

Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor

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We have detected nuclear localization signals within the 795 amino acid rat glucocorticoid receptor. Using a transient expression assay, we monitored by immunofluorescence the subcellular distribution of receptor derivatives and β -galactosidase-receptor fusion proteins. Two distinct nuclear localization signals, NL1 and NL2, were defined. NL1 maps to a 28 amino acid segment closely associated, but not coincident with the DNA binding domain; NL2 resides within a 256 amino acid region that also includes the hormone binding domain. Most importantly, nuclear localization of fusion proteins containing either the full-length receptor or the NL2 region alone is fully hormone-dependent; similar results were obtained with the wild-type receptor, provided the analysis was performed in medium lacking serum and phenol red. The rate of hormone-induced nuclear localization of an NL2-containing fusion protein is consistent with the rapid kinetics of hormone-regulated transcription mediated by the receptor. Thus, hormonal control of nuclear localization contributes to the modulation of glucocorticoid receptor transcriptional regulatory activity.

Key words: nuclear localization/glucocorticoid receptor/signal transduction/steroid hormones

Introduction

The glucocorticoid receptor is an intracellular protein that associates specifically with cognate hormonal ligands, and mediates signal transduction. The hormone–receptor interaction facilitates ‘receptor transformation’, a poorly understood alteration in the receptor that culminates in its functional association with specific DNA sequences termed glucocorticoid response elements (GREs) within the cell nucleus; such hormone–receptor–GRE complexes regulate the rate of transcription initiation from nearby promoters (for review see Yamamoto, 1985). Manipulation and expression of the sequence encoding the 795 amino acid rat glucocorticoid receptor revealed that the steroid binding domain resides within the C-terminal third of the molecule, while an 86 amino acid segment between residues 440 and 525 appears sufficient for specific DNA binding and for GRE-mediated transcriptional regulation (Godowski *et al.*, 1987; Rusconi and Yamamoto, 1987; Miesfeld *et al.*, 1987a,b). Similar conclusions are emerging from investigations of human (Giguère *et al.*, 1986; Green and Chambon, 1987; Hollenberg *et al.*, 1987) and mouse (Danielson *et al.*, 1986) glucocorticoid receptors.

The rat glucocorticoid receptor has a deduced monomer mol. wt of 87.5 kd (Miesfeld *et al.*, 1986), which exceeds the apparent exclusion limit of ~70 kd for passive diffusion through nuclear pores (Paine *et al.*, 1975; Bonner, 1975; Lang *et al.*, 1986). Nuclear accumulation of proteins appears to be mediated by

nuclear localization signals (for review see Dingwall and Laskey, 1986; Silver and Hall, 1987), protein segments thought either to facilitate diffusion and intranuclear retention, or to interact with putative active transport machinery (see also Bonner, 1978; De Robertis, 1983). Thus, it seemed likely that the glucocorticoid receptor might contain one or more nuclear localization signals. Conceivably, modulation of the activity of such signals might play a role in the hormone-dependent receptor transformation process. According to this view, the interaction of the receptor with GREs and other intranuclear components might be prevented in the absence of hormone by excluding the receptor from the nuclear compartment.

Previous studies of the equilibrium intracellular distribution of the glucocorticoid receptor in the absence of added hormone have yielded conflicting results (see for example Govindan, 1980; Papamichael *et al.*, 1980; Antakly and Eisen, 1984; Fuxe *et al.*, 1985; Welshons *et al.*, 1985; Wikström *et al.*, 1987; see also Mendel *et al.*, 1986). In the present report, we first determined the subcellular localization of the receptor and receptor fragments upon transient expression of cloned receptor cDNA sequences in transfected cells. We then expressed specific subregions of the receptor coding sequence as β -galactosidase fusion proteins, thus enabling further characterization of nuclear localization signals.

Results

Experimental strategy

Our general approach was to assess the subcellular distribution of various portions of the glucocorticoid receptor or β -galactosidase-receptor fusion proteins expressed transiently from recombinant plasmids in transfected cells. The transfected cultures were incubated in media containing or lacking steroid ligands, and the receptor derivatives visualized in fixed cells by indirect immunofluorescence. Monkey COS7 or CV-1 cells were utilized as transfection recipients; these lines (which gave similar results) are well suited for this type of analysis because they lack detectable endogenous receptor. Only some of the cells within a transfected culture appeared to take up and express the exogenous DNA as judged by their strong immunofluorescence staining; other cells remained relatively free of staining. This provided a useful internal control for the specificity of the immunoassays.

Hormone-dependent nuclear localization of intact receptor

We first examined the subcellular distribution of the intact glucocorticoid receptor, using for the immunoassays monoclonal antibody 250 (Okret *et al.*, 1984), which recognizes an epitope in the N-terminal portion of the receptor (Okret *et al.*, 1984; Rusconi and Yamamoto, 1987). These initial experiments revealed that receptor localization is strongly affected by the cell culture conditions. We focused in particular on the fetal calf serum and the phenol red pH indicator included in the growth medium; phenol red has been shown to interact with the estrogen receptor and displays weak agonist activity (Berthois *et al.*, 1986). Indeed, when cells transfected with sequences encoding the in-

