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Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors

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Multiple proto-signals (p-NLSs) for nuclear targeting, none of which suffices on its own, cooperate in the estrogen (ER) and progesterone (PR) receptors. In the ER, an estrogen-inducible p-NLS was found in the hormone binding domain (HBD), in addition to three lysine/arginine-rich motifs resembling prototype constitutive nuclear localization signals (NLSs). The inducible and the constitutive ER p-NLSs cooperate in the presence of estrogen and hydroxy-tamoxifen, but not in the presence of ICI 164,384. In the PR, three p-NLSs, two of which are located within and directly adjacent to the second zinc finger, cooperate with each other and a weak hormone-inducible p-NLS in the PR HBD. No 'masking' of p-NLSs by the HBD was observed for ER and PR, while the ligand-free glucocorticoid receptor HBD inhibited the activity of both homologous and heterologous NLSs. Nuclear co-translocation experiments indicated that *in vivo* the stability of ER and PR dimers is hormonally controlled, but that, in the absence of the cognate ligand, ER dimers are more stable than PR dimers. This is likely to account for the differential hormone requirement of ER and PR DNA binding *in vitro*.

Key words: DNA binding/steroid receptor/nuclear localization signal/cooperation

Introduction

The intracellular steroid hormone receptors are members of a superfamily of inducible transcriptional trans-regulators which, in response to binding of cognate ligands, specifically recognize response elements of target genes (enhancers in the case of positive regulation). The structure of these receptors is modular and there are separate hormone and DNA binding domains [HBD (region E) and DBD (region C) respectively]. Both the hormone-binding domain and the N-terminal region A/B contain independent transcriptional activation functions which exhibit promoter- and cell-specificity, and may exert their action through currently unidentified transcriptional intermediary factors (for reviews and references see Beato, 1989; Evans, 1988; Green and Chambon, 1988; Gronemeyer, 1991).

The classical concept of cytoplasmic to nuclear translocation following activation was modified when intranuclear localization of the unliganded human estrogen (hER) and progesterone (hPR) receptors was demonstrated (Gasc *et al.*, 1984; King and Green, 1984; Welshons *et al.*, 1984; Ylikomi *et al.*, 1985). 'Activation' refers to a not yet fully understood structural alteration of the receptor which occurs upon ligand binding (Gronemeyer, 1991). The intracellular localization of ligand-free glucocorticoid receptor (GR) is, however, still controversial (Antakly *et al.*, 1989; Gasc *et al.*, 1989; Picard and Yamamoto, 1987; Wikstrom *et al.*, 1987) The molecular weight of the monomeric hER is ~65 kDa (Green *et al.*, 1986); this might be small enough to allow passive diffusion through nuclear pores (~40–60 kDa; see Lang *et al.*, 1986; Paine *et al.*, 1975), while the molecular weights of PR (Kastner *et al.*, 1990; Misrahi *et al.*, 1987) and GR (Hollenberg *et al.*, 1985) clearly exceed this limit. There is evidence that specific nuclear localization signals (NLSs) are responsible for size-independent active nuclear targeting of proteins, which appears to involve two distinct steps, nuclear pore binding and energy-consuming translocation (Dingwall and Laskey, 1986; Newmeyer and Forbes, 1988; Richardson *et al.*, 1988; Roberts, 1989). Proteins that bind specifically to NLSs have been detected (Adam *et al.*, 1989; Yamasaki *et al.*, 1989). Nuclear targeting of β-galactosidase fusion proteins has provided evidence for the existence in the hER and rat GR of a constitutive NLS resembling the SV40 T-antigen NLS prototype (Picard *et al.*, 1990; Picard and Yamamoto, 1987). In addition, the existence of a hormone-dependent NLS has been reported for the rat GR (Picard and Yamamoto, 1987), but no such signal was found in hER (Picard *et al.*, 1990).

In this study we have chosen to analyze steroid hormone receptor NLSs in their natural amino acid sequence context for the following reasons. (i) It is known that the efficiency of NLSs is sensitive to variations in the protein context; thus, a given NLS may become inactive when placed in a different environment even within the same protein (Roberts *et al.*, 1987). (ii) The β-galactosidase marker that is commonly used for heterologous nuclear targeting studies is a tetrameric protein, while steroid receptors are assumed to be dimers (Eriksson and Wrange, 1990; Kumar and Chambon, 1988; Sabbah *et al.*, 1989; Tsai *et al.*, 1989) and the consequences of multimerization on NLS efficiency is unknown. (iii) The possible cooperation between several nuclear targeting sequences and the effect of other domains of the protein on their activity can be assessed only in their natural environment.

We present here a detailed analysis of the signals that trigger the intracellular compartmentalization of ER, PR and GR, in both the absence and presence of their ligands. We report the identification and cooperation of three constitutive and one estrogen-inducible hER nuclear targeting sequence which we have termed proto-NLSs (p-NLSs), as all of these sequences have characteristics of 'classical' NLSs (see Discussion), while none of them individually (nor even two

of them together) was sufficient for wild-type nuclear targeting of the receptor. Three constitutively active proto-NLSs cooperate in the chicken PR and one of these signals is located in the second zinc finger of the DBD. In addition, the PR contains a weak inducible p-NLS. We report also that in the absence of ligand the intracellular localization of GR is different from that of ER and PR, because the strong constitutive GR NLS is masked by the ligand-free GR HBD. Finally, we present the results of nuclear co-translocation experiments aimed at investigating the hormone requirement for ER and PR dimerization.

Results

Experimental design

The intracellular accumulation of wild-type and mutant hER and cPR, transiently expressed in HeLa cells grown in a phenol-red free medium containing charcoal-stripped fetal calf serum, was investigated by using immunohistochemical techniques with previously described anti-hER monoclonal antibodies (kindly given by Abbot) and polyclonal or monoclonal anti-cPR antibodies (M.T.Bocquel, Y.Lutz and H.Gronemeyer, unpublished results). Receptors were visualized with biotinylated secondary antibody and avidin–biotin–peroxidase complex. No staining was observed in non-transfected HeLa cells or when using non-specific IgG as primary antibody (data not shown). Furthermore, cells expressing hER did not stain with anti-cPR antibodies, cells expressing cPR did not stain with anti-hER antibodies, and no differences in subcellular localization were seen when the amount of transfected expression vector was varied between 1 and 10 μg (data not shown). A crosslinking fixative (paraformaldehyde) and a precipitating fixative (methanol/acetone) both gave identical results, as did several modifications of the basic procedure (Materials and methods).

All data were derived from at least two independent transfections with independent receptor expression vector preparations. Serum deprivation (20 h before fixation) did not affect receptor compartmentalization (data not shown). For each transfection at least 250 stained cells were scored, and data were expressed as percentages of cells that fall into one of the four categories described in the legend to Figure 1. To characterize mutants we use the terms 'cytoplasmic' and 'wild-type nuclear' for nuclear scores (category N) of $<5\%$ and $>85\%$ (see HE0 and HE17 in Figure 1), respectively. The number of cells counted in each category and their immunocytochemical presentation is exemplified in Figure 1, showing the wild-type nuclear localization of hER (HE0) and cPR (cPR1), and no preferential subcellular compartmentalization of the majority of a hER (HE17, see Figure 2) and a cPR (cPR6, see Figure 4) 'cytoplasmic' mutant.

The hER contains several estrogen-independent p-NLSs located outside the 'zinc finger' region and the hormone binding domain

In the absence of hormone, wild-type hER is predominantly ($N > 80\%$) found in the nuclei of transfected cells [Figures 1 and 2; both HE0 and HEG0 (Tora *et al.*, 1989) gave identical results]. Truncation of either the HBD in HE21, HE39 or HE38B, or the N-terminal region A/B (HE19) still yielded mutants exhibiting wild-type nuclear localization (Figure 2, Table I). However, HE15B, HE16 and HE17 were cytoplasmic mutants (Figure 2, Table I), thus defining a NLS function between amino acids 265 and 303 (i.e. the sequence added to HE15B in order to obtain HE38B, Figure 2). This sequence is contained within the region (amino acids 256–303) that has been previously shown to be sufficient to target a heterologous protein, *Escherichia coli* β-galactosidase, into the nucleus (Picard *et al.*, 1990). Interestingly, and in contrast to other steroid receptors (see below), this region contains three basic motifs (designated as p-NLS1, 2 and 3 in Figure 2) which exhibit some similarity with the prototype SV40 T-antigen NLS. On their own, however, these NLS-like motifs were unable to direct heterologous proteins to the nucleus (Picard *et al.*, 1990). Thus, the significance of these putative NLSs was investigated in their natural protein context.

Removal of lysines 302 and 303 (HE38, Figure 2) reduced the number of exclusively nuclear stained cells by ~30% (Table I, compare HE38 with HE38A and B). Note that this deletion affects the basic motif 299-KRSKK (p-NLS1 in Figure 2). Since HE38A and HE15A showed similar

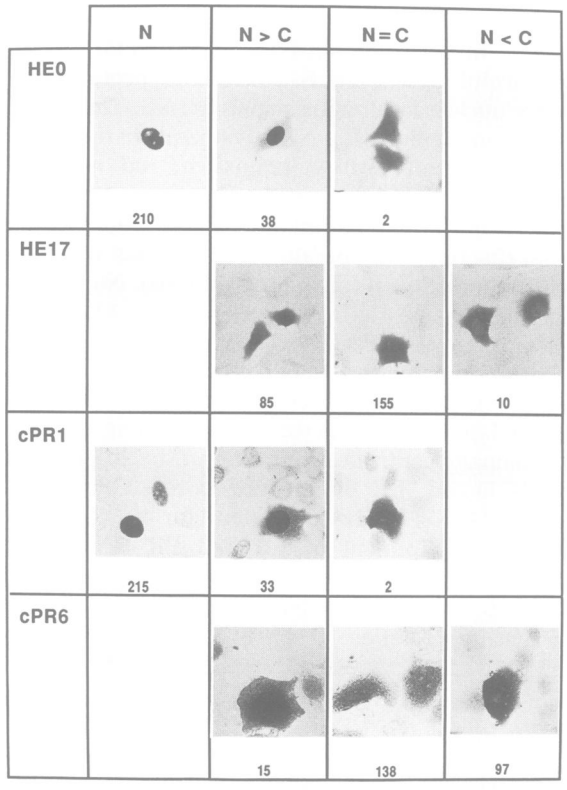


Fig. 1. Classification of immunohistochemical staining patterns after transfecting HeLa cells with different human estrogen receptor (hER) and chicken progesterone receptor (cPR) mutants. Cells were transfected with wild-type receptor expression vectors (HE0, HEG0 or cPR1) or C-terminal deletion mutant (HE17 or cPR6) expression vectors in which the entire NLSs are deleted (with the exception of those in the zinc finger domain; see text). Cells were stained using monoclonal ER or polyclonal PR antibodies. The staining was classified into four categories: N, cells which contain only nuclear staining; N > C, cells which contain also cytoplasmic staining; N = C, cells in which the intensity of nuclear and cytoplasmic staining is equal; N < C, cells in which the intensity of cytoplasmic staining exceeds nuclear staining. The numbers given here are the actual numbers of cells in a representative experiment falling into these categories with a total of 250 transfected cells scored. Throughout the paper these data are reported as percentages of cells falling into each category, derived from two to 15 independent experiments.

intracellular distribution, the sequences between 298 and 274 appear to have no NLS function. However, further deletion to amino acid 264 generated the cytoplasmic mutant HE15B (Figure 2, Table I; see also HE16), thus indicating the presence of at least one additional NLS function, located N-terminally of amino acid 274. There are two NLS-like motifs between amino acids 256 and 273 [denoted by p-NLS2 (266-KHKRQR) and p-NLS3 (256-RKDRR)]. In order to assess their individual contribution to nuclear targeting, selective deletions were introduced into HE39 (wild-type nuclear localization). Deletion of amino acids 261–271 which contain p-NLS2 (yielding HE39A, Figure 2) resulted in a drastic decrease in nuclear localization when compared with HE39 (Table I). In contrast, deletion of a sequence containing p-NLS3 yielded a mutant (HE39B, Figure 2) that still exhibited exclusive nuclear staining in 16% of all transfected cells (Table I). Moreover, the remaining 84% showed more intense staining in the nucleus than in the cytoplasm (Table I). Interestingly, an additional truncation in HE39B which deleted p-NLS1 (HE246, Figure 2) reduced the number of exclusively nuclear stained cells to 1%, with 85% of the cells still exhibiting nuclear accumulation (Table I).

