 μg/ml Hoechst 33258 in PBS, preparations were mounted in 1 mg/ml p-phenylene diamine (Sigma) in 75% glycerol in PBS.

hGR transfected cells, grown on coverslips, were treated with hormone for 24 h, washed twice in PBS, fixed 10 min in 2% formaldehyde in PBS, washed twice in PBS, permeabilized 10 min in 0.5% Nonidet P-40, and washed twice in PBS. Cells were incubated 2 × 10 min in PBG (0.5% BSA, 0.1% gelatin in PBS), followed by overnight incubation with a mouse monoclonal antibody mAb7 [Okret et al., 1984] (1 μg/ml) or monoclonal antibody $GR_{788-795}$ (undiluted hybridoma culture supernatant) directed against the hGR. Western blotting and immunofluorescence labeling of several hGR deletion mutants demonstrated that the epitope of mAb7 is situated in region 9-205 (data not shown). Antibody $GR_{788-795}$ is directed against the C-terminal amino acid residues 788–795 of the rat GR [Flach et al., 1992], which are identical to amino acid residues 770–777 of the hGR. After 6 × 5 min washing in PBG, cells were incubated 1 h in PBG with biotin-conjugated sheep-antimouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by FITC-conjugated streptavidin (Gibco, Breda, The Netherlands) for 1 h. After 6 × 5 min washing in PBG, preparations were stained with Hoechst 33258 and mounted as described above. The same procedure was followed for labeling of permeabilized cells and nuclear matrices.

Cells transfected with the βgalNLS fusion protein were fixed and stained as with hAR-transfected cells, using monoclonal antibody 12B3 against β-galactosidase.

Permeabilization and Nuclear Matrix Preparation

All incubations were carried out in situ on monolayer cultures of transfected cells. For permeabilization, transfected and hormone-treated cells were washed twice in CSK buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5 mM benzamidin, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml aprotinin), incubated 3 min with 0.5% Nonidet P-40 in CSK with (method B) or without (method A) 0.5 mM NaTT and then 5 min with CSK with or without NaTT, respectively, and washed twice briefly with CSK. Then cells were incubated 85 min in CSK or subjected to extractions to obtain nuclear matrices.

For nuclear matrix preparations, cells permeabilized in the absence of NaTT were incubated 10 min with 0.5 mM NaTT in CSK (method A), and cells permeabilized in the presence of NaTT were incubated 10 min in CSK (method B). Subsequently, all cells were washed once with CSK and once with digestion buffer (CSK with 50 mM NaCl instead of 100 mM NaCl), followed by incubation for 1 h with 250 μg/ml DNase I (type IV; Sigma) in digestion buffer. Then cells were extracted with 0.25 M $(NH_4)_2SO_4$ in digestion buffer for 10 min and washed twice with CSK. By phase-contrast microscopy it was confirmed that more than 80% of the cells remained attached to the substratum during the in situ extractions. Permeabilized cells and nuclear matrices in petri dishes were collected in 200 μl sample buffer (2% SDS, 50 mM Tris (pH 6.8), 2% β-mercapto ethanol, 10% glycerol) and heated at 95°C for 5 min. Total cell protein samples were obtained by washing transfected and hormone treated cells twice in CSK, after which they were collected and heated in 200 μl sample buffer. Permeabilized cells and nuclear matrices on coverslips were prepared further for immunofluorescence labeling.

Polyacrylamide Gel Electrophoresis and Western Blotting

Protein samples from approximately 2×10^5 cell equivalents were separated on 8% polyacrylamide gels in the presence of SDS and transferred to nitrocellulose membranes [Towbin et al., 1979]. Membranes were incubated 16 h with 1% blocking reagent (Boehringer, Mannheim, Germany), followed by one of the antibodies mentioned above, followed by alkaline phosphatase conjugated goat-antimouse (Jackson ImmunoResearch Laboratories) or goat-antirabbit antibody (BioRad). Blots were stained using the BCIP/NBT method (BioRad). Antibodies were diluted in 0.1% gelatin, 0.5% bovine serum albumine, 0.05% Tween-20, and 300 mM NaCl in PBS. After staining, all blots were reprobed with monoclonal antibody 101-B7 against lamin B (Matritech, Cambridge, MA) and alkaline phosphatase conjugated goat-antimouse antibody. In case of the receptor mutants I488* and I550*,

lamin B probing was carried out on a separate, identical blot because these receptor mutants have approximately the same apparent molecular size as lamin B.

For semiquantification of the amount of receptor bound to nuclei and nuclear matrices, Western blots were made for each receptor mutant of equivalent amounts of nuclei and nuclear matrices and a dilution series of a total cell protein sample from the same experiment (not shown). Receptor band intensities of nuclei and nuclear matrices were compared by eye with the dilution series of the total cell preparation to estimate the approximate percentage of cellular receptor that was retained in nuclei and nuclear matrices. This analysis was carried out in duplicate or triplicate for each receptor mutant.

RESULTS

Nuclear Localization of Wild Type and Mutant hAR and hGR

To investigate the interaction of steroid receptors with the cell nucleus and the nuclear matrix, COS-1 cells were transiently transfected with expression vectors coding for wild type and mutated hAR and hGR. Binding to nuclei and nuclear matrices was examined by subjecting transfected cells to several in situ extractions, followed by Western blot analysis to estimate the amount of receptor that remained bound to nuclei or nuclear matrices. Evidently, only receptor mutants that were located in the cell nucleus after expression in COS-1 cells could be used to study binding to the nucleus and nuclear matrix. Therefore, we first investigated the subcellular localization of wild type and mutated receptor proteins by indirect immunofluorescence microscopy, using specific antibodies against these receptors.