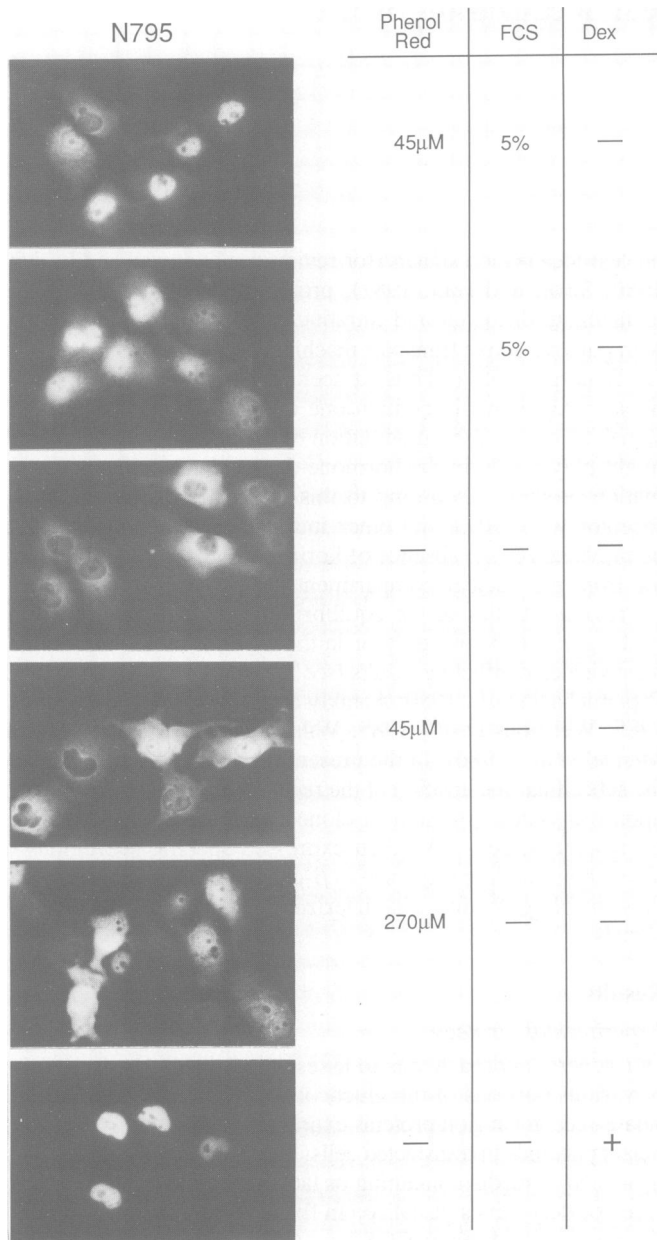


Fig. 1. Culture conditions affect subcellular distribution of the glucocorticoid receptor. Representative micrographs of the immunofluorescence analysis are shown. COS7 cells were transfected with the receptor expression vector N795 in growth medium lacking phenol red; after 24 h, cells were washed and further incubated for 18 h in medium supplemented with phenol red, fetal calf serum (FCS), and 0.1 μ M dexamethasone (Dex) as indicated.

tact receptor (N795; for description of nomenclature, see Materials and methods) were incubated under standard growth conditions — Dulbecco's modified Eagle's (DME) medium (which includes 45 μ M phenol red) supplemented with 5% fetal calf serum — the receptor was predominantly nuclear in about two-thirds of the fluorescence-positive cells (Figures 1 and 2).

Given these findings, we analyzed independently the contributions of phenol red and fetal calf serum, as well as the effects of steroid ligands on receptor localization to the nucleus. Thus, N795 was transfected into multiple culture dishes in medium containing 5% fetal calf serum but lacking phenol red; after 24 h, subsets of dishes were shifted for 18 h to serum-free medium alone, or to serum-free medium containing various concentra-

tions of phenol red. In each case, the overall level of receptor expression was indistinguishable from that observed in control cultures incubated in standard medium (data not shown).

As shown in Figure 1 and summarized in Figure 2, the unliganded receptor was predominantly cytoplasmic in the absence of serum and phenol red, whereas addition of either component alone increased the level of nuclear accumulation of the receptor: at 5% fetal calf serum, about 2/3 of the fluorescence-positive cells displayed nuclear staining; the effect was somewhat less pronounced with 45 μ M phenol red alone, although at 270 μ M phenol red, the receptor was predominantly nuclear in about half of the stained cells. Notably, the endogenous cortisol levels in the medium were about 2 nM, only 1% of the concentration required for half-maximal receptor binding (Rousseau *et al.*, 1972). Thus, the serum factors that induce localization have not been identified, but it is intriguing that only the intact receptor, containing the wild-type hormone binding domain, was sensitive to those factors (Figure 2 and data not shown).

Significantly, addition of 0.1 μ M dexamethasone provoked complete nuclear localization of the receptor in 100% of the stained cells even in medium lacking both serum and phenol red (Figures 1 and 2). Thus, we conclude that nuclear localization of the intact receptor is partially induced by certain components in standard growth medium, but that dexamethasone induces complete nuclear localization even in the absence of both serum and phenol red. Interestingly, 0.3 μ M RU486, a strong glucocorticoid antagonist (Bourgeois *et al.*, 1984; Becker *et al.*, 1986), also triggered efficient accumulation of receptor in the nucleus (Figure 2); this suggests that receptor 'transformation' involves more than just ligand-induced nuclear localization (see Discussion).