The above results indicate that none of the three NLS-like motifs is an efficient NLS on its own. However, a mutant (HE246) containing only p-NLS2 exhibited residual activity in its natural sequence context [note that it could not direct β-galactosidase into the nucleus (Picard *et al.*, 1990)]. Moreover, p-NLS2 can apparently cooperate with p-NLS3 and/or p-NLS1, since p-NLS2 together with either p-NLS1 (HE39B) or p-NLS3 (HE15A) gave a much higher level of nuclear accumulation than that observed with any of the isolated NLSs. In contrast, the p-NLS1/p-NLS3 association (HE39A) did not generate an efficient NLS. As the three hER NLSs have similar sequences to that of the 'prototype' NLSs (e.g. the SV40 T-antigen NLS; see Discussion), it is likely that each of them can bind to cognate receptors of the nuclear pore complex. Taken individually, however, these bindings are insufficient for wild-type nuclear localization, and hER p-NLS1, -2 and -3 have to cooperate to become efficient in nuclear targeting. Thus, we consider them as 'proto-NLSs'.

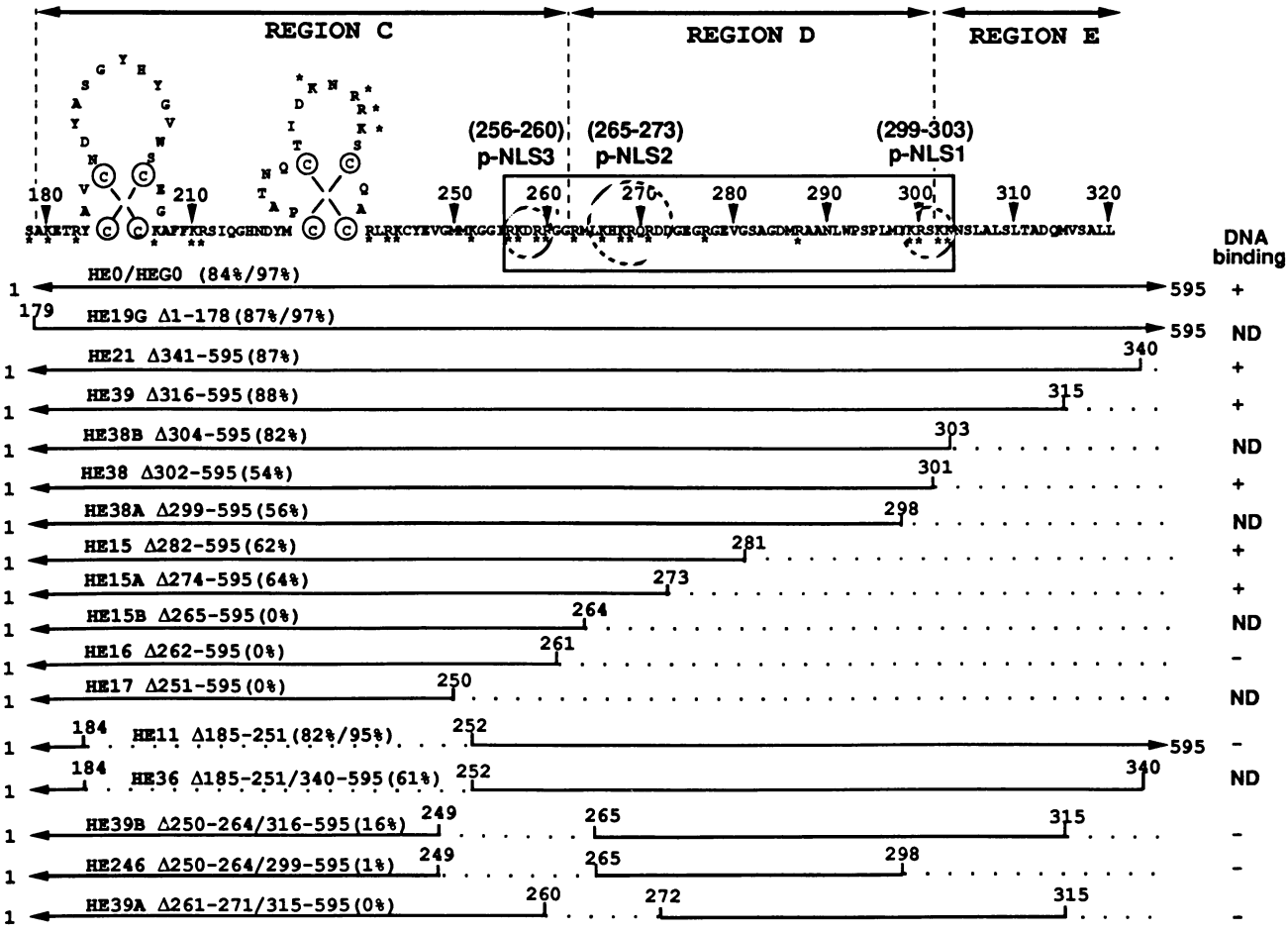


Fig. 2. Schematic illustration and intracellular localization of full-length (HE0 and HEG0) and C-terminally truncated mutant estrogen receptors. Regions C and E contain the DNA and steroid binding domain, respectively. The putative zinc fingers in the DNA-binding domain are indicated by loops. For details concerning nomenclature and architecture of receptors, see Introduction and references therein. Sequences expressed in the constructs are represented by horizontal lines with the first and last amino acid numbered. Deleted sequences are indicated by dotted lines, the amino acid residues missing are given after the mutant names. Percentages in brackets indicate the fraction of cells that exhibit exclusively nuclear staining (category N in Figure 1) in the absence of estradiol; or in the absence and presence of estradiol where two percentages are given. The box containing residues 256–303 indicates the sequence that is sufficient to target β-galactosidase to the nucleus (Picard *et al.*, 1989). Circled sequences (p-NLS1 to 3) indicate the hER p-NLS as described in the text. HE0 contains a valine at position 400, whereas HEG0 has a glycine at the same position and corresponds to the wild-type receptor (Tora *et al.*, 1989).

Table I. Intracellular localization of estrogen receptor mutants lacking the hormone binding domain

	Deletion	Intracellular localization			
		N	N > C	N = C	N < C
HE0/HEG0		84	16		
HE19G	1–178	87	13		
HE21	341–595	87	13		
HE39	316–595	88	12		
HE38B	304–595	82	14	4	
HE38	302–595	54	40	6	
HE38A	299–595	56	41	3	
HE15	282–595	62	37	1	
HE15A	274–595	64	33	3	
HE15B	265–595	0	36	64	
HE16	262–595	0	36	63	1
HE17	251–595	0	34	62	4
HE11	185–251	82	16	2	
HE36	185–251/340–595	61	39		
HE39B	250–264/316–595	16	84		
HE246	250–264/299–595	1	85	14	
HE39A	261–271/315–595	0	54	46	
AB-NLS		0	30	62	7

Wild-type or mutant ERs (deleted residues are given; for an illustration of the mutants, see Figure 2) were transiently transfected into HeLa cells, the immunohistochemical staining of at least 250 cells for each mutant was monitored, and these data were expressed as percentages of cells that fall into one of the following four categories (compare Figure 1): exclusively nuclear staining (N), nuclear staining stronger than cytoplasmic staining (N > C), nuclear staining equal (N = C) or lower N < C) than cytoplasmic staining. No differences were observed between the intracellular localization of HE0 (hERVal400) and HEG0 (hERGly400). AB-NLS refers to a chimera containing the SV40 T-antigen NLS linked to the ER region AB.

The ER HBD contains an estrogen-inducible p-NLS that cooperates with the constitutive p-NLSs

Exposure to estradiol of cells transfected with HE0 or HEG0 resulted in a small but reproducible increase in nuclear accumulation of hER (Figure 2). This effect was not related to a putative 'unmasking' of the DBD after hormone binding, since HE11, which does not to bind DNA, was indistinguishable from HEG0 with respect to intracellular compartmentalization (Figure 2, Table II). This hormonal effect on hER intracellular location was most obvious when one or several p-NLSs were deleted. For example, HE252G (in which p-NLS2 is deleted) was not nuclear in the absence of estrogen. Upon exposure to hormone, however, this mutant was found exclusively in the nucleus in 87% of the transfected cells (Figure 3 and Table II). Similar effects were seen for mutants HE243G (p-NLS3 deleted; 0–27%), HE244G (p-NLS1 and p-NLS2 deleted; 0–22%), HE248G (p-NLS1 deleted; 36–95%), HE251G (p-NLS3 deleted; 0–78%), HE256G (p-NLS2 and p-NLS3 deleted; 0–13%) and HE257G (p-NLS2 and p-NLS3 deleted; 0–8%) (Figure 3 and Table II). The hormone-dependent nuclear accumulation of HE249G, which lacks both the N-terminal A/B and the zinc finger domain (Figure 3), further supported the conclusion that DNA binding was not involved in this ligand effect. Moreover, HE243G, HE251G and HE4, which do not bind to an estrogen response element (ERE) *in vitro*, all exhibited the hormonal effect on nuclear localization (Figure 3, Table II).

It is interesting to note that the deletion of p-NLS2 and/or p-NLS3 in mutants lacking an HBD (HE16, HE17, HE39A and B; Figure 2) abolished binding to an ERE *in vitro* (data not shown), which further supports our previous suggestion (Kumar and Chambon, 1988) that this highly charged region is a component of the DBD. In fact, analysis of the formation and stability of the ER–ERE complex demonstrated that full-length receptors with selective deletion of p-NLS3 (HE251G and HE243G; Figure 3) did not bind to an ERE, while mutants having p-NLS2 deleted but not p-NLS3 (HE252G and HE244G) formed significantly less stable ER–ERE complexes (data not shown).