The subcellular distribution of the wild type and a number of mutant hGRs is shown in Figure 1a–h. The wild type hGR was both cytoplasmic and nuclear in the absence of steroid hormone (Fig. 1a). After addition of the GR agonist dexamethasone (DEX), wild type and most mutant glucocorticoid receptors that were tested displayed an exclusively nuclear staining (Fig. 1b–h). Two hGR deletion mutants Δ451-487 and Δ420-777 remained cytoplasmic in the presence of DEX (not shown). The nuclear localization of hGR mutants I488* and I550* was not dependent on steroid, because these constructs lack the hormone binding domain. Based on their nuclear localization a number of hGR mutants were selected for the nuclear and nuclear matrix binding studies. These mutants are depicted in Figure 2a. Because mutant Δ1-420 was expressed at a very low level compared to the wild type receptor, binding studies were carried out with the almost identical mutant Δ9-385, which had a normal expression level.

The subcellular localization of various hAR mutants was published previously [Jenster et al., 1993]. Based on these data we selected a number of mutants that were localized in the nucleus after hormone stimulation. We tested the localization of an additional hAR mutant (AR67), which lacks both the DBD and most of the N-terminal domain. After stimulation with the AR agonist R1881, this mutant was localized mainly in the nucleus, but also some cytoplasmic labeling was present (Fig. 1i). The hAR mutants that were found to be localized in the nucleus after hormone stimulation are summarized in Figure 2b.

Isolation of Nuclei and Nuclear Matrices and Analysis of Receptor Binding

Binding of steroid receptors to the cell nucleus and the nuclear matrix was tested by in situ extraction of COS-1 cells expressing wild type or mutant hAR or hGR. Two different methods for the isolation of nuclear matrices were compared (Fig. 3). In method A, cells were permeabilized with a nonionic detergent and then stabilized with sodium tetrathionate (NaTT), digested with DNase I, and extracted with 0.25 M ammonium sulphate. Method B was identical to method A except that the permeabilization and the stabilization were combined in one single step. NaTT is known to stabilize the nuclear matrix structure [Kaufmann et al., 1981; Kaufmann and Shaper, 1984] and the binding of the glucocorticoid receptor to the nuclear matrix [Kaufmann et al., 1986], most likely by introducing disulfide bridges. Components which have a weak (i.e., rapidly dissociating) interaction with the nuclear matrix may be lost during permeabilization. Immediate addition of NaTT during permeabilization may result in stabilization of some of these weak interactions. Therefore, it was expected that method B would result in retention of more proteins in the nuclear matrix fraction than method A. We analyzed receptor binding in permeabilized cells and in nuclear matrices that were obtained with both methods.

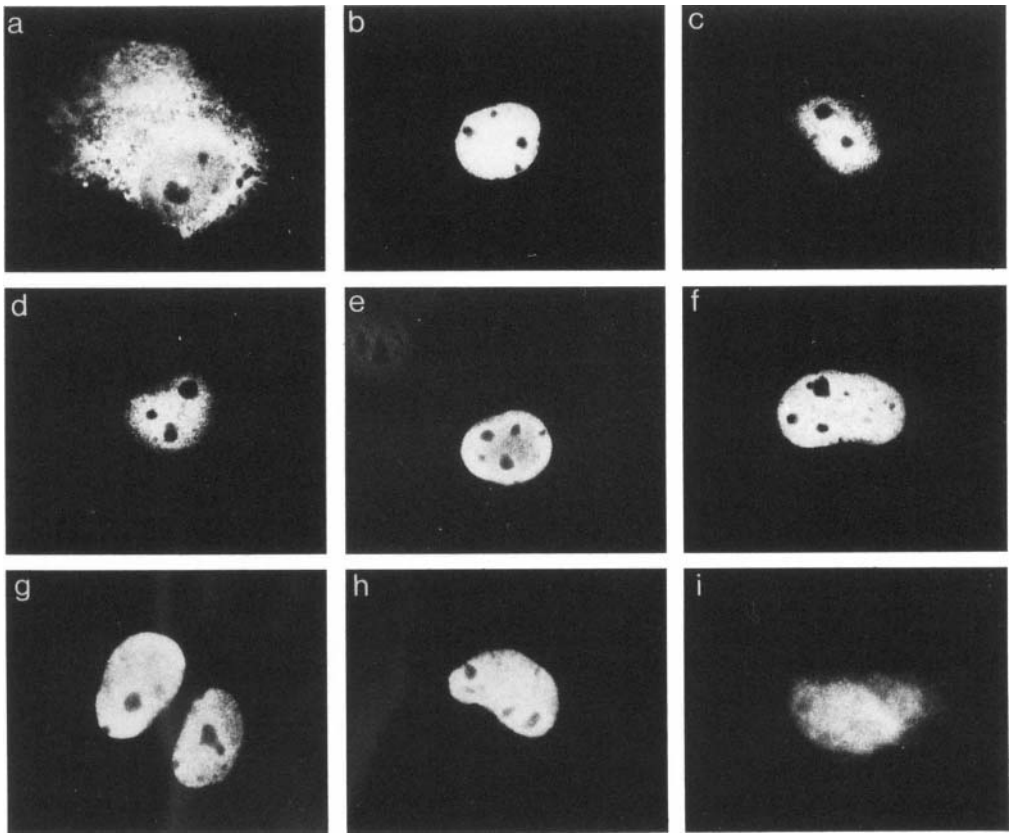

Fig. 1. Subcellular localization of wild type and mutated hGR and hAR. Immunofluorescence microscopy of cells expressing various hGR and hAR mutants. COS-1 cells were transfected with wild type hGR (**a,b**), hGR mutants G438 (**c**), Δ420-451 (**d**), I550* (**e**), I488* (**f**), Δ9-205 (**g**), or Δ1-420 (**h**) or hAR mutant AR67 (**i**). After growing 24 h on microscope coverslips in the absence of hormone (a) or in the presence of 10^{-8} M DEX (b–h) or 10^{-8} M R1881 (i), cells were formaldehyde-fixed and labeled with mouse monoclonal antibody mAb7 (a–f) or $GR_{788-795}$ (g,h) against the hGR or rabbit antiserum SP066 (i) against the hAR, followed by biotinylated sheep-antimouse antibody and FITC-labeled streptavidin (a–h) or FITC-labeled goat-antirabbit antibody (i).