Detection of two nuclear localization signals within the glucocorticoid receptor

To examine in more detail the steroid-dependent nuclear localization of the glucocorticoid receptor, recombinant plasmids bearing deletions in various portions of the receptor coding region were transfected into COS7 cells. Monoclonal antibody 250 was used as described before to detect the receptor derivatives diagrammed in Figure 2; in each case the epitope is retained. The eight C-terminal deletion mutants shown, whose steroid binding is abolished by truncation of 27 (in N768) to 558 amino acids, fell into three subclasses when subcellular distribution was examined. First, deletion derivatives N768, N694 and N671 were on average predominantly cytoplasmic or evenly distributed between nucleus and cytoplasm, similar to the behavior of the intact receptor in the absence of hormone. In contrast, N615 and N525 were nuclear, suggesting that a C-terminal portion of the intact unliganded receptor may inhibit nuclear localization (see Discussion). Finally, N508, N464 and N237. β (which contains receptor amino acids 1–237 fused to the last 48 amino acids of rabbit β -globin) were predominantly cytoplasmic, consistent with the view that a nuclear localization signal, which we denote NL1, resides N-terminal of amino acid 525. The cytoplasmic distribution of N508 is notable because this derivative retains considerable DNA binding activity *in vitro* (Rusconi and Yamamoto, 1987), implying that DNA binding alone cannot account for efficient nuclear localization.

To test for nuclear localization activity independent of both NL1 and the DNA binding domain, we constructed N237.540C, an internal deletion derivative lacking amino acids 238–539. Rusconi and Yamamoto (1987) showed that the receptor segment from amino acid 547 to the C-terminus retains hormone binding activity at elevated concentrations of dexamethasone. Indeed,














	receptor	LOCALIZATION	
		-Dex	+Dex
N795	1  795	- C>N (few C=N) ¹ - N>C (2/3) ²	³ N
N768	 768	C>N (1/2) ^{1, 2}	C>N (1/2) ⁴
N694	 694	C>N (few C=N)	n.d.
N671	 671	C>N to N>C	n.d.
N615	 615	N	n.d.
N525	 525	N	n.d.
N508	 508	C>N	n.d.
N464	 464	C>N (few C=N)	n.d.
N237.β	 237 	C>N	C>N ⁴
N237.540C	 237  540  795	C>N (few C=N) ^{1, 2}	N>C to N=C ⁴

Fig. 2. Subcellular distribution of receptor deletion mutants. The results of immunofluorescence analyses are summarized. Receptor derivatives are schematically represented by stippled boxes with numbers indicating amino acid positions; see Materials and methods for description of nomenclature. N795 is the wild-type receptor. In recombinant N237.β, the 237 N-terminal amino acids of the receptor are fused to the last 48 amino acids of the rabbit β-globin (hatched box). To allow alignment of all derivatives, sequences deleted in recombinant N237.540C are represented by a thin line. Dex, dexamethasone; C and N, cytoplasmic and nuclear fluorescence, respectively; C>N, predominantly cytoplasmic fluorescence accompanied by some nuclear staining; N>C, predominantly nuclear fluorescence accompanied by some cytoplasmic staining; C=N or N=C, equal cytoplasmic and nuclear staining; C>N (few C=N), vast majority of stained cells are C>N, while a few cells are C=N; N>C (2/3), about two thirds of stained cells are N>C, while other third is N=C to C>N; C>N (1/2), half of stained cells is C>N while other half is C=N to N>C; n.d., not determined. Except where indicated, medium contained phenol red and FCS; note that these results and others (not shown) demonstrate that phenol red and FCS effect partial nuclear localization of only the intact receptor. Superscripts: 1 and 2, cells were cultured as indicated in the legend to Figure 1 in the absence and in the presence, respectively, of 45 μM phenol red and 5% FCS; 3, medium plus 0.1 μM dexamethasone or 0.3 μM RU486; 4, 10 μM dexamethasone.

N237.540C is predominantly cytoplasmic in the absence of hormone, and accumulates in the nucleus at 10 μM dexamethasone (Figure 2). Thus, this derivative defines a second nuclear localization signal, NL2, which appears to be hormone-dependent for activity. Because N237.β is predominantly cytoplasmic in the absence and presence of hormone, we conclude provisionally that NL2 resides within the fragment that encompasses the steroid binding domain.

Nuclear localization of β-galactosidase-receptor fusion proteins

NL1 and NL2 were characterized further by constructing plasmids encoding protein fusions of *Escherichia coli* β-galactosidase and specific portions of the receptor. This strategy has several useful features: first, β-galactosidase is not intrinsically destined for the nucleus (see below); second, antibodies specific for the β-galactosidase moiety could be used for immunoassays independent of receptor sequences; third, β-galactosidase is a large protein (116 kd monomer), thus limiting to less than a factor of two the mol. wt differences between the largest and the smallest β-galactosidase-receptor fusion derivatives, and maintaining all derivatives well above the apparent exclusion limit for passive entry through nuclear pores (Paine *et al.*, 1975; Bonner, 1975; Lang *et al.*, 1986).