Although the HBD is obviously involved in the estrogen-induced nuclear accumulation, it does not itself contain an efficient NLS, as the intracellular location of HE247G was not affected by the presence of the hormone (Figure 3, Table II). Even HE14 and HE255G, both of which contain p-NLS1, did not show any exclusive nuclear staining in the

Table II. Ligand effects on the intracellular localization of estrogen receptor mutants

	Deletion	Ligand	Intracellular localization		
			N	N > C	N ≤ C
HE0/HEG0		–	84	16	
		E2	97	3	
		OHT	92	8	
		ICI	83	17	
HE4	199–264	–	6	92	2
		E2	58	42	
		OHT	54	46	
		ICI	6	93	1
HE241G	250–303	–	0	28	72
		E2	0	61	39
HE5	265–330		0	93	7
HE248G	299–303	–	36	64	
		E2	95	5	
HE251G	250–264	–	0	98	2
		E2	78	22	
HE256G	250–270	–	0	95	5
		E2	13	86	1
HE257G	250–274	–	0	95	5
		E2	8	92	
HE252G	261–271	–	0	96	4
		E2	87	13	
		OHT	50	50	
		ICI	0	94	6
HE243G	250–264/299–303	–	0	89	11
		E2	27	71	2
HE244G	261–271/299–303	–	0	85	15
		E2	22	78	
HE249G	1–240	–	34	66	
		E2	78	22	
HE255G	1–274	–	0	59	41
		E2	0	76	24
HE14	1–281	–	0	66	34
		E2	0	92	8
		OHT	0	53	47
		ICI	0	47	53
HE247G	1–281/299–303	–	0	78	22
		E2	0	77	23
NLS-HE14	1–240	–	11	87	2
		E2	74	26	
		OHT	74	26	
		ICI	18	80	2

ER mutants containing the HBD were classified as described in the legend to Table I without ('–') or after exposure to 10 nM estradiol ('E2'), hydroxy-tamoxifen ('OHT') or ICI 164,384 ('ICI'). Only three categories are listed.

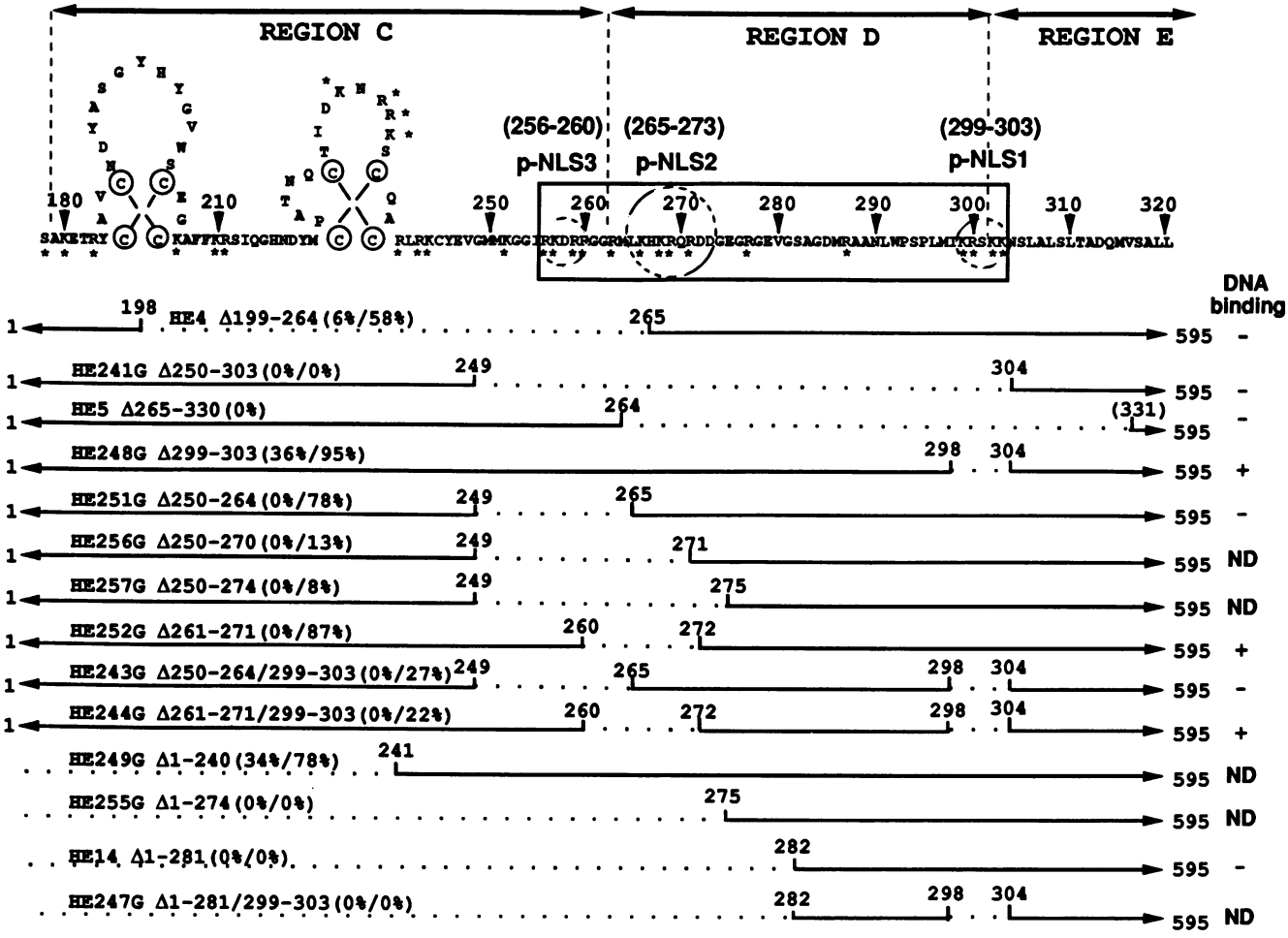


Fig. 3. Effect of hormone binding on the intracellular location of mutant estrogen receptors expressing the HBD but lacking individual or multiple p-NLSs. Illustration as in Figure 2. Percentages in brackets indicate the fraction of cells that exhibit exclusively nuclear staining (category N in Figure 1) in the absence and presence of estradiol. All constructs designated as HE . . . G contain a glycine at position 400.

presence of estrogen (note, however, that the hormone induced an increase in the N > C category; Table II). In agreement with these results, we note that exposure to estradiol did not alter the cytoplasmic location of fusion proteins between β-galactosidase and the hER HBD (corresponding to HE14) (Picard *et al.*, 1990). Thus, two possibilities have to be considered which may account for the hormonal effect: either the hER HBD contains an estrogen-inducible p-NLS, which has to cooperate with other p-NLSs to become efficient, or the unliganded HBD masks p-NLSs 1–3 to some extent.

We reasoned that if the hormonal effect resulted only from the unmasking of p-NLSs, then in the absence of hormone, mutants lacking the HBD should accumulate more efficiently in the nucleus than the corresponding mutants that contain the HBD. Alternatively, if a hormone-inducible proto-signal existed in the HBD and cooperated with other p-NLSs, then the nuclear levels of mutants containing one (or several) of these p-NLSs and the HBD should increase in the presence of hormone when compared with the nuclear levels of the same mutants lacking the HBD. In fact, this was the case when mutant pairs expressing only p-NLS2 (HE246 and HE243G), only p-NLS3 (HE16 and HE244G) or p-NLS1 plus p-NLS3 (HE39A and HE252G) were analyzed and compared (Figures 2 and 3). In all cases the liganded HBD contributed positively to nuclear accumulation, since nuclear levels were always higher with the HBD-expressing mutants in the presence of estradiol (Tables I and II). Note that HE248G exhibited almost wild-type nuclear localization in the presence of the hormone, indicating that the inducible p-NLS in the HBD can efficiently substitute for p-NLS1 (compare HE39 and HE248G plus hormone with HE38A, Tables I and II, Figures 2 and 3).

Interestingly, the hormone-inducible p-NLS appears to be able to cooperate with heterologous NLSs. When the SV40 T-antigen NLS was attached to HE14, yielding NLS-HE14, this chimera exhibited only partial nuclear localization (N = 11%; Table II). In the presence of hormone, however, there was a large increase in the nuclear accumulation of NLS-HE14 (N = 74%; Table II). Though we cannot exclude the possibility that the unliganded HBD masks the T-antigen NLS, we believe that the low level of nuclear accumulation in the absence of estrogen reflects an intrinsic weakness of the T-antigen NLS in this construct. This is supported by the observation that the nuclear level of AB-NLS, in which the identical NLS is linked to the ER region A/B, is even lower (Table I).

Some NLS masking by the unliganded HBD may, however, occur with mutants containing only p-NLS1 and p-NLS2 (compare HE39B and HE251G) or p-NLS2 and p-NLS3 (compare HE38A and HE248G) (Figures 2 and 3). Even with these mutants, though, unmasking could not be the sole cause of the hormonal effect, since the nuclear levels of HE251G and HE248G in the presence of estradiol were

far higher than those of the corresponding mutants (HE39B and HE38A) lacking the HBD (Tables I and II). Moreover, no masking was apparent when all three constitutive p-NLSs were present in the wild-type hER: ligand-free HEG0 was as nuclear as HE21, HE39 and HE38B, which all lack the HBD (Table I).

In conclusion, several lines of evidence point to the existence of a ligand-inducible p-NLSs in the hER HBD, which on its own is not sufficient for ensuring efficient nuclear accumulation, but can cooperate with constitutive hER p-NLSs or a heterologous NLS. This inducible p-NLS apparently contributes to the nuclear accumulation of the wild-type receptor in the presence of hormone (Figure 2). Some masking of the constitutive p-NLSs by the ligand-free HBD was observed with some hER mutants; however, this mechanism does not seem to operate in the case of the intact receptor.

Is there an additional p-NLS in the ER zinc finger domain?

In hER mutants that lack the HBD, the presence of the zinc finger domain has a positive effect on nuclear accumulation, as HE36 was less nuclear (61% in category N) than HE21 (87%), which binds to DNA (Figure 2, Table I; see also Kumar and Chambon, 1989). This appears to be contradictory to results obtained with mutants containing the HBD and all p-NLSs, since no difference was observed between those carrying and lacking the zinc finger region, either in the absence or in the presence of hormone (compare HEG0/HE0 with HE11; Figure 2). However, it has to be considered that these proteins with truncated HBDs may possibly diffuse through the nuclear membrane due to their small size, and the presence of a functional DBD may alter the cytoplasmic/nuclear equilibrium, thus possibly accounting for the different distribution of HE21 and HE36. We also cannot rigorously exclude the presence of an additional p-NLS (see also below the discussion for the PR), located between amino acids 184 and 252 which would be weaker than p-NLS2 or p-NLS3 [since both HE39A and HE39B were much more cytoplasmic than HE36 (Figure 2, Table I)], and whose deletion has no effect on the nuclear accumulation of the full receptor when the other p-NLSs are present (compare HE0 or HEG0 with HE11 in Figure 2).