Proteins from intact cells and from the insoluble fractions after extraction were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed for the presence of receptor, using specific antibodies against hAR or hGR. The apparent molecular weight of all hGR mutants, as judged from the Western blots, was close to the molecular size that can be predicted from the sequences of the constructs (data not shown). The apparent molecular weight of the wild type hAR and of the mutants AR13, AR64, and AR65 was 10–12 kDa higher than the predicted value, as was noticed previously [Jenster et al., 1991]. To verify that equivalent amounts of cells and nuclear matrices were loaded, blots were also probed with a monoclonal antibody against the 65 kDa nuclear matrix protein lamin B, which served as an internal standard. Lamin B fractionates completely in the insoluble nuclear fraction [Kaufmann and Shaper, 1984]. The amount of receptor that was bound to nuclei and nuclear matrices was determined semiquantitatively as described in Materials and Methods.

Only receptor mutants that were located in the nucleus after steroid treatment were included in the binding studies. Therefore, receptors that remained associated with the insoluble cell fractions after permeabilization or after matrix preparation were assumed to be bound to the nucleus and the nuclear matrix, respectively. To exclude the possibility that the extractions caused dissociation of receptors from the nucleus and subsequent trapping by residual cytoplasmic structures, we checked the localization of each receptor mutant after extraction by immunofluorescence microscopy. In no case was cytoplasmic labeling observed (data not shown). Thus, mutant receptors that were found by West-

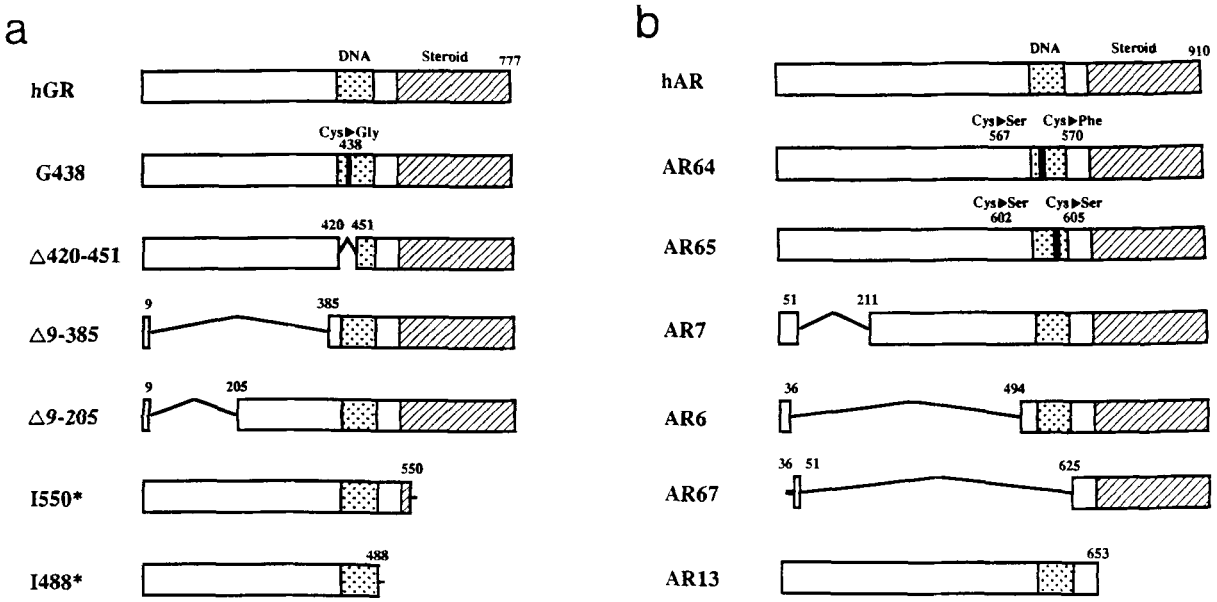

Fig. 2. Scheme of receptor mutants showing predominant nuclear localization. Schematic representation of hGR (**a**) and hAR (**b**) mutants that were tested for binding to nuclei and nuclear matrices. Nomenclature of the receptor mutants was taken from the publications in which they were described first [Hollenberg et al., 1987; Hollenberg and Evans, 1988; Jenster et al., 1993].