Results for 13 such fusion derivatives are summarized in Figure 3B, and representative micrographs are shown in Figure 3A. As

expected, β-galactosidase itself and fusion derivative Z.4-445, in which β-galactosidase is joined to the N-terminal half of the receptor, are predominantly cytoplasmic both in the presence and in the absence of dexamethasone. In contrast, fusions that included either the entire receptor, or only its C-terminal half, were almost exclusively cytoplasmic in the absence of hormone and nuclear upon dexamethasone addition. This behavior was observed when the receptor sequences were fused either to the N-terminus or to the C-terminus of β-galactosidase. Moreover, these results were obtained even in standard growth medium; therefore, studies of the fusion derivatives were carried out in the presence of serum and phenol red. We do not understand the apparent insensitivity to the culture conditions of the fusion proteins compared to the receptor alone, but it is conceivable that their relatively high mol. wts increase the stringency of the requirements for nuclear localization. The largest of the fusion proteins are >200 kd, suggesting a native molecular weight of >800 kd if β-galactosidase tetramers were formed. In any case, it is clear that the hormone-dependent nuclear localization activity of the receptor mediates efficient nuclear entry and retention even of large proteins not normally destined for the nucleus.

NL1 was mapped in greater detail by testing fusions containing subfragments of the C-terminal half of the receptor (Figure 3). Extending the preliminary conclusion from studies of C-terminal deletions of the receptor alone, we found that NL1 ac-

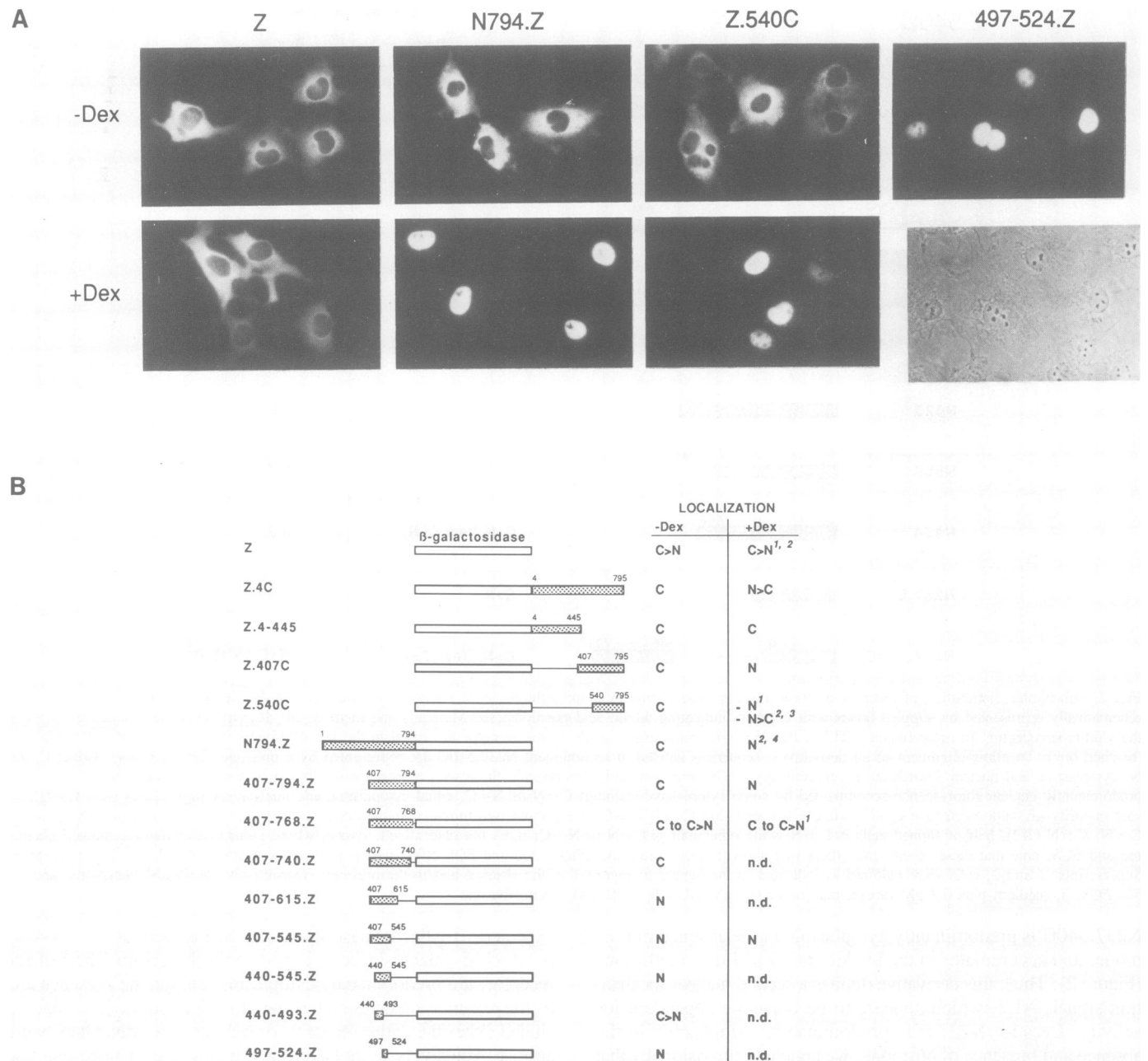


Fig. 3. Subcellular distribution of β -galactosidase-receptor fusion derivatives. **(A)** Immunofluorescence micrographs of representative recombinants (see also summarized results in panel B). Recombinant proteins Z, N794.Z, and Z.540C were expressed in COS7 cells in the absence (-Dex) or presence (+Dex) of dexamethasone. For recombinant 497-524.Z, both the immunofluorescence (-Dex) and the phase contrast (lower right) micrographs of the same field are shown. **(B)** Summary of immunofluorescence analyses. Receptor portions and β -galactosidase are represented by stippled and open boxes, respectively. Numbers above stippled boxes indicate N- and C-terminal amino acid positions of receptor moieties. To allow alignment of all derivatives, receptor sequences deleted are represented by a thin line. Assessment of the fluorescence staining was as described in the legend to Figure 2. Except where indicated, 0.1 μ M dexamethasone was used for the '+Dex' condition. Superscripts: 1, 10 μ M dexamethasone; 2, 0.1 μ M dexamethasone; 3, 10 μ M RU486; 4, 0.3 μ M RU486.