Differential induction of the HBD p-NLS by the anti-estrogens hydroxy-tamoxifen and ICI 164,384

ER mutants whose intracellular localization was markedly affected by the presence of estradiol responded similarly

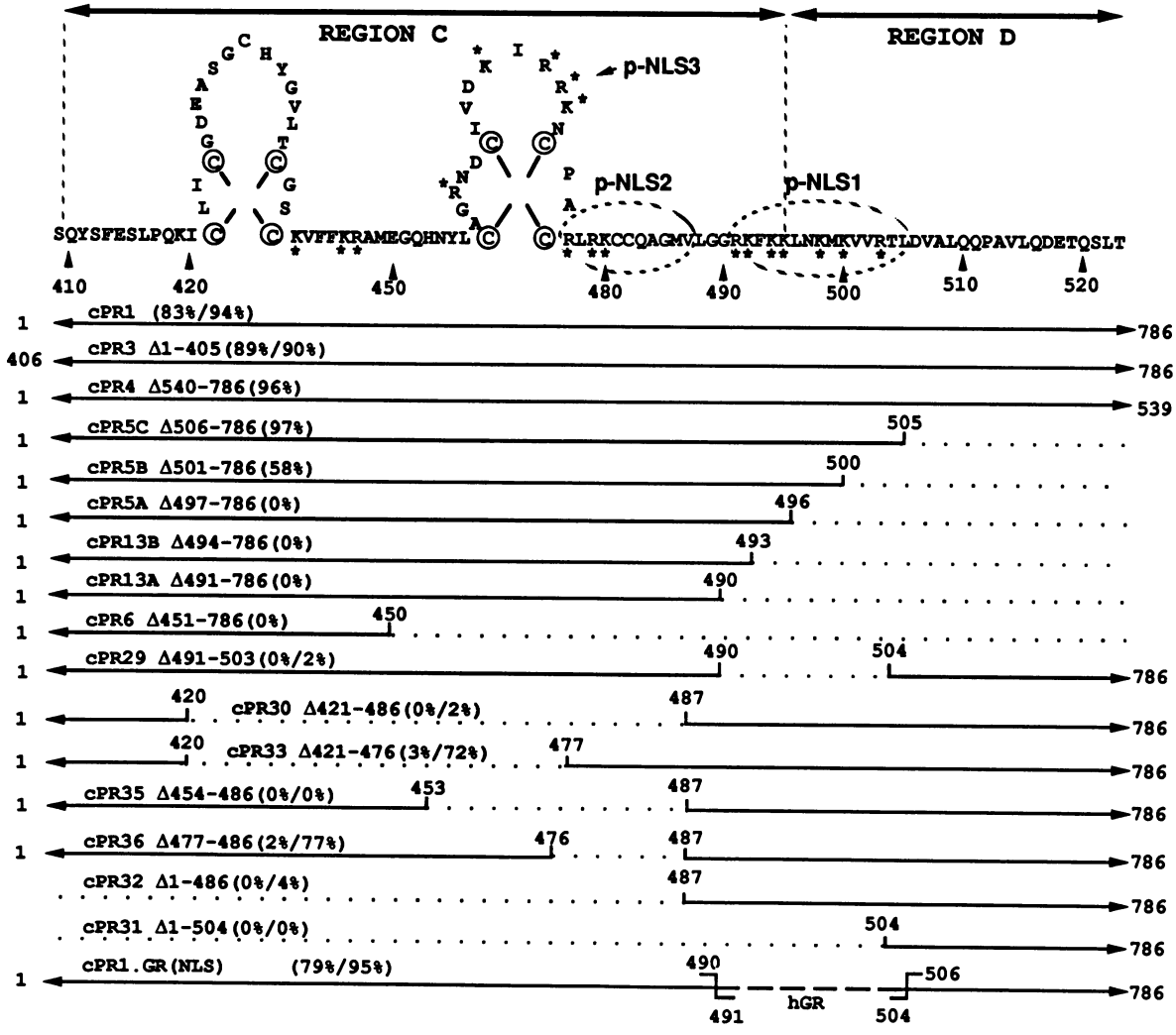


Fig. 4. Schematic illustration as in Figure 2 of wild-type and mutant progesterone receptors analyzed in this study. The sequence surrounded by the broken lines indicates p-NLSs 1 and 2. A third signal (p-NLS3) is present in the C-terminal zinc finger (see text). Percentages in brackets indicate the fraction of cells that exhibit only nuclear staining (category N in Figure 1) in the absence of R5020, or in the absence and presence of R5020 where two percentages are given.

to the anti-estrogen hydroxy-tamoxifen (OHT), but the anti-estrogen ICI 164,384 (ICI) had no effect on their localization (Table II). The difference between the two anti-estrogens was observed for HE252G (lacking p-NLS2), for HE4 (lacking p-NLS3 and the DBD) and also for the chimeric NLS-HE14 (Figure 3, Table II). Since NLS-HE14 and HE4 lack the DBD, a differential DNA binding capability of hER in the presence of OHT and ICI cannot account for these differences, and we conclude that estrogen and OHT induce the p-NLS of the hER HBD, whereas ICI is unable to do so.

Three constitutive and one hormone-inducible p-NLS in the progesterone receptor

The PRs of various species contain a stretch of basic amino acids (in cPR encompassing amino acids 491 and 503) at the junction of regions C and D (Figures 4 and 6). Deletion mapping analysis demonstrated that this region contains a nuclear targeting sequence that is necessary, but not sufficient, for the nuclear localization of the receptor. While cPR1, 3, 4 and 5, like hER, have an almost exclusive nuclear localization, cPR5B, which lacks only one basic residue of the 491-RKFKKLNKMKVVR motif, was exclusively nuclear in only 58% of transfected HeLa cells (Figure 4, Table III). Removal of two further lysines from the C terminus creates a mutant (cPR5A) that still accumulated in the nucleus, but all transfected cells also showed cytoplasmic staining (Figure 4, Table III). A truncation including the entire R/K-rich motif (either cPR13B, 13A or 6) yielded receptors that did not accumulate at all in the nucleus of the transfected cells (Figure 4, Table III). Furthermore, deletion of only this sequence from the full-length cPR demonstrated that amino acids 491–503 are essential for nuclear accumulation of the ligand-free receptor (cPR29; Figure 4, Table III). This nuclear targeting signal is, however, not sufficient for nuclear accumulation, since cPR32 (which lacks the region A/B and the DBD) was never exclusively nuclear (Figure 4). Thus, an additional cooperating signal(s) must be located in regions A/B or C. Again, this signal(s) cannot be an efficient NLS on its own, since cPR29 is not nuclear (Figure 4 and Table III).

The finding that cPR3 exhibited wild-type behavior indicates that the region A/B does not contain the additional nuclear targeting sequences. However, deletion of the cPR region C (cPR30) or of the two zinc fingers (cPR33) severely affected nuclear accumulation even though the motif at positions 491–503 was present. Deletion of only the C-terminal zinc finger and of the adjacent sequence 477-RLRK yielded a mutant (cPR35) that did not accumulate in the nucleus, whereas deletion of the N-terminal zinc finger was reported to be without effect (Guiochon-Mantel *et al.*, 1989). Surprisingly, there are apparently two nuclear targeting signals in the region that has been deleted in cPR34, as both cPR33 and cPR36 (in which the regions containing the basic motifs 467-KIRRK or 477-RLRK have been deleted) show, in contrast to cPR35, some nuclear targeting activity (2–3%; Figure 4, Table III). More importantly, in the presence of hormone, both cPR33 and cPR36, but not cPR35, efficiently accumulate in the nucleus, indicating that these two signals can cooperate with an additional hormone-inducible one. Thus, we conclude that three constitutive p-NLSs are present in the PR (p-NLS1–3 in Figure 4; note that p-NLS3 has not been rigorously defined, but is likely to encompass the basic sequence 467-KIRRK).

A hormonal effect was observed on the nuclear localization of the wild-type cPR and this effect was even more pronounced for mutants in which one of the PR p-NLSs was deleted (cPR29, cPR33 and cPR36). However, the distribution of cPR31, which contains only the HBD, was not affected by hormone (Figure 4, Table III). Since cPR1, 3, 4 and 5C exhibited very similar nuclear accumulation, it is unlikely that masking of the p-NLSs by the unliganded HBD could be responsible for the hormonal effect. Thus, as is the case for the ER, a hormone-inducible p-NLS appears to be located in the cPR HBD. In the presence of p-NLS1, this inducible signal can efficiently cooperate with either p-NLS3 or p-NLS2 (compare hormonal effects in cPR36 and cPR33, respectively), while cooperation is weak with the latter two alone (compare cPR29). Also p-NLS1 alone cannot efficiently cooperate with the inducible signal, since the hormone has only a minor effect on the intracellular distribution of cPR30.

The constitutive nuclear targeting sequences of PR and GR are equally potent, but the activity of the constitutive GR NLS is inhibited by the ligand-free HBD

In the rat GR, a constitutively active nuclear targeting sequence, encompassing amino acids 497–524, has been shown to be sufficient to target β-galactosidase into the

Table III. Intracellular location of wild-type (cPR1) and mutant progesterone receptors in the absence ('–') and presence ('R') of 10 nM R5020

	Deletion	Ligand	Intracellular localization			
			N	N > C	N = C	N < C
cPR1		–	83	17		
		R	94	6		
cPR2	1–163	–	88	11		
		R	96	12		
cPR3	1–405	–	89	4		
		R	90	10		
cPR4	540–786		96	4		
cPR5C	506–786		97	3		
cPR5B	501–786		58	42		
cPR5A	497–786		0	82	18	
cPR13B	494–786		0	15	79	6
cPR13A	491–786		0	7	66	27
cPR6	451–786		0	6	55	39
cPR29	491–503	–	0	3	65	32
		R	2	67	26	5
cPR30	421–486	–	0	5	73	22
		R	2	67	26	5
cPR33	421–476	–	3	80	17	22
		R	72	28		
cPR35	454–486	–	0	0	82	18
		R	0	66	29	5
cPR36	477–486	–	2	74	24	
		R	77	23		
cPR32	1–486	–	0	33	66	1
		R	4	85	10	
cPR31	1–504	–	0	47	53	
		R	0	48	52	
cPR1.GR(NLS)		–	79	21		
		R	95	5		

The classification of PR mutants (see Figure 4 for an illustration) is as described in Table I. cPR1.GR(NLS) refers to a mutant in which cPR p-NLS2 was replaced with the corresponding sequences of the human glucocorticoid receptor.