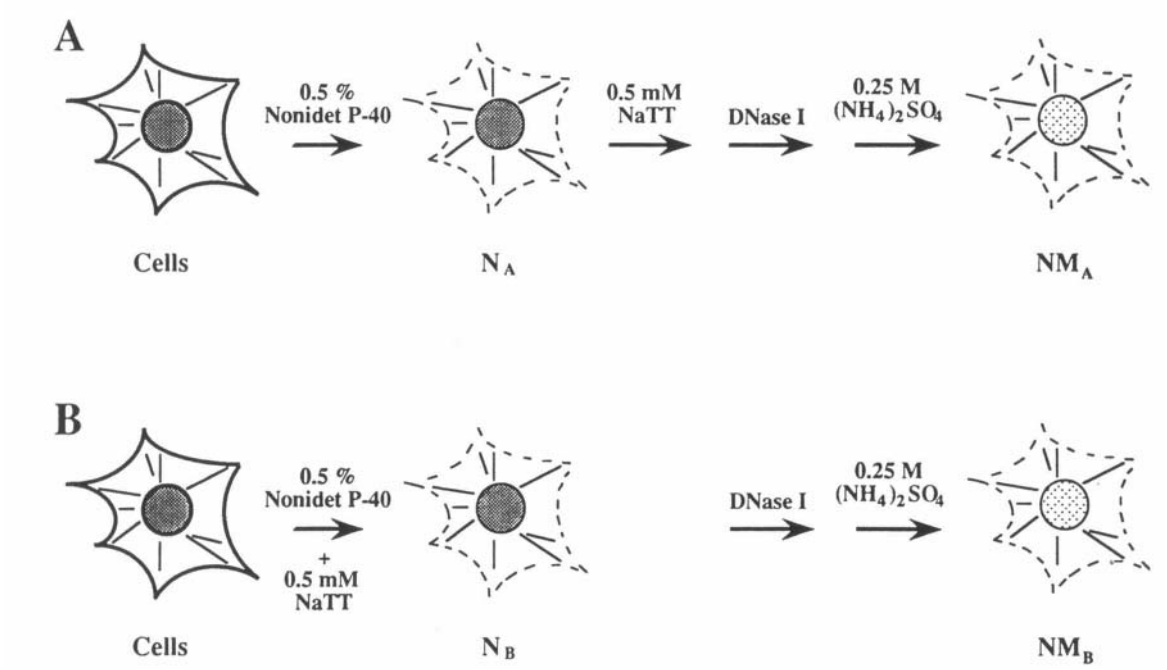

Fig. 3. Scheme of the extraction methods **A** and **B**. Schematic outline of the two in situ extraction methods A and B to isolate nuclear (N_A and N_B) and nuclear matrix (NM_A and NM_B) fractions from transfected cells. Method B was identical to method A, except that in method B the Nonidet P-40 permeabilization and the NaTT stabilization were combined in one single incubation step (see also Materials and Methods).

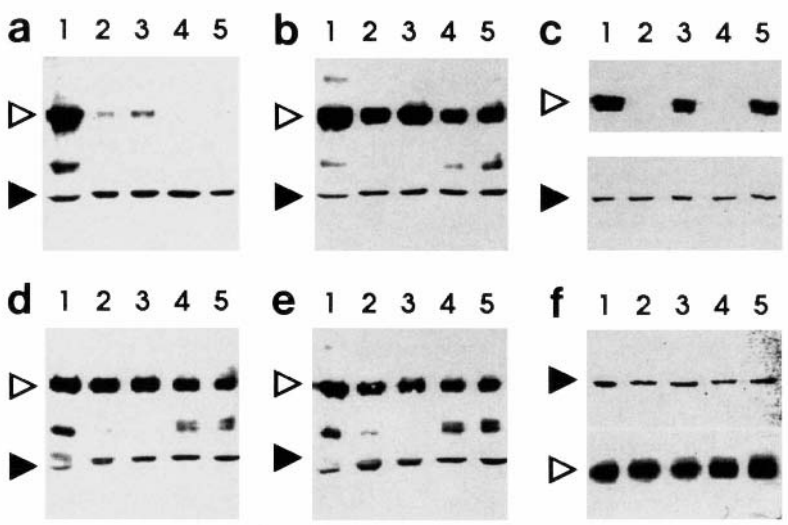

Fig. 4. Western blot analysis of hAR binding to nuclei and nuclear matrices. Cells expressing wild type hAR (**a,b**), AR13 (**c**), AR64 (**d**), AR65 (**e**), or AR67 (**f**) were grown in the absence (a) or presence (b–f) of 10^{-8} M R1881 and extracted as described in the text and Figure 3 to obtain nuclei or nuclear matrices. Protein samples from equivalent numbers of cells, nuclei, or nuclear matrices were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with monoclonal antibody F39.4.1 (a–e) or rabbit antiserum SP066 (f), followed by alkaline phosphatase-conjugated antimouse or antirabbit antibody, respectively. Blots were also probed with monoclonal antibody 101-B7 against lamin B, which served as an internal standard. Open arrowhead, band representing (mutant) receptor; solid arrowhead, band representing lamin B. Other bands may be hAR degradation products or represent alternative translational initiation products, because they were not observed in mock-transfected cells (data not shown). Lane 1: Cells, Lanes 2, 3: Nuclei, Lanes 4, 5: Nuclear matrices. Lanes 2, 4, extraction method A; lanes 3,5, extraction method B.

ern blot analysis to be resistant to permeabilization and nuclear matrix extraction were bound to nuclear structures and not to cytoplasmic components (e.g., the cytoskeleton).

Binding of Wild Type and Mutated hAR to the Nucleus and Nuclear Matrix

When cells expressing the wild type hAR and stimulated with R1881 were permeabilized (method A), approximately 15–40% of the receptor molecules remained bound to the nucleus (Fig. 4b; Table I). When NaTT was added during permeabilization (method B) 40–100% of the hAR remained bound to the nucleus. Subsequent isolation of nuclear matrices from these preparations did not result in any significant further extraction of receptor, indicating that most of the wild type hAR that is bound to the nucleus is also associated with the nuclear matrix. In the absence of hormone, less than 5% of the hAR molecules was bound to nuclei or nuclear matrices with either method A or method B (Fig. 4a). Thus, binding of the hAR to the nucleus and the nuclear matrix is hormone-dependent and is enhanced two- to threefold when NaTT is present during permeabilization of the cells.

TABLE I. Semiquantitative Analysis of Wild Type and Mutant hAR Binding to Nuclei and Nuclear Matrices*

	[R1881] (M)	N_A	NM_A	N_B	NM_B
hAR	0	–	–	–	–
hAR	10^{-8}	++	++	+++	+++
AR64	10^{-8}	++	++	++	++
AR65	10^{-8}	++	++	++	++
AR7	10^{-8}	n.d.	n.d.	++	++
AR6	10^{-8}	++	++	++	++
AR67	10^{-8}	+++	+++	+++	+++
AR13	10^{-8}	–	–	++	++

*The amount of receptor bound to nuclei (N_A and N_B) and nuclear matrices (NM_A and NM_B) prepared according to method A (N_A and NM_A) or method B (N_B and NM_B) was determined by semiquantitative Western blot analysis as described in Materials and Methods and was calculated as the percentage of the total amount of each mutant receptor in the cell. – = less than 5%; + = 5–15%; ++ = 15–40%; +++ = 40–100%; n.d. = not determined.