tivity is retained in derivative 497-524.Z, which carries only 28 receptor amino acids; this fusion protein is localized efficiently to the nucleus even in the absence of hormone. In addition, the results shown in Figure 3 confirm that NL2 resides within the C-terminal 256 amino acids between 540 and 795. Thus, fusion derivative Z.540C displayed dexamethasone-dependent nuclear localization similar to that seen with N237.540C; moreover, the antagonist RU486 also elicited this behavior.

Kinetics of NL2-mediated nuclear localization

To estimate the kinetics of nuclear localization, we expressed

fusion derivative Z.540C in the absence of hormone, and then activated NL2 subcellular localization by addition of dexamethasone 42 h after transfection. Subcellular distribution of the large (140 kd monomer; 560 kd tetramer) fusion protein was then monitored at various times thereafter. As shown in Figure 4, nuclear localization occurred with a $t_{1/2}$ of roughly 5 min, and was virtually complete within 15-30 min. This localization rate appears sufficient to account for the kinetics of specific transcriptional regulation by the hormone-receptor complex, which proceeds with a $t_{1/2}$ of 8-9 min after addition of hormone (Ucker and Yamamoto, 1984).

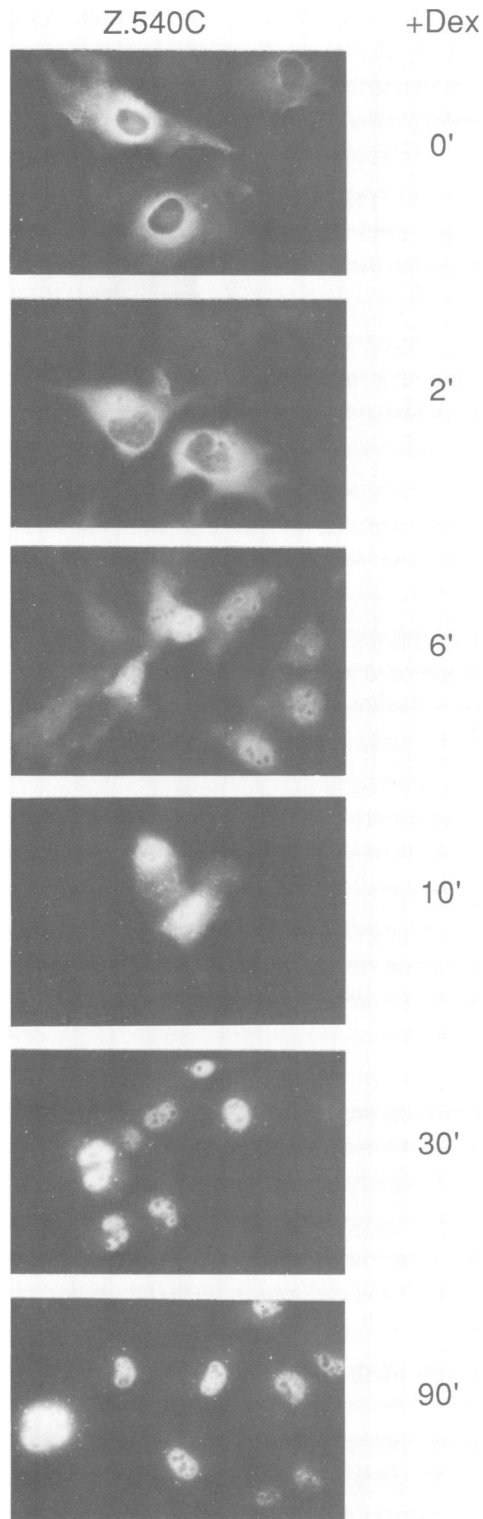


Fig. 4. Time-course of NL2-mediated nuclear localization. Nuclear localization of derivative Z.540C was triggered 42 h after transfection by addition of 10 μ M dexamethasone (Dex) for 2, 6, 10, 30 and 90 min (see also Figure 3A for distribution of Z.540C after long term dexamethasone treatment); cells were then fixed and analysed by immunofluorescence.