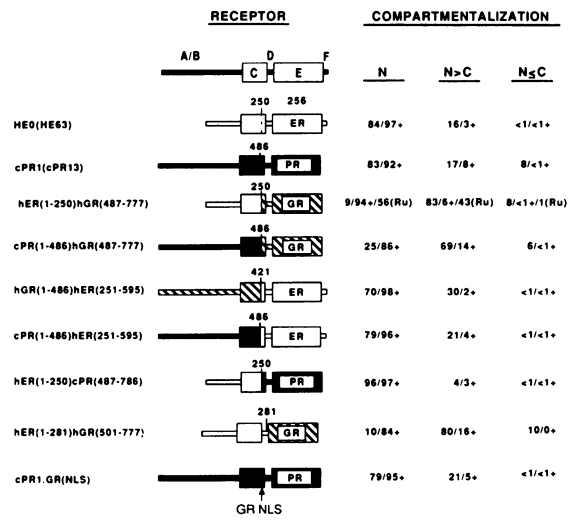


Fig. 5. Effect of hormones and anti-hormones on the intracellular distribution of steroid receptors and chimeric constructs. Receptors are illustrated on the left, boxes indicate DNA and HBDs; hER sequences, white; hGR sequences, stippled; cPR sequences, black. For definition of categories N, N > C and N < C, see Figure 1. The first number in each category represents the percentage of cells belonging to this category when grown in the absence of hormone; numbers followed by + indicate hormone exposure. Numbers suffixed by (RU) give intracellular distribution of the corresponding chimera in the presence of RU486.

nucleus (Picard and Yamamoto, 1987). This region contains two sequence motifs at positions equivalent (Figure 6) to that of PR p-NLS1 and 2 [note that it is not known whether the basic GR motif (shaded in Figure 6) is an efficient NLS on its own]. In addition, the GR HBD contains an inducible NLS which, when attached to β-galactosidase, transports the fusion protein into the nucleus in a hormone-inducible manner (Picard and Yamamoto, 1987). To compare the relative strengths of the constitutive nuclear targeting sequences of hGR, hER and cPR, and in order to monitor the effect of hormone binding in a receptor background, chimeric receptors were constructed in which the sequences C-terminal of the 66 amino acid core of region C were exchanged with each other receptor (as illustrated in Figure 5). The intracellular location of HE0 (or HE63), hER(1−250)cPR(487−786) and hER(1−250)hGR(487−777) was determined using a monoclonal anti-hER antibody, while an anti-cPR antibody was used for cPR13, cPR(1−486) hER(251−595) and cPR(1−486)hGR(487−777). All chimeras were expressed at similar levels and were functionally active with respect to hormone-dependent transcriptional activation of cognate reporter genes (Turcotte *et al.*, 1990). As expected, the nuclear localizations of hER(1−250)cPR(487−786) and cPR(1−486)hER(251−595) were similar to those of cPR and hER, respectively (Figure 5).

In the absence of glucocorticoids, most of the cells

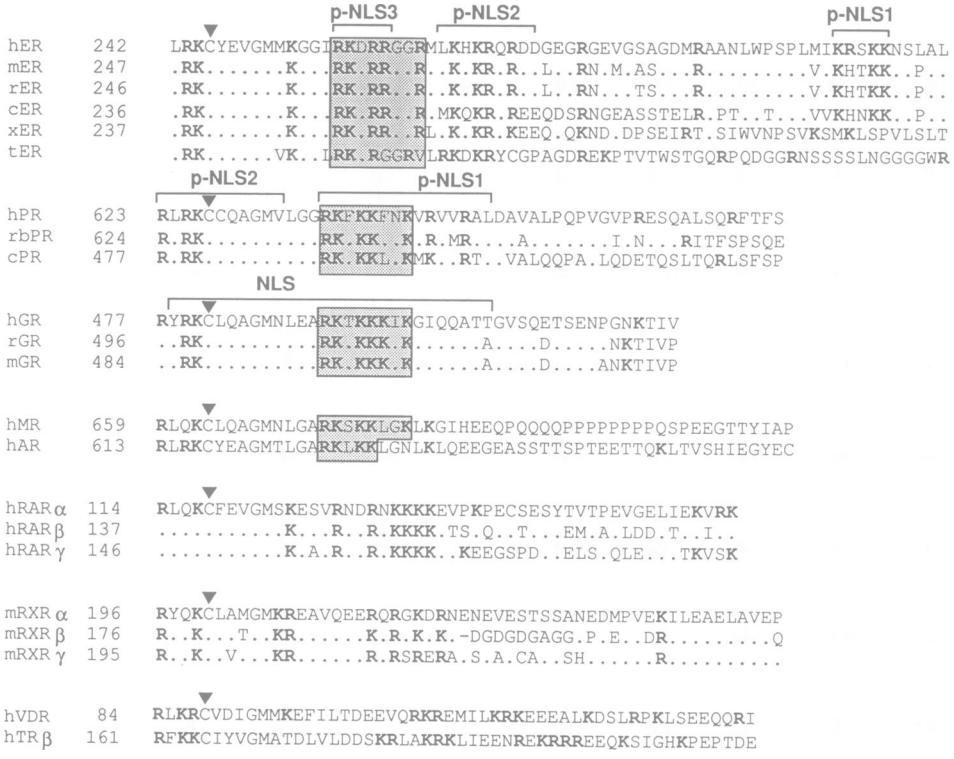
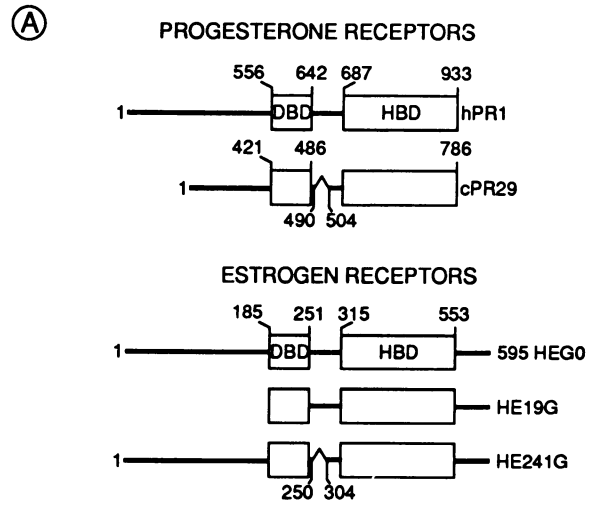


Fig. 6. Sequence alignment of different steroid, thyroid and retinoic acid receptors at the C/D domain junction. The last cysteines in region C are indicated by solid triangles. Positively charged amino acids (lysine and arginine) in bold. The shaded area corresponds to a positively charged motif that is conserved throughout the steroid receptor subfamily. Above the sequences the nuclear localization signals as mapped in this study or by Picard and Yamamoto (1987) are indicated. hER (Green *et al.*, 1986), human; mER (White *et al.*, 1987) mouse; rER (Koike *et al.*, 1987), rat; cER (Krust *et al.*, 1986; Maxwell *et al.*, 1987), chick; and xER (Weiler *et al.*, 1987), *Xenopus laevis* estrogen receptors. hPR (Kastner *et al.*, 1990), human; rPR (Loosfelt *et al.*, 1986), rabbit; and cPR (Gronemeyer *et al.*, 1987), chicken progesterone receptors. hGR (Govindan *et al.*, 1985; Hollenberg *et al.*, 1985), human; rGR (Rusconi and Yamamoto, 1987), rat; and mGR (Danielsen *et al.*, 1986), mouse glucocorticoid receptors. hMR (Arriza *et al.*, 1987), human mineralocorticoid receptor; hAR (Chawnshang *et al.*, 1988), human androgen receptor; hVDR (Baker *et al.*, 1988), human vitamin D receptor; hTRβ (Weinberger *et al.*, 1986) human β-thyroid receptor; hRARs, human retinoic acid receptors (Giguere *et al.*, 1987; Petkovitch *et al.*, 1987; de The *et al.*, 1987; Krust *et al.*, 1989); mRXRs, mouse retinoid X receptors (Leid *et al.*, 1992; Mangelsdorf *et al.*, 1990).

expressing chimeras that contained the constitutive NLS and the HBD of hGR exhibited cytoplasmic staining: exclusive nuclear staining was seen in only 9% and 25% of HeLa cells expressing hER(1−250)hGR(487−777) or cPR(1−486) hGR(487−777), respectively (Figure 5). Thus the replacement in the GR of regions A/B and C with those of the hER or cPR does not alter its cytoplasmic location in the absence of hormone. To support further the conclusion that the N-terminal half of the hGR has no role in the nuclear targeting of hGR, the chimeric receptor hGR(1−486) hER(251−595) was analyzed and shown to exhibit hER wild-type characteristics (Figure 5). Only upon hormone exposure ('+') did the ER−GR and PR−GR chimeras become as nuclear as the wild-type ER or PR (Figure 5). We then replaced the constitutive PR p-NLS2 with the corresponding GR targeting sequence to see whether, in the background of a non-liganded PR, the GR sequence would be less efficient than its PR counterpart. The behavior of cPR1.GR(NLS) was indistinguishable from that of the wild-type cPR (Figures 5 and 6, Table III). We conclude that these PR and GR sequences are of equal strengths and, therefore, that the ligand-free GR HBD probably masks or inactivates the GR NLS sequence. To support the masking hypothesis further, we tested whether the unliganded GR HBD would decrease the efficiency of a heterologous NLS. As expected, the chimera hER(1−281)hGR(501−777), which corresponds to HE15 (Figure 2, Table I) linked to the GR HBD, was significantly less nuclear (10%) than HE15 (62%) in the absence of glucocorticoid (Figure 5). In all cases exposure of those chimeras carrying the GR nuclear targeting sequences to dexamethasone resulted in nuclear staining that was indistinguishable from that of the chimeras carrying the targeting sequences of hER or cPR (Figure 5). Interestingly, the anti-hormone RU486 only partially induced the nuclear accumulation that was observed with the agonist (Figure 5, compare '+' and 'RU').

We thus conclude that (i) heterologous constitutive NLSs are masked by the ligand-free HBD of GR, but not by those of PR or ER and (ii) the constitutive GR NLS itself has a strength comparable to that of PR. Together with the observations that an HBD-truncated rat GR is nuclear while a large fraction of the ligand-free hGR molecules are located in the cytoplasm (Picard and Yamamoto, 1987), these results indicate that the cytoplasmic location of GR in the absence of ligand is due to masking of its constitutive nuclear targeting sequences.