To identify the domains of the hAR that are involved in binding to the nuclear matrix, we tested the effect of several mutations on the binding of the hAR to permeabilized nuclei and nuclear matrices prepared according to method A or method B. Representative Western blots are shown in Figure 4c–f; the results of semi-

quantitative analysis (see Materials and Methods) of these and other blots are summarized in Table I.

When cells were extracted according to method A, deletion of the N-terminal domain (AR6) or disruption of either one of the two zinc fingers in the DBD (AR64 and AR65) had no significant effect on nuclear and nuclear matrix binding. However, deletion of the C-terminal domain (AR13) resulted in complete dissociation of the hAR during permeabilization of the cells. Complementary to this, a hAR fragment lacking both the DBD and almost the entire N-terminal domain (AR67) remained bound to the cell nucleus and the nuclear matrix. We conclude that binding of the hAR to the nucleus and the nuclear matrix prepared according to method A is mediated by the C-terminal domain.

Different results were obtained with nuclear matrices isolated according to method B. With this method, all mutants tested (including AR13) remained bound to the nuclear matrix. This indicates that addition of NaTT during permeabilization affects the interaction of the hAR with the nuclear matrix not only quantitatively, but also qualitatively. The interaction of the hAR with the nuclear matrix prepared by method B is most likely mediated by more than one domain.

Binding of Wild Type and Mutated hGR to the Nucleus and Nuclear Matrix

In contrast to the wild type hAR, the DEX-stimulated wild type hGR was completely extracted by permeabilization in the absence of NaTT (method A). Thus the hGR is not tightly bound to the nucleus under these conditions (Fig. 5b). Evidently, under these conditions the hGR is also not bound to the nuclear matrix either (data not shown). However, when NaTT was added during permeabilization (method B) 15–40% of the hGR molecules resisted extraction. The same amount of receptor was retained in nuclear matrices isolated according to method B, indicating that all nuclear bound hGR was associated with the nuclear matrix. In the absence of hormone, no hGR binding to nuclei or nuclear matrices was observed (Fig. 5a; Table II). Thus, binding of the hGR to the nuclear matrix is hormone-dependent and is only observed when NaTT is present during permeabilization of the cells.

Several mutant hGRs were tested for binding to nuclei and nuclear matrices. Representative Western blots are shown in Figure 5c–f; the results of semiquantitative analysis are summarized in Table II. After permeabilization in the absence of NaTT (method A), none of the hGR

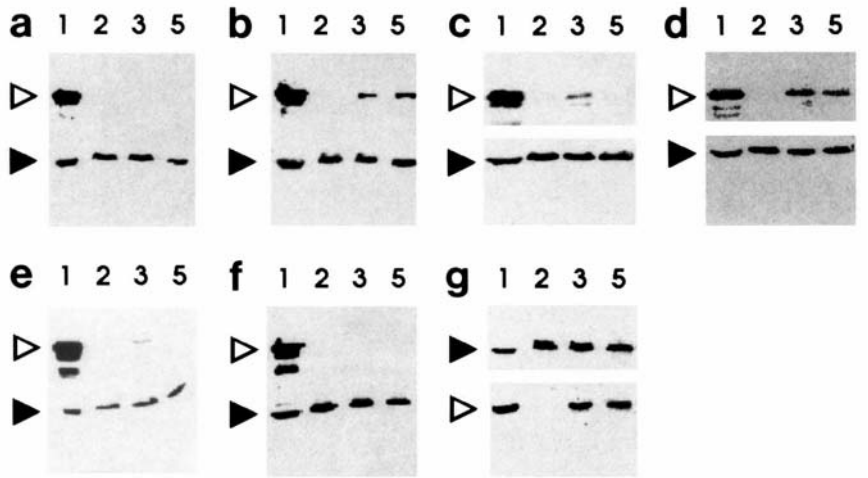

Fig. 5. Western blot analysis of hGR binding to nuclei and nuclear matrices. Cells expressing wild type hGR (**a,b**), I550* (**c**), I488* (**d**), Δ420-451 (**e**), G438 (**f**), or Δ9-385 (**g**) were grown in the absence (a) or presence (b–g) of 10^{-8} M DEX and extracted as described in the text and Figure 3 to obtain nuclei or nuclear matrices. Protein samples from equivalent numbers of cells, nuclei, or nuclear matrices were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with monoclonal antibody mAb7 (a–f) or $GR_{788-795}$ (g), followed by alkaline phosphatase-conjugated antimouse antibody. Blots were also probed with monoclonal antibody 101-B7 against lamin B, which served as an internal standard. Open arrowhead, band representing (mutant) receptor; solid arrowhead, band representing lamin B. Lane 1: Cells, Lanes 2, 3: Nuclei, Lane 5: nuclear matrices. Lane 2, extraction method A; lanes 3, 5, extraction method B.