Discussion

Two signals for nuclear localization

We have shown that the glucocorticoid receptor accumulates in nuclei in a hormone-dependent fashion, presumably mediated by the two distinct localization signals that we have defined, NL1

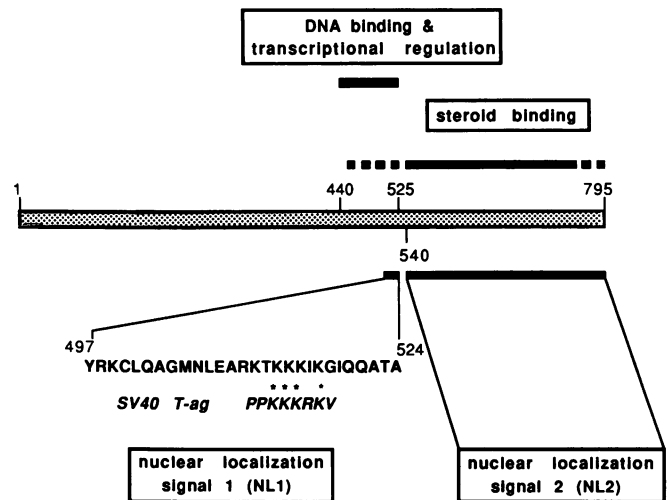


Fig. 5. Functional domains of the rat glucocorticoid receptor. The 795 amino acids of the receptor are represented by the stippled box. Solid bars above and below it indicate functional domains that have been mapped previously (Miesfeld *et al.*, 1986, 1987a,b; Godowski *et al.*, 1987; Rusconi and Yamamoto, 1987) and in the present work, respectively. The nuclear localization signal of the SV40 T-antigen (Kalderon *et al.*, 1984a,b) is shown in italics and its homology to NL1 sequences indicated by asterisks.

and NL2. NL1 is distinct from the hormone binding region, and is constitutively active when the two are uncoupled, whereas NL2 activity has not been separated from the ligand binding domain. Neither NL1 nor NL2 binds DNA *in vitro* (Rusconi and Yamamoto, 1987), suggesting that association of the receptor with nuclei does not simply reflect an increase in its DNA binding activity upon 'transformation', the alteration in receptor properties triggered by hormone binding. The notion that DNA binding and nuclear localization are distinct and separable functions is further corroborated by the recent isolation of clustered point mutations that retain DNA binding activity but are defective in nuclear localization (D.P. and P. Godowski, unpublished).

NL1, which maps to amino acids 497–524 (Figure 5), resides in a basic, potentially α -helical segment containing a subregion that is 50% homologous to a nuclear localization signal of SV40 large T-antigen (Kalderon *et al.*, 1984a,b); indeed, the arginine within the T-antigen signal has been changed to isoleucine (thus producing a 5 of 8 match with the receptor sequence) with little effect on nuclear localization (Smith *et al.*, 1985). The entire NL1 sequence is conserved perfectly in the human (Weinberger *et al.*, 1985) and the mouse (Danielsen *et al.*, 1986) glucocorticoid receptors, perhaps reflecting the fact that NL1 is included in a portion of the receptor that mediates multiple receptor activities (Rusconi and Yamamoto, 1987; Godowski *et al.*, 1987; Miesfeld *et al.*, 1987a,b). Further genetic analyses will be essential to define precisely the residues involved in NL1 function. Analyses of other nuclear proteins have revealed that localization signals display complex sequence patterns; in fact, several sequences unrelated to the SV40 signal have been described (for review see Dingwall and Laskey, 1986; Silver and Hall, 1987), but none of these is found within the glucocorticoid receptor.

We have not yet succeeded in further delimiting NL2; similar difficulties were encountered in mapping the steroid binding domain within this same 256 amino acid region (Rusconi and Yamamoto, 1987). Conceivably, this might indicate that the NL2 signal is 'assembled' from noncontiguous residues that are juxtaposed only upon hormone binding.

Several proteins have been shown to contain multiple nuclear localization signals (Hall *et al.*, 1984; Kalderon *et al.*, 1984a,b;

Welsh *et al.*, 1986; Richardson *et al.*, 1986; M.N.Hall, C.Craik, G.Mullenbach and Y.Hiraoka, personal communication), and it has been suggested that the rate (Lanford *et al.*, 1986) of localization may increase with multiple signals, presumably reflecting a rate-limiting interaction with the transport machinery (Goldfarb *et al.*, 1986). In the case of the receptor, we measured localization rates upon hormone addition, and found that fusion derivative Z.407C, which contains both nuclear localization signals, displays the same rapid kinetics of localization as Z.540C, which contains only NL2 (data not shown). This implies that NL2 is the major kinetic determinant for receptor localization.

Nuclear localization is hormone-dependent

A novel feature of the intact receptor is that nuclear localization activity is fully dependent upon prior hormone binding, a requirement that is relieved by deletion of > 180 amino acids from the C-terminus. Thus, in the absence of hormone, residues in this C-terminal domain normally may inhibit localization activity. Several models could account for this finding. First, unliganded receptor might be physically 'anchored' by association with a cytoplasmic component. In fact, the untransformed receptor is detected as a large protein complex in crude extracts under nondenaturing conditions, whereas the transformed hormone-receptor complex appears monomeric (Holbrook *et al.*, 1983; Sherman *et al.* 1983; Vedeckis, 1983; Raaka and Samuels, 1983). The 90 kd heat-shock protein is one of the factors found associated with the unliganded receptor (Sanchez *et al.*, 1985), and is therefore a potential candidate for an anchoring factor. This scheme is generally similar to that suggested by Nigg *et al.* (1985) for the catalytic subunit of the type II cAMP-dependent protein kinase; the kinase subunit localizes to the nucleus upon cAMP-induced release from its regulatory subunit, which remains associated with the Golgi complex. An alternative possibility is that NL1 and NL2 are simply masked in the absence of hormone, either by receptor sequences themselves, or by association with other cellular factors. In this case, hormone binding might effect a conformational change in the receptor that unmasks the nuclear localization signals. A third model is that the localization signals might themselves be 'assembled' as a result of the hormone-induced conformational change in the receptor; for NL1, this scheme seems less likely since it would require that multiple deletions all adventitiously induce a conformational change that yields a functional signal.