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		INTRACELLULAR LOCALIZATION		
	LIGAND	N	N > C	N ≤ C
cPR29	-	0	5	95
	R	2	67	31
cPR29+hPR0	-	0	40	53
	R	30	70	
HE241G	-	0	28	72
	E	0	61	39
	OHT	0	58	42
	ICI	0	30	70
HE241G+HE19G	-	40	60	
	E	70	30	
	OHT	59	42	
	ICI	28	72	

Fig. 7. Co-translocation of translocation-deficient estrogen or progesterone receptors by co-expression of translocation-competent receptors to assess the degree of ER and PR dimerization *in vivo* in the absence and presence of ligands. **A.** Illustration of the human estrogen and human and chicken progesterone receptor mutants used in the nuclear co-translocation experiments. Boxes indicate the DNA and hormone-binding domains (DBD and HBD respectively); residues flanking these domains and the deletions, are given. **B.** Translocation-deficient receptors (cPR29, HE241G) were transiently expressed in HeLa cells, either alone or in the presence of co-expressed translocation-competent receptors (hPR0, HE19G) and nuclear accumulation of the translocation-deficient receptors in the absence and presence of ligands was analyzed by immunohistochemical staining (the anti-cPR antibodies do not recognize the human PR; HE19G does not contain the epitope for the antibody H226, which, however recognizes HE241G).

Co-translocation studies demonstrate different degrees of hormone-dependent and hormone-independent dimerization of hER and cPR in vivo

It has been shown previously that the rabbit PR can co-translocate NLS- (and DBD)-deficient PR mutants into the nucleus in a hormone-dependent manner. These results have been taken as strong evidence for hormone-induced dimerization of the rabbit PR (Guiochon-Mantel *et al.*, 1989) We used this approach to investigate whether the ER exists as a dimer *in vivo*, and whether or not the hormone is required for dimerization. In addition, we asked whether hormone-induced dimerization of the PR involves structures that have been conserved in the chicken and human homologues. To this end we made use of an antibody directed against the N-terminal region of cPR which does not crossreact with the human PR. While co-expression of cPR29 and wild-type hPR (hPR0) resulted in a small increase of nuclear cPR29, addition of the agonist R5020 greatly increased its nuclear accumulation (N = 30%, Figure 7) over that seen in the absence of hPR0 (N = 0%, Figure 7). We conclude that cPR and hPR can form heterodimers *in vivo* and that the formation of these dimers is greatly increased in the presence of hormone. Note, however, the increase in the N > C category when cPR29 and hPR0 were co-expressed in the absence of R5020, indicating that PR dimers are also formed, albeit less efficiently, in the absence of hormone (Figure 7).

To study the co-translocation of the ER, we chose the cytoplasmic mutant HE241G, which does not present any exclusive nuclear localization when expressed in HeLa cells, irrespective of whether or not the cells were exposed to estrogen (Figures 3 and 7). Immunostaining was done with an antibody directed against the N-terminal region A/B which is missing in the nuclear translocation-competent mutant HE19G (Figure 2, Table I). Interestingly, co-expression of HE19G in the absence of estrogen resulted in a greatly increased nuclear accumulation of HE241G (from N = 0% to N = 40%; Figure 7). Moreover, a further increase was seen in the presence of estrogen (N = 70%; Figure 7), to an extent not far from that seen with wild-type hER. Similar hormone-induced co-translocation was observed when HE243G or HE244G were co-expressed with HE19G (data not shown). Therefore, *in vivo* the dimerization domain that has been identified in the HBDs of human and mouse ER (Fawell *et al.*, 1990a; Kumar and Chambon, 1988) is constitutively active but acquires its optimal activity only in the presence of the hormone. Interestingly, OHT also stimulated the co-translocation of HE241G, albeit somewhat less efficiently than estrogen, while ICI was inactive. However, it is noteworthy that ICI did not greatly affect the constitutive level of HE19G-mediated translocation of HE241G (Figure 7).

Discussion

Cooperativity and masking of nuclear targeting signals is dependent on the protein content

Although no consensus sequence has emerged from a number of studies of sequences which are sufficient on their own for nuclear targeting of proteins ('intrinsic' NLSs), the motif Lys−X2−Lys/Arg appears to be shared by the vast majority of such sequences. The first lysine residue is of central importance, since its replacement even with an arginine residue in the most studied prototype NLS of the SV40 T-antigen (Lys128) severely affected nuclear accumulation and substitution by any other amino acid abrogated nuclear transport (Lanford and Butel, 1984). However, the above mentioned motif is not sufficient for nuclear transport. Additional nearby lysine and/or arginine residues are required, and the nature of the residues flanking the Lys/Arg cluster is also important. For example, the minimal SV40 T-antigen NLS that is necessary for T-antigen transport and sufficient for nuclear accumulation of a number of heterologous proteins is Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu (Roberts, 1989). However, the presence of this sequence does not necessarily lead to efficient nuclear accumulation, as the protein context is crucial to such an extent that the SV40 T-antigen NLS may no longer function in an inappropriate environment (Roberts *et al.*, 1987). This has been taken as an indication for certain structural requirements for an NLS to function, either in the sense that a signal may be masked (i.e. unable to bind to the nuclear pore complex due to steric hindrance by other parts of the protein) or in that an NLS has to be embedded in a protein domain with specific structural characteristics. In fact, secondary structure prediction of the SV40 T-antigen suggested an α-helical NLS domain (Roberts *et al.*, 1987).

Thus, in order to demonstrate that an amino acid sequence is the NLS of a given protein, it is not sufficient to show that it can function as an efficient nuclear targeting sequence when appended to an otherwise cytoplasmic protein. In fact, sequences identified by this approach may not even be necessary for nuclear targeting of the cognate protein (Tratner and Verma, 1991). This approach identifies potential signals for nuclear accumulation, but ignores essential effects, such as masking and cooperativity, that result from the protein context. These considerations led us to investigate, in its original protein context, the function of amino acids 256−303 in hER, which was previously identified as a potential hER NLS (Picard *et al.*, 1990).

Multiple p-NLSs cooperate in the ER

Amino acids 256−303 of hER contain three Lys/Arg-rich motifs (p-NLS1, 2 and 3 in Figures 2 and 6) which resemble the prototype SV40 T-antigen NLS, but none of them is sufficient to target heterologous proteins to the nucleus (Picard *et al.*, 1990). Similarly, neither one nor two of these p-NLSs were strong enough to translocate wild-type levels of ER mutants with truncated HBDs into the nucleus. However, in contrast to the results obtained with heterologous proteins, each of these sequences exerted some residual targeting activity within their natural sequence context. This effect was even greater when two such sequences were present in an HBD-truncated mutant, such as HE39B (containing p-NLS1 and 2; compare with the cytoplasmic mutants HE16, HE17 and HE15B; Table I). Note that even combining two of these p-NLSs is insufficient for nuclear targeting of β-galactosidase (Picard *et al.*, 1990).

In addition to the three constitutively active ER p-NLSs, we detected a fourth signal that was active only in the presence of estrogen. Again, this signal was undetectable in chimeric constructs (Picard *et al.*, 1990). Interestingly, on its own, this hormone-inducible p-NLS does not affect intracellular compartmentalization (see HE247G; Figure 3, Table II), but can cooperate with one or, even more efficiently, with two other p-NLSs (compare, for example, HE243G and HE246; Figures 2 and 3). By comparing the constitutive nuclear accumulation levels of pairs of mutants that differ only by the presence of the HBD, we have ruled out the possibility that unmasking of the residual p-NLSs by the ligand-free HBD could be responsible for the hormonal effect.

In some mutants the presence of the ER DBD positively affects nuclear accumulation (see above). We cannot exclude the possibility that this is due to the effect of basic sequences in the ER which correspond to PR p-NLS2 and 3 (see below). However, it is obvious from a comparison of HE11 and cPR30 that p-NLSs 1−3 are the major targeting sequences of the ER and that the zinc finger region plays a minor, if any, role in the nuclear accumulation of wild-type ER.

Cooperating p-NLSs of the PR

Similarly as for the ER, we also detected multiple PR p-NLSs that cooperatively generated nuclear targeting. One PR sequence (p-NLS1) corresponded in its position to ER p-NLS2 and 3 (Figure 6). No targeting signal corresponding to ER p-NLS1 exists in the PR, but two p-NLSs were found in the DBD, located within the C-terminal zinc finger and in the flanking amino acid 477−486 region (p-NLS3 and 2, respectively, in Figure 4). Though we did not map these signals precisely, it is most likely that they comprise the basic motifs 467-KIRRK and 477-RLRK. Clearly, these two motifs are distinct p-NLSs, as shown by their residual targeting activity in cPR33 and cPR36 when compared with

a mutant (cPR35) lacking both sequences. In addition, we have detected a fourth, hormone-inducible nuclear targeting signal in the PR HBD, which can cooperate efficiently with p-NLS1 and either one of the two other p-NLSs. Note that masking of PR p-NLSs cannot be responsible for the hormonal effect, as HBD-truncated receptors containing p-NLS2 and 3 accumulate even less in the nucleus than those expressing one of these signals and the HBD. Also, masking of p-NLS1 does not explain the hormonal effect seen with cPR33 and cPR36, since cPR30 exhibits a very weak hormonal response (Figure 4). Using a different approach, Guiochon-Mantel *et al.* (1991) also concluded that the second zinc finger region of PR contained a karyophilic signal, although they did not realize that it is actually composed of two signals (p-NLS2 and p-NLS3). Moreover, they interpreted their data as demonstrating masking of this signal by the ligand-free HBD. However, our data clearly rule out the masking phenomenon (see above) and show that cooperativity between the ligand-inducible PR p-NLS and the constitutive p-NLSs is responsible for the observed hormonal effect on nuclear accumulation.

Alignment of the corresponding sequences of steroid receptors from various species reveals that sequences similar to p-NLS3 of hER can be found in all these receptors (shaded boxes in Figure 6; it is unknown whether the lack of one basic residue in the human androgen receptor affects its nuclear accumulation) at a constant distance from the receptors (the last Cys residue of this region is indicated by a triangle in Figure 6). Note that ER p-NLS1 seems to have appeared late in evolution, as it is completely missing in trout and frog ERs (the significance of the additional arginine in hER p-NLS1 has not been investigated), while the final motifs for ER p-NLSs 2 and 3 originated after the ancestors of frogs and fishes diverged. A cluster of basic amino acids is found at a similar distance from the zinc fingers in the retinoic acid receptors (RARs; motif $KX_3RX_2RXK_4$), while no sequences corresponding to ER p-NLS3 are present in the regions D of the vitamin D3 (hVDR) and thyroid (hTR3) receptors. Surprisingly, the recently described retinoid X receptors (RXRs), which heterodimerize with RARs (Leid *et al.*, 1992; Yu *et al.*, 1992) and bind a stereoisomer of retinoic acid (Levin *et al.*, 1992), do not seem to contain any sequence resembling a constitutive NLS, neither at the region C/D junction, nor elsewhere. It is not known whether the ligand-free RXR is a cytoplasmic protein, but a RAR-dependent nuclear co-translocation may have to be considered for its mechanism of action.