TABLE II. Semiquantitative Analysis of Wild Type and Mutant hGR Binding to Nuclei and Nuclear Matrices*

	[DEX] (M)	N_A	N_B	NM_B
hGR	0	–	–	–
hGR	10^{-8}	–	++	++
G438	10^{-8}	–	–	–
Δ420-451	10^{-8}	–	–	–
Δ9-385	10^{-8}	–	++	++
Δ9-205	10^{-8}	–	++	++
I550*	10^{-8}	–	++	–
I488*	10^{-8}	–	++	+

*The amount of receptor bound to nuclei (N_A and N_B) and nuclear matrices (NM_B) prepared according to method A (N_A) or method B (N_B and NM_B) was determined by semiquantitative Western blot analysis as described in Materials and Methods and was calculated as the percentage of the total amount of each mutant receptor in the cell. – = less than 5%; + = 5–15%; ++ = 15–40%; +++ = 40–100%; n.d. = not determined.

mutants showed nuclear binding. This is consistent with the behaviour of the wild type hGR. When cells were extracted according to method B, deletion of part or most of the N-terminal domain (Δ9-205 and Δ9-385) did not affect binding to nuclei and nuclear matrices. However, deletion of the first zinc finger of the DBD (Δ420-451), or disruption of the first zinc finger by replacement of the cysteine residue at position 438 by glycine (G438), resulted in a striking decrease of receptor binding to nuclei and nuclear matrices. Deletion of the C-terminal domain (I488* and I550*) had no effect on nuclear binding but resulted in a partial (I488*) or complete (I550*) loss of nuclear matrix binding. These results demonstrate that the intact DBD of the hGR is required for binding of the hGR to the nucleus and nuclear matrix. The C-terminal domain is not essential for binding to the nucleus but is required for optimal binding to the nuclear matrix.

Effect of NaTT on Matrix Binding of Nuclear Proteins

The results described above clearly show that NaTT has a stabilizing effect on the nuclear binding of steroid receptors. The observation that mutation of a single cysteine in the DBD of the hGR (mutant G438) causes a dramatic decrease in binding to NaTT-stabilized nuclei and nuclear matrices indicates that the stabilizing effect of NaTT is highly selective. Most likely, after treatment with NaTT only a specific set of nuclear proteins is associated with the nuclear matrix. We tested this further by measuring nuclear matrix binding of a protein that has no known interaction with any nuclear component and contains several SH groups. For this purpose, COS-1 cells were transfected with an expression vector coding for a fusion protein (βgalNLS) consisting of β-galactosidase linked to the nuclear localization signal (NLS) of the SV40 large T antigen [Schreiber et al., 1992]. The βgalNLS protein contains 16 cysteine residues, of which the sulfhydryl groups are potential targets for oxidation by NaTT. Due to the presence of the NLS, the fusion protein is readily transported into the nucleus, as was confirmed by immunofluorescence microscopy using a monoclonal antibody against β-galactosidase (Fig. 6a). Despite its exclusive nuclear localization, the βgalNLS protein showed no detectable binding to nuclei or nuclear matrices, irrespective of the presence or absence of NaTT during permeabilization (Fig. 6b). This observation supports the view that the nuclear interactions of the hAR and hGR presented here are not artefacts induced by random sulfhydryl cross-linking by NaTT.

DISCUSSION

The nuclear matrix plays an important role in nuclear organization and may be involved in the regulation of transcription [de Jong et al., 1990; van Driel et al., 1991]. Steroid receptors bind to the nuclear matrix after hormone stimulation [Kaufmann et al., 1986; Barrack, 1987]. This binding has been reported to be saturable, indicating that specific acceptors for steroid receptors are present in the nuclear matrix [Barrack, 1983; Colvard and Wilson, 1984; Belisle et al., 1989; Metzger and Korach, 1990; Schuchard et al., 1991]. These acceptors may consist of residual matrix DNA, specific matrix proteins, or both.

To obtain insight in the mechanism of binding of steroid receptors to the nuclear matrix, we set out to identify protein domains in the hAR and the hGR that are involved in the interaction with the nuclear matrix. For this purpose we isolated nuclei and nuclear matrices from cells expressing wild type or mutant receptors. Binding of these receptors to nuclei and nuclear matrices was determined by semiquantitative Western blotting.

The internal fibrogranular network of the nuclear matrix is relatively labile and is in many