Gross genetic manipulations, such as these described here, do not distinguish these models. Indeed, a detailed understanding of the hormone dependence of receptor nuclear localization will require not only further genetic studies, but also biochemical approaches. For example, Newmeyer *et al.*, (1986a,b) have shown that ATP is required for transport of at least one nuclear protein (nucleoplasmin) *in vitro*, suggesting that nuclear localization may involve active transport. It has not been established whether this is the case for receptor, but it has long been known that nuclear association of glucocorticoid receptor can be mimicked under cell-free conditions (Baxter *et al.*, 1972).

Clearly, the hormone-dependence of nuclear localization of the receptor should prove to be a useful characteristic. It is particularly intriguing that NL2 can be separated from the rest of the receptor as a hormone-dependent 'nuclear localization cassette', and may provide a general tool for rapidly shuttling proteins of interest between the cytoplasmic and the nuclear compartments in a highly regulated fashion.

Nuclear localization is necessary but not sufficient for transcription regulation

The equilibrium distribution of receptors for glucocorticoids (e.g., see Govindan, 1980; Papamichael *et al.*, 1980; Antakly and Eisen, 1984; Fuxe *et al.*, 1985; Welshons *et al.*, 1985; Wikström *et al.*, 1987; see also Mendel *et al.*, 1986) and for other steroids (King and Green, 1984; Welshons *et al.*, 1984, 1985; Perrot-Aplanat *et al.*, 1985, 1986) in the apparent absence of bound ligands has been a matter of considerable controversy. Immunoassays of glucocorticoid receptors in tissues from adrenalectomized rats showed a marked reduction in nuclear staining (Antakly and Eisen, 1984; Fuxe *et al.*, 1985), consistent with the view that the hormone-free receptor is predominantly cytoplasmic under physiological conditions.

In our standard cell culture medium, β -galactosidase-receptor fusion proteins, but not intact receptor itself, were cytoplasmic in the absence of added hormones; when fetal calf serum and phenol red were removed from the medium, even the intact receptor remained cytoplasmic, indicating that components in standard media may artifactually induce partial nuclear localization of the receptor, unless it is 'constrained' by fusion to β -galactosidase. Moreover, receptors localized to nuclei in standard media appeared nonfunctional with respect to transcriptional regulation at GRE-linked promoters. Similarly, RU486 efficiently elicits nuclear localization of the receptor, but failed to trigger GRE function (Bourgeois *et al.*, 1984); even at the high intracellular concentrations of receptor present in our transfected cells, RU486 displays only very weak agonist activity (D.Sakai and K.R.Y., unpublished results). Finally, we found that receptor derivative N615 is localized constitutively to the nucleus; this receptor mutant binds GRE-containing DNA specifically *in vitro* (Rusconi and Yamamoto, 1987), but is unable to activate detectably transcription from a GRE-regulated promoter *in vivo* (Godowski *et al.*, 1987).

Taken together, these findings demonstrate that nuclear localization alone is not sufficient for receptor-mediated transcriptional regulation. This implies that receptor 'transformation' is a complex process, perhaps involving multiple steps that separately affect receptor competence for nuclear localization, DNA binding and transcriptional regulation. In any case, we have established clearly that acquisition of competence for nuclear localization is a strongly hormone-dependent event, suggesting that this may be a critical point in the signal transduction pathway by which the hormonal ligand ultimately modulates the expression of specific genes.

Materials and methods

Plasmids

Deletion mutants of the rat glucocorticoid receptor and β -galactosidase fusion proteins are denoted according to the following nomenclature: derivatives named with N or C contain receptor amino acids extending from the N-terminus or C-terminus, respectively, through the given amino acid number (for example, N795 refers to the intact receptor); names with two hyphenated numbers denote derivatives that include the receptor segment delineated by the given amino acid numbers; fusions of non-contiguous receptor sequences, and of receptor sequences to β -galactosidase (Z) or to rabbit β -globin (β) are symbolized by a decimal point.

Receptor derivatives N768, N694, N671, N615, N525, N508 and N464 as well as their cloning into the SV40 expression vector pSV7d have been described previously (Godowski *et al.*, 1987).

The expression vector for constructs described below was derived from a recombinant with the SV40 enhancer, the human α 1-globin promoter, and the second intron and the polyadenylation site of the rabbit β -globin gene (Pääbo *et al.*, 1986) by recloning into the plasmid pSP64. In the present study this expression vector was also used for expression of the intact receptor, N795.

The recombinant N237. β was obtained by an in-frame ligation of the *Bgl*III site of the receptor at amino acid 237 to the *Bam*HI site of the rabbit β -globin gene (Maniatis *et al.*, 1978; van Ooyen *et al.*, 1979) at amino acid 100.

The receptor deletion mutant N237.540C was constructed by an in-frame fusion of the *Bgl*III site at amino acid 237 to the *Pst*I site at amino acid 540 of the receptor using synthetic linkers.