In contrast to the p-NLSs of ER and PR, the constitutive NLS of GR is masked by the ligand-free HBD

The hormone-inducible p-NLSs are of minor importance for the wild-type ER and PR, since these proteins are already nuclear in the absence of hormone. Moreover, the hER HBD does not carry a sequence that is strong enough to target β-galactosidase to the nucleus (Picard *et al.*, 1990). In contrast, a chimera between β-galactosidase and the GR HBD accumulated in the nucleus in a hormone-dependent manner (Picard and Yamamoto, 1987). In addition, a second constitutively acting targeting signal has been mapped in the GR (NLS in Figure 6; note that it contains basic residues corresponding to PR p-NLS2 and that it is unknown whether the shaded motif is sufficient for nuclear targeting). Despite the finding of a constitutive GR NLS that is sufficient to target a heterologous protein to the nucleus, the ligand-free GR is, in contrast to ER and PR, found to a considerable degree in the cytoplasm (Sanchez *et al.*, 1990; Antakly *et al.*, 1989; Gasc *et al.*, 1989; Picard and Yamamoto, 1987; Wikstrom *et al.*, 1987). Again, this illustrates the dependence of a targeting sequence on the protein environment.

In order to compare the efficiency of the two GR NLSs relative to those of ER and PR, we constructed chimeric receptors (Figure 5). This allowed us to use the same antibody for the wild-type and for the chimeric receptors, thus ruling out antibody-specific differences. Our data show that in the absence of ligand the region of the GR containing the previously described NLSs (Picard and Yamamoto, 1987) is clearly less efficient in nuclear transport than the corresponding regions of ER or PR. This is not due to a weaker activity of the basic motif in the GR NLS, since the GR sequence from amino acid 491 to 504 can perfectly substitute PR p-NLS1 [compare cPR1 and cPR1.GR(NLS) in Figure 4], thus suggesting that the ligand-free GR HBD is masking the activity of the constitutive GR NLS. In fact, the ligand-free GR HBD strongly inhibited even the activity of the heterologous p-NLS2 and p-NLS3 in the ER−GR chimera hER(1−182)hGR(501−777), as the corresponding mutant without the GR HBD (HE15, Figure 2) accumulates more efficiently in the nucleus than the chimera. Upon hormone binding this masking is apparently relieved and both the constitutive and the inducible NLSs can exert their action.

The mechanism by which the ligand-free GR HBD, in contrast to that of the ER and PR, masks (p-)NLSs is not known but may be related to a different 3D structure of these HBDs. Alternatively, secondary binding proteins may be involved; we note in this respect the differential interaction *in vitro* of GR and ER with hsp90 (Chambraud *et al.*, 1990; Pratt *et al.*, 1988).

Differential effects of hydroxy-tamoxifen and ICI 164,384 on the hormone-inducible p-NLSs of ER

In those hER mutants that accumulate in the nucleus upon hormone binding, the two anti-estrogens OHT and ICI provoke different effects. While OHT acts similarly to estradiol, ICI is not able to induce the p-NLS in the HBD (see HEG0, HE0, HE252G and NLS-HE14 under the various hormonal treatments in Table III). We take this as an indication that the ICI HBD structure is different from that of the OHT HBD, which itself differs from the estradiol HBD structure. In agreement with this proposal, we note that these complexes migrate differently in native gel electrophoresis (Fawell *et al.*, 1990b; Kumar and Chambon, 1988). Note, however, that the inability of ICI to induce the HBD p-NLS has no consequence for the wild-type receptor location, since hER is nuclear even in the absence of ligand, and the presence of ICI does not modify this location (Figure 2).

In contrast, for GR the anti-hormone RU486 should affect compartmentalization of the wild-type receptor, since chimeras containing the GR NLS and HBD showed a different intracellular location when exposed to hormone or RU486 (Figure 5), suggesting that RU486 is unable to appropriately expose the hormone-inducible GR NLS described by Picard *et al.* (1987).

Differential hormone dependency for dimerization of ER and PR in vivo

A number of reports address the dimeric status of steroid hormone receptors, and there is considerable controversy as to whether receptors exist as dimers *in vivo* and whether or not the ligand induces dimerization [for references and a discussion of this topic, see Gronemeyer (1991)]. With one exception all of these reports are based on *in vitro* techniques, such as gel retardation or co-immunoprecipitation. Guiochon-Mantel *et al.* (1989), however, demonstrated hormone-dependent co-translocation of translocation-deficient rabbit PR mutants into the nucleus when a translocation-competent rabbit PR mutant was co-expressed. These results are the most convincing evidence for the existence of a ligand-induced dimerization domain. We have compared the hormonal requirement for the *in vivo* dimerization of ER and PR using the co-translocation technique. For PR we observed a low level of co-translocation in the absence of hormone, indicating that the stability of ligand-free PR dimers is low. In striking contrast, even in the absence of hormone, the translocation-deficient ER mutant HE241G (which lacks p-NLSs 1 to 3, see Figure 3) accumulated in the nucleus upon co-expression with the translocation-competent HE19G (see Figures 2 and 7). These results are consistent with the interpretation that ligand-free dimers are formed by the ER, but not the PR. Interestingly, similar differences in the stability of ER and PR dimers have been predicted from the *in vitro* analysis of the cooperative DNA binding of these receptors (Notides *et al.*, 1981; Skafar, 1991).

From the above results one would predict a differential DNA binding characteristic of ER and PR *in vitro*. In particular, due to the low stability of PR dimers in the absence of hormone, no efficient interaction with a cognate response element is expected, while ER may bind under these conditions. In fact, this is exactly what has been observed: wild-type ER, but not PR, binds *in vitro* to its cognate palindromic element in the absence of hormone, while PR requires hormone for *in vitro* DNA binding (D.Metzger, M.Berry and P.Chambon, in preparation; Meyer *et al.*, 1990). In addition, it has been observed that DNA complexes of PR are less stable than those of ER (Ponglikitmongkol *et al.*, 1990). Obviously, the higher stability of ER dimers accounts for this difference. Since ER dimerization is further enhanced by estrogen, a common mechanism may nevertheless be responsible for ER and PR dimerization. Interestingly, OHT was only slightly less efficient than estrogen with respect to ligand-induced dimerization, while ICI was inactive. Importantly, however, *in vivo* ICI did not significantly reduce the level of dimerization that was seen in the absence of ligand (Figure 7), in contrast to what has been proposed recently from *in vitro* experiments (Fawell *et al.*, 1990b). Thus, our results are in line with other *in vitro* and *in vivo* data which indicate that ICI does not act by inhibiting DNA binding of ER (D.Metzger, M.Berry and P.Chambon, in preparation; Sabbah *et al.*, 1991; Wrenn and Katzenellenbogen, 1990).

Conclusion

We have demonstrated that multiple proto-signals of different strengths are cooperatively involved in the nuclear targeting of steroid hormone receptors. This is not a peculiarity of this family of proteins, because several other proteins have been reported to contain more than one nuclear targeting signal (Dang and Lee, 1988; Eckhard *et al.*, 1991; Morin *et al.*, 1989; Robbins *et al.*, 1991; Tratner and Verma, 1991). Moreover, the ability of 'defective' signals to co-operate has been shown previously, when it was found that three copies of a mutant SV40 T-antigen NLS (carrying Arg128) were sufficient for nuclear targeting of pyruvate kinase, irrespective of their location in the protein, while one copy was not active and two copies resulted in partial nuclear accumulation (Roberts *et al.*, 1987). Note that even the wild-type SV40 T-antigen NLS is not sufficient for nuclear targeting of some T-antigen mutants (Schneider *et al.*, 1988) and that another non-karyophilic T-antigen mutant required the addition of three copies of the T-antigen NLS to restore wild-type nuclear accumulation (Fischer-Fantuzzi and Vesco, 1988). Thus, we propose that cooperation between p-NLSs is a common theme among nuclear proteins and that it can be investigated only in the natural protein context. The presence of multiple cooperating p-NLSs supports the proposed cooperation of basic clusters to bind to a single receptor of the nuclear pore complex (Robbins *et al.*, 1991). Two recently characterized nuclear pore complex proteins (Davis and Fink, 1990; Nehrbass *et al.*, 1990) have central domains consisting of a series of degenerate repeats. These proteins are candidate receptors and it is possible that the p-NLSs of ER and PR interact with repeated structures of such receptor(s) in a cooperative manner.

Materials and methods

Expression vectors

All hER and cPR wild-type and mutant expression vectors apart from those mentioned below have been described (Berry *et al.*, 1990; Bocquel *et al.*, 1989; Chambraud *et al.*, 1990; Gronemeyer *et al.*, 1987; Kumar and Chambon, 1988; Kumar *et al.*, 1986, 1987) [note that no differences were observed in this study between hERGly400 (HEG0) and hERVal400 (HE0) (Tora *et al.*, 1989). Note also that only HE4, HE11 and HE14 contain Val400; all other mutants expressing a functional HBD contain Gly400]. HE38A and HE38B were constructed by cleaving HE39 at the *BclI* site and ligating synthetic oligonucleotides with a stop codon following amino acids 298 and 303, respectively. HE15A and HE248G, and HE39A and HE39B were constructed by site-directed mutagenesis of HEG0 and HE39, respectively (the G in HE248G and in the following constructs indicates the presence of a glycine at position 400). HE15B was constructed by PCR amplification (with HEG0 as a template) of a fragment corresponding to amino acids 185–264, using primers which incorporate a 5′ *KpnI* and a 3′ *XhoI* preceded by a stop codon, such that this fragment could be inserted into the corresponding sites of HE60 (Green *et al.*, 1988b). HE241G was constructed from HE251G by amplifying with PCR a region corresponding to amino acids 89–289 and substitution of the corresponding sequence in HE248G. HE242G was similarly constructed by using HE252G as PCR template. HE243G was generated by adding the PCR-derived fragment corresponding to amino acids 265–298 into HE241G; HE244G was constructed by adding a fragment corresponding to amino acids 271–298 into HE242G. HE246G was made by PCR by introducing a stop after codon 298. HE247G was constructed from HE248G by PCR amplification of a region corresponding to amino acids 282–595 using primers which introduce an *EcoRI* site and an in-frame Kozak sequence (Kozak, 1986) at the 5′ end and a *BamHI* site at the 3′ end. The *EcoRI–BamHI* fragment was inserted at the corresponding sites into pSG5 (Green *et al.*, 1988a). HE249G originates from HE250G by addition of an oligonucleotide adaptor resulting in an extension of the N terminus. AB-NLS is described by Tasset *et al.* (1990). NLS-HE14 was a gift from D.Tasset and was constructed by inserting oligonucleotides encoding the same SV40 T-antigen NLS peptide as AB-NLS into the *BamHI* site of HE14. cPR5C is a derivative of cPR5 with a stop codon following amino acid 505. cPR29 was constructed by site-directed mutagenesis of cPR21 [identical to cPR1 (Gronemeyer *et al.*, 1987), but using pSG5 (Green *et al.*, 1988a) as expression vector], resulting in a deletion of amino acids 491–503. cPR13 was constructed by site-directed muta-

genesis introducing *Kpn*I and *Bam*HI sites flanking a PR cassette which can be exchanged with the equivalent cassettes in HE63 and HG7 (Kumar *et al.*, 1987). cPR13A and cPR13B were constructed by deleting the *Bam*HI–*Hin*dIII fragment of cPR13 and ligating a *Hin*dIII–*Bam*HI adaptor with a stop codon after amino acids 490 and 493, respectively. cPR30 and cPR.GR(NLS) were constructed from cPR21 by site-directed mutagenesis; in cPR30 there is a *Kpn*I site in place of the deleted sequence. cPR31 was constructed by PCR amplification of the cPR1 sequence encoding amino acids 504–786 and by introducing an *Eco*RI site and a Kozak sequence at the 5′ end and a *Bgl*II site at the 3′ end, and by insertion into pSG5. To generate cPR32 the *Bam*HI–*Bam*HI vector fragment of cPR13 was ligated to an oligonucleotide adaptor containing an in-frame Kozak sequence and destroying the 3′ *Bam*HI site. Subsequently, the *Bam*HI– *Bam*HI fragment of cPR1, containing the rabbit β-globin splice site was re-introduced. cPR33 was constructed by adding a *Kpn*I-flanked PCR-amplified fragment encoding amino acids 477–486 in-frame into the *Kpn*I site of cPR30. To generate cPR35 and cPR36, *Kpn*I-flanked PCR-amplified fragments encoding amino acids 421–453 and 421–476, respectively, were similarly inserted into the *Kpn*I site of cPR30. The chimeric receptors illustrated in Figure 5 were obtained by exchanging *Bam*HI fragments between cPR13, HE63 and HG7. hER(1–281) hGR(501–777) was generated by substituting the *Bam*HI–*Xho*I fragment from HG1 with the corresponding one from HE15. All chimeras were active with respect to transcriptional activation of cognate reporter genes (Turcotte *et al.*, 1990). All mutations generated by PCR or site-directed mutagenesis were verified by sequencing.

Transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% charcoal-stripped fetal calf serum (FCS) lacking phenol red. Cells were plated at 20–30% confluence in Petri dishes containing a microscope slide. The cells were allowed to attach for 8–16 h and transfected with a total of 5 μg DNA, using the calcium phosphate co-precipitation technique. The precipitate was removed 24 h later and cells were incubated for a further 24 h. When indicated, steroids were added 2–4 h before fixation at a final concentration of 10 nM (for estradiol, OHT, ICI and R5020) or 20 nM (for PR). There was no change in the results whether the cells were exposed to steroids for only 15 min or 24 h).

Gel retardation

Gel retardation was done entirely as described by Kumar and Chambon (1988). 'Off curves' were according to Eul *et al.* (1989) by incubating with labelled ERE for 15 min at room temperature, followed by a chase with 100 pmol non-radioactive ERE. The complexes were incubated for 0, 10, 20 or 40 min or 1 h at room temperature and loaded onto a running polyacrylamide gel.

Histological techniques

Transfected cells were washed with phosphate-buffered saline (PBS) and fixed with paraformaldehyde (crosslinking fixative) or methanol/acetone (precipitating fixative). Cells were incubated in 4% paraformaldehyde (in PBS) for 15 min at room temperature (RT) and washed in PBS. To ensure the entry of antibodies, cells were permeabilized by incubating in 0.5% Triton X-100 (in PBS) for 40 min at RT. If permeabilization was omitted, no staining was seen. For methanol/acetone fixation, cells were first incubated in methanol(−20°C) for 4 min and then in acetone (−20°C) for 2 min. In this case, the permeabilization step was omitted. After fixation, cells were incubated in PBS for 10 min. Both techniques were used throughout this study and gave similar results.

Several modifications of this basic procedure were tested to investigate whether experimental conditions affect the distribution of receptors between cytoplasm and nucleus. Omitting the PBS wash before fixation or extending it up to 15 min did not affect receptor distribution, and we did not observe different results when cells were 'pre-fixed' by adding paraformaldehyde directly to the Petri dish (at a final concentration of 1.5%) and continuing incubation for 5 min at 37°C before final fixation in 4% paraformaldehyde (10 min at RT). The effect of FCS on receptor distribution was studied by changing to medium lacking FCS 20 h prior to fixation (the medium was changed several times to wash out traces of FCS). No effect of FCS on intracellular receptor location was observed in these experiments including the analysis of cPR(1–486)hGR(487–777) (see also Picard and Yamamoto, 1987).

After fixation and washing with PBS, cells were incubated for 20 min in 3% goat serum to saturate non-specific binding sites, followed by incubation with specific antibodies for 1–2 h at RT or overnight at 4°C, and washing in PBS. Specifically bound antibodies were detected by biotinylated secondary antibodies [1 h incubation at RT followed by PBS washes before incubation with avidin–biotin–peroxidase (Vectastain reagents, Vector Laboratories, Burlingame, CA)] for 1 h at RT followed by a final PBS wash. Peroxidase was visualized using diaminobenzidine as a substrate. For hER receptor four different (primary) monoclonal antibodies were used [H226 (epitope in region A/B), D75 (epitope in region F) and H222 (epitope in the HBD) (Greene *et al.*, 1980; Kumar *et al.*, 1987)], for cPR we used two different polyclonal antibodies directed against region A/B (Bocquel *et al.*, 1989; Tuohimaa *et al.*, 1984). Histological controls included staining with non-specific antibodies, staining of untransfected cells, and staining of cells transfected with ER by using anti-PR antibodies and vice versa.

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References

Adam,S.A., Lobl,T.J., Mitchell,M.A. and Gerace,L. (1989) *Nature*, **337**, 276–279.

Antakly,T., Thompson,E.B. and O'Donnell,D. (1989) *Cancer Res.*, **49**, 2230s–2234s.

Arriza,J.L., Weinberger,C., Cerelli,G., Glaser,T.M., Handelin,B.L., Housman,D.E. and Evans,R.M. (1987) *Science*, **237**, 268–275.

Baker,A.B., McDonnell,D.P., Hughes,M., Crisp,T.M., Mangelsdorf,D.J., Haussler,M.R., Pike,J.W., Shine,J. and O'Malley,B.W. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3294–3298.

Beato,M. (1989) *Cell*, **56**, 335–344.

Berry,M., Metzger,D. and Chambon,P. (1990) *EMBO J.*, **9**, 2811–2818.

Bocquel,M.T., Kumar,V., Stricker,C., Chambon,P. and Gronemeyer,H. (1989) *Nucleic Acids Res.*, **17**, 2581–2595.

Chambraud,B., Berry,M., Redeuilh,G., Chambon,P. and Baulieu,E.E. (1990) *J. Biol. Chem.*, **265**, 20686–20691.

Chawnshang,C., Kokontis,J. and Liao,S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7211–7215.

Dang,C.V. and Lee,W.M. (1988) *Mol. Cell. Biol.*, **8**, 4048–4054.

Danielsen,M., Northrop,J.P. and Ringold,G.M. (1986) *EMBO J.*, **5**, 2513–2527.

Danielsen,M., Hinck,L. and Ringold,G.M. (1989) *Cell*, **57**, 1131–1138.

Davis,L.I. and Fink,G.R. (1990) *Cell*, **61**, 965–978.

de The,H., Marchio,A., Tiollais,P. and Dejean,A. (1987) *Nature*, **330**, 667–670.

Dingwall,C. and Laskey,R.A. (1986) *Annu. Rev. Cell Biol.*, **2**, 367–390.

Eckhard,S.G., Milich,D.R. and McLachlan,A. (1991) *J. Virol.*, **65**, 575–582.

Eriksson,P. and Wrange,O. (1990) *J. Biol. Chem.*, **265**, 3535–3542.

Eul,J., Meyer,M.E., Tora,L., Bocquel,M.T., Quirin-Stricker,C., Chambon,P. and Gronemeyer,H. (1989) *EMBO J.*, **8**, 83–90.

Evans,R.M. (1988) *Science*, **240**, 889–895.

Fawell,S.E., Lees,J.A., White,R. and Parker,M.G. (1990a) *Cell*, **60**, 953–962.

Fawell,S.E., White,R., Hoare,S., Sydenham,M., Page,M. and Parker,M.G. (1990b) *Proc. Natl. Acad. Sci. USA*, **87**, 6883–6887.

Fischer-Fantuzzi,L. and Vesco,C. (1988) *Mol. Cell. Biol.*, **8**, 5495–5503.

Gasc,J., Renoir,J., Radanvi,C., Joab,I., Tuohima,P. and Baulieu,E. (1984) *J. Cell Biol.*, **99**, 1193–1201.

Gasc,J.M., Delahaye,F. and Baulieu,E.E. (1989) *Exp. Cell Res.*, **181**, 492–504.

Giguere,V., Ong,E.S., Segui,P.S. and Evans,R.M. (1987) *Nature*, **330**, 624–629.

Govindan,M.V., Devic,M., Green,S., Gronemeyer,H. and Chambon,P. (1985) *Nucleic Acids Res.*, **13**, 8293–8304.

Green,S. and Chambon,P. (1988) *Trends Genet.*, **4**, 309–314.

Green,S., Walter,P., Kumar,V., Krust,A., Bornert,J.M., Argos,P. and Chambon,P. (1986) *Nature*, **320**, 134–139.

Green,S., Issemann,I. and Sheer,E. (1988a) *Nucleic Acids Res.*, **10**, 369.

Green,S., Kumar,V., Theulaz,I., Wahli,W. and Chambon,P. (1988b) *EMBO J.*, **7**, 3037–3044.
Greene,G.L., Nolan,C., Engler,J.P. and Jensen,E.V. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5115–5119.
Gronemeyer,H. (1991) *Annu. Rev. Genet.*, **25**, 89–123.
Gronemeyer,H., Turcotte,B., Quirin-Stricker,C., Bocquel,M.T., Meyer,M.E., Krozowski,Z., Jeltsch,J.M., Lerouge,T., Garnier,J.M. and Chambon,P. (1987) *EMBO J.*, **6**, 3985–3994.
Guiochon-Mantel,A., Loosfelt,H., Lescop,P., Sar,S., Atger,M., Perrot-Applanat,M. and Milgrom,E. (1989) *Cell*, **57**, 1147–1154.
Guiochon-Mantel,A., Lescop,P., Christin-Maitre,S., Loosfelt,H., Perrot-Applanat,M. and Milgrom,E. (1991) *EMBO J.*, **10**, 3851–3859.
Hollenberg,S.M., Weinberger,C., Ong,E.S., Cerelli,G., Oro,A., Lebo,R., Thompson,E.B., Rosenfeld,M.G. and Evans,R.M. (1985) *Nature*, **318**, 635–641.
Kastner,P., Krust,A., Turcotte,B., Stropp,U., Tora,L., Gronemeyer,H. and Chambon,P. (1990) *EMBO J.*, **9**, 1603–1614.
King,W.J. and Greene,G.L. (1984) *Nature*, **307**, 745–747.
Koike,S., Sakai,M. and Muramatsu,M. (1987) *Nucleic Acids Res.*, **15**, 2499–2513.
Kozak,M. (1986) *Cell*, **44**, 283