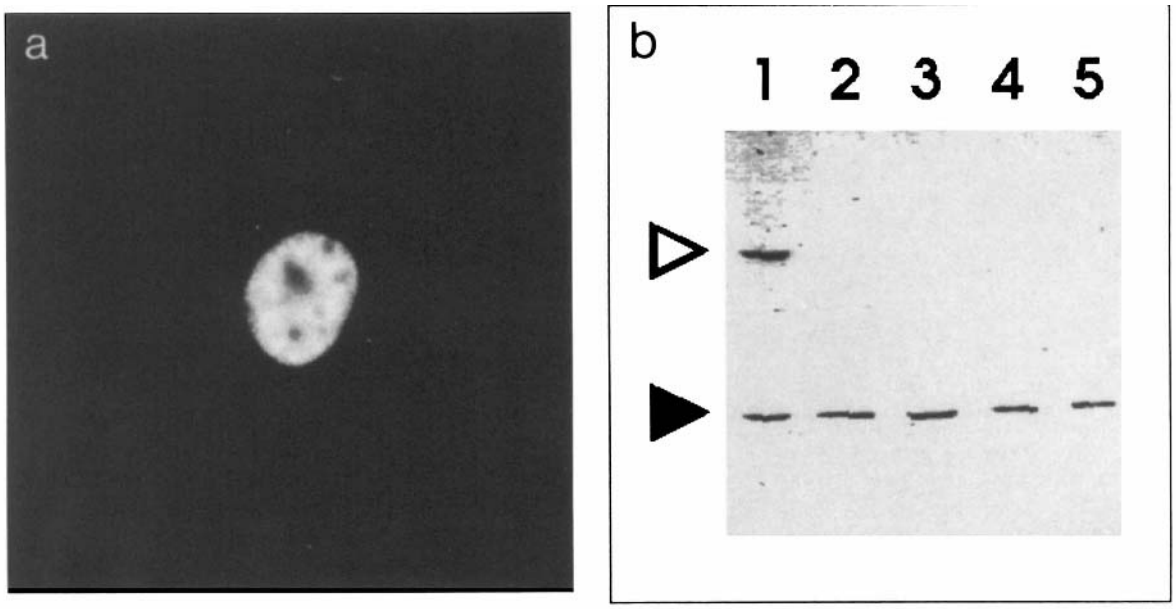

Fig. 6. Analysis of nuclear and nuclear matrix binding of a βgalNLS fusion protein. **a:** Subcellular localization of βgalNLS protein. COS-1 cells were transfected with plasmid pCHSV coding for the βgalNSL protein, grown on coverslips, fixed in 3.7% formaldehyde, and labeled with monoclonal antibody 12B3 against β-galactosidase, followed by FITC-labeled goat-antimouse antibody. No labeling was observed in cells transfected with empty expression vector. **b:** Western blot analysis nuclear and nuclear matrix binding of βgalNLS. Cells expressing βgalNLS were extracted as described in the text and Figure 3 to obtain nuclei or nuclear matrices. Protein samples from equivalent numbers of cells, nuclei, or nuclear matrices were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with monoclonal antibody 12B3, followed by alkaline phosphatase-conjugated antimouse antibody. The blot was also probed with monoclonal antibody 101-B7 against lamin B, which served as an internal standard. Open arrowhead, band representing βgalNLS; solid arrowhead, band representing lamin B. Lane 1: Cells. Lanes 2, 3: nuclei, Lanes 4, 5: Nuclear matrices, Lane 2, 4, extraction method A; Lane 3, 5, extraction method B.

cell types only found when a stabilization step is included in the isolation protocol. Various stabilization methods have been described, such as fixation with acrolein [de Graaf et al., 1991], incubation at 37 or 42°C [Mirkovitch et al., 1984; McConnell et al., 1987], treatment with Cu^{2+} [Kaufmann et al., 1981; Lebkowski and Laemmli, 1982], and oxidation with sodium tetrathionate (NaTT) [Kaufmann and Shaper, 1984]. The molecular events responsible for stabilization in most of these procedures are poorly understood. Stabilization by NaTT is most likely due to the formation of intra- and interprotein S-S bridges [Kaufmann and Shaper, 1984]. However, the NaTT-stabilized matrix is not a continuous network of disulfide bridged proteins [Stuurman et al., 1992]. This implies that other interactions than disulfides are important for matrix integrity too.

In the present study we utilized NaTT to stabilize the nuclear matrix. NaTT treatment was included either during permeabilization or after permeabilization of the cells (Fig. 3). When NaTT is present during permeabilization (method B) it rapidly stabilizes the nuclear matrix. We call the nuclear matrices prepared according to method B complete matrices. When NaTT is added after permeabilization (method A), some weakly associated matrix proteins may be extracted during the permeabilization step. Therefore, these nuclear matrices are called depleted matrices.

Our observations can be summarized as follows. Depleted matrices contained 15–40% of the wild type hAR protein (Table I). Evidently, this receptor fraction is tightly bound to the nuclear matrix. The interaction of these receptor molecules with the depleted matrix required an intact C-terminal domain. The N-terminal domain and the DNA-binding domain were not required. In contrast, complete matrices retained any of the mutant hAR proteins. We interpret these results as follows. Binding of the hAR to depleted nuclear matrices involves a tight interaction with an acceptor protein via the C-terminal hAR domain. In complete matrices, at least one other interaction of the hAR with the nuclear matrix must take place, since

deletion of the C-terminal domain does not abolish binding. This interaction is relatively weak, as it becomes evident only after NaTT stabilization. This interaction may be mediated by an acceptor that differs from the acceptor that binds to the C-terminal domain of the hAR. In conclusion, the C-terminal domain and at least one other domain of the hAR are involved in matrix binding.

The hGR behaved unlike the hAR. Neither wild type nor mutated hGR proteins were found in depleted matrix preparations. Complete matrices, however, contained 15–40% of the wild type hGR protein (Table II). These results indicate that under conditions that the matrix structure is not stabilized during permeabilization, either the hGR acceptors dissociate from the matrix, or the hGR-acceptor interaction itself is a weak interaction. Treatment with NaTT evidently stabilizes such interactions. It has been reported that the glucocorticoid receptor in NaTT-stabilized rat liver nuclear matrices is covalently bound to other matrix components through intermolecular disulfide bridges [Kaufmann et al., 1986]. This suggests that at least part of the stabilizing effect of NaTT results from direct cross-linking of the receptor to its nuclear matrix acceptors.

Disruption of the DBD of the hGR was sufficient to completely abolish interaction with complete matrices. Therefore, either residual matrix DNA or a matrix protein that specifically binds to the DBD may function as an acceptor for the hGR. The C-terminal domain of the hGR also contributed to matrix binding. One mutant, lacking most of this domain (I550*), was not retained by the matrix at all, whereas another C-terminal deletion mutant (I488*) bound only poorly. Presumably the C-terminal domain interacts with an acceptor protein. The observation that both the DBD and the C-terminal domain are required suggests that these two domains bind to the nuclear matrix in a cooperative fashion. The N-terminal domain of the hGR is not important for matrix binding under our experimental conditions.

The hAR and the hGR show different matrix binding characteristics. First, the hAR binds both to depleted and complete matrices, whereas the hGR binds only to complete matrix preparations. Second, for binding to complete matrices the hGR requires both the DBD and the C-terminal domain, whereas in the hAR disruption of either of these two domains does not affect binding to complete matrices. These differences clearly demonstrate that the hAR and the hGR are associated with the nuclear matrix through different molecular interactions. Most likely, the nuclear matrix contains distinct acceptors for the hAR and the hGR.