Recombinant Z, the β -galactosidase expression vector, was constructed by joining a segment encoding 5' untranslated sequences and the first three codons of herpes simplex virus thymidine kinase (see clone pTK3 in Rusconi and Yamamoto, 1987) to the *lacZ* coding region from clone pMC1871 (Casadaban *et al.*, 1983). The same segment of the thymidine kinase gene provided the translation start site for all 5' deletion mutants of the receptor (see Rusconi and Yamamoto, 1987). As an equivalent alternative, pRSV- β Gal (Edlund *et al.*, 1985) was used as an expression vector for β -galactosidase.

Recombinants 407-768.Z, 407-615.Z, 407-545.Z, 440-545.Z and 440-493.Z were constructed by in-frame fusion of receptor sequences from recombinants X766, X616, X546, 440C and X546, and 440C and X492, respectively (described by Rusconi and Yamamoto, 1987), to the 5' end of the *lacZ* gene from clone pMC1871. For recombinant 497-524.Z, a fragment from plasmid 497C (Rusconi and Yamamoto, 1987) encoding receptor amino acids 497-524 was ligated to receptor sequences at amino acid 542 of recombinant 407-545.Z by an in-frame junction of two *Pst*I sites.

Recombinants Z.4C, Z.407C and Z.4-445 were obtained by in-frame fusions of receptor sequences from recombinants 4C (S.Rusconi, unpublished), X795 (Rusconi and Yamamoto, 1987), N445 (R.Miesfeld and K.R.Y., unpublished) and 4C, respectively, to the *Bam*HI site at the 3' end of the *lacZ* gene from clone pUR291 (Rüther and Müller-Hill, 1983). For recombinant Z.540C, receptor sequences downstream of the *Pst*I site at amino acid 540 were fused in-frame to the *Pst*I site at the 3' end of the *lacZ* gene from clone pUR292 (Rüther and Müller-Hill, 1983).

To allow in-frame fusion of the entire steroid binding domain to β -galactosidase, untranslated 3' trailer sequences were deleted by nuclease *Bal*31 resection and a synthetic *Bam*HI linker was ligated at the terminus. The deletion mutant lacking two C-terminal amino acids retains full hormone binding capacity (Rusconi and Yamamoto, 1987). We used a *Bal*31 deletion mutant lacking only one amino acid at the C-terminus of the receptor to construct N794.Z and 407-794.Z; the receptor sequences indicated were ligated to a *Bam*HI site at the 5' end of the *lacZ* gene from clone pMC1871. Recombinant plasmid 407-740.Z was derived from 407-794.Z by in-frame ligation of the *Hind*III site at receptor amino acid 740 to the *Hind*III site at amino acid 792.

Cell culture and transfection

Monkey kidney COS-7 (Gluzman, 1981) and CV-1 cell lines were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal calf serum (Hyclone).

Cells were transfected with 5 μ g DNA on 60 mm dishes by the calcium phosphate coprecipitation technique (Graham and van der Eb, 1973; Wigler *et al.*, 1978) with the following modifications: after incubating the cells with the coprecipitate for 15-18 h, cultures were washed with 5 ml TBS (25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM Na₂HPO₄, pH 7.4; Kimura and Dulbecco, 1972) to dissolve the precipitate, and then were incubated with fresh medium. To screen large numbers of recombinants, we routinely isolated plasmid DNA from 3 ml bacterial cultures by the alkaline lysis procedure (Birboim and Doly, 1979), followed by RNase treatment, extraction with phenol/methylene chloride (1:1), and selective isopropanol precipitation. For transfection using such DNA preparations, approximately 1 μ g was mixed with 4 μ g of a carrier plasmid DNA purified by CsCl-EtBr and CsCl density gradient centrifugation. Using this procedure, these relatively crude DNA preparations transfected as efficiently as a highly purified plasmid DNA (data not shown). Where indicated, dexamethasone was added concomitantly with the DNA, or, without noticeable difference, 15-24 h later. Cells were fixed for immunoassay 42 h after transfection.

Immunofluorescence assay

Indirect immunofluorescence analysis was carried out as described by Banerji *et al.* (1981) with the following modifications: PBS (7.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl) was used throughout the procedure, and cells were generally fixed on 60 mm tissue culture dishes with 70% (v/v) methanol, 30% (v/v) acetone (kept at -20°C). We have obtained identical results in an analysis of the intact receptor (N795), β -galactosidase (Z), and a fusion derivative (N794.Z) using the procedure of Wikström *et al.* (1987), in which the cells are fixed with 3.7% (w/v) formaldehyde in PBS for 30 min followed by permeabilization with 0.5% (v/v) Triton X-100 in PBS for 30 min (data not shown). The first antibody was added for 2 h at room temperature. The mouse monoclonal antibody raised against *E. coli* β -galactosidase (a gift from J.Partaletis and T.Mason, provided by M.N.Hall) was diluted 1:10; the monoclonal antibody

250 against rat glucocorticoid receptor (Okret *et al.*, 1984) was diluted 1:2500. These antibodies gave identical results in an analysis of fusion protein N794.Z, which contains both epitopes (data not shown). The second antibody, rhodamine-conjugated F(ab')₂ fragment of goat anti-mouse IgG (heavy and light chain specific; Cappel), was added at a dilution of 1:200 for 1 h at room temperature.

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