An important issue is the mechanism of stabilization of matrix protein interactions by NaTT. One might argue that the interactions of steroid receptors with the nuclear matrix are artefacts caused by nonspecific formation of disulfide bridges by treatment with NaTT. A number of observations argue against this possibility. First, disruption of the DBD by a single amino acid residue substitution results in loss of binding of the hGR to the nuclear matrix, indicating that the interaction is specific. Second, we show that the fusion protein βgalNLS does not bind to the nucleus or to the nuclear matrix in the presence of NaTT, although it is exclusively located in the nucleus and it contains 16 cysteine residues. Third, Stuurman et al. [1992b], have demonstrated that only a very limited set of nuclear matrix proteins is actually covalently cross-linked after treatment with NaTT. Fourth, it has been shown that in NaTT-stabilized nuclear matrices the polyoma large T antigen is covalently cross-linked to a small number of proteins [Humphrey and Pigiet, 1987]. These proteins are probably nearest neighbours of T antigen in the nucleus. Clearly, the stabilizing effect of NaTT is selective.

The role of different domains of the hAR and hGR in the activation of transcription has been extensively characterized [Hollenberg et al., 1987; Jenster et al., 1991]. Comparison of our results with these transactivition activity data shows that there is no positive correlation between nuclear matrix binding and activation of transcription by mutant receptors. For instance, the N-terminal domains of the hAR and the hGR are required for maximal transcriptional activation [Jenster et al., 1991; Hollenberg et al., 1987] but are not essential for nuclear matrix binding. Conversely, hGR mutant I550*, which lacks most of the C-terminal domain, still shows 40% of the wild type transactivation activity [Hollenberg and Evans, 1988] but does not bind to the nuclear matrix. Also the C-terminal domain of the hAR interacts with the nuclear matrix, but deletion of this domain does not result in a decrease in transactivation of a reporter gene construct in COS-1 cells [Jenster et al., 1991]. This lack of correlation between ma-

trix binding and transactivation activity suggests that matrix binding is not directly involved in the regulation of transcription by steroid receptors. However, it should be emphasized that the available data on the role of steroid receptor domains in the regulation of transcription are based on measurements using artificial reporter plasmids, which have only a poorly defined chromatin structure. It remains possible that steroid receptor-matrix interactions are required for the transcriptional activation of genes in their proper chromatin context.

We showed by immunofluorescence microscopy that all hGR mutants that were tested for nuclear matrix binding were localized in the nucleus after steroid treatment (Fig. 1). These localization data are consistent with previously reported results [Cadepond et al., 1992]. In general, we observed predominant nuclear localization for mutant proteins that contained the intact bipartite nuclear localization sequence (NLS). In the hGR this NLS consists of two conserved stretches of basic amino-acid residues at positions 479–480 and 491–495, separated by a spacer of ten residues [Dingwall and Laskey, 1991]. Although the truncated hGR mutant I488* lacked the C-terminal part of the bipartite NLS, we found this mutant to be located exclusively in the nucleus. In agreement with this observation Cadepond et al. [1992] have reported the predominantly nuclear localization of a similar mutant (I491*), which is truncated after position 491. Apparently the conserved NLS basic residues at position 491–495 are not absolutely required for nuclear import of these hGR mutants.

We also found the hAR mutant AR67 to be mainly nuclear, despite complete deletion of the bipartite NLS at position 608–625 [Jenster et al., 1993]. Because of its small molecular size (~35 kDa), this mutant protein may be able to traverse the nuclear pore without requiring a NLS [Nigg et al., 1991]. The nuclear accumulation of the AR67 protein is probably the result of its interaction with the nuclear matrix.

It has been reported that disruption of the DBD of the hAR causes a dramatic change in the subnuclear distribution of this receptor [Jenster et al., 1993]. After hormone stimulation, the wild type hAR is homogeneously distributed throughout the nucleoplasm, whereas mutants AR64 and AR65 are concentrated in large clusters inside the nucleus. Also, in nuclear matrix preparations we found a homogeneous distribution for the wild type hAR and a clustered distribution for AR64 and AR65 (data not shown). These clusters may be either large aggregates of these mutant receptors due to reduced solubility or caused by an interaction of the hAR mutants with specific nuclear substructures, such as nuclear bodies [Stuurman et al., 1992a]. In both cases the altered distribution indicates that mutant receptors AR64 and AR65 display nuclear interactions which are not manifest for the wild type receptor. Thus, caution should be taken with the interpretation of binding data of these mutants. All other mutants showed a normal wild type–like subnuclear distribution.

In conclusion, we show that specific protein domains of the hAR and hGR are involved in binding to the nuclear matrix. The binding characteristics of both receptors depend on the precise method that is used to isolate the nuclear matrix. Evidently, the interaction of certain receptor domains with the nuclear matrix is relatively labile. Our results show that the hAR and the hGR are attached to the nuclear matrix through different molecular interactions. The physiological relevance of these receptor-matrix interactions remains to be elucidated. Matrix binding of the various receptor mutants is not clearly correlated to their potency to activate transcription of an artificial reporter gene construct. However, receptor-matrix interactions may play a significant role in the activation of genes in their proper chromatin context. Alternatively, matrix binding may be involved in other receptor functions, such as inhibition of the expression of specific genes, or intranuclear transport or storage of receptors. Identification of the nuclear matrix acceptors that interact with steroid receptors will be an important step towards understanding the function of matrix binding.

ACKNOWLEDGMENTS

B.v.S. and G.J. made equal contributions to the work described in this article. We thank Dr. R.M. Evans for the wild type and mutant hGR plasmids, Dr. J. Ménissier-de Murcia for the pCHSV plasmid, Dr. J.-Å. Gustafsson for the mAb7 antibody, Dr. H.M. Westphal for the $GR_{788-795}$ antibody, and Dr. J. Trapman for the 12B3 antibody. We thank Dr. E.R. de Kloet for stimulating discussions and Erica van Binnendijk for excellent technical assistance. This work was supported by the medical council (GB-

MW) of the Dutch Organization for Scientific Research (B.v.S. and G.J.).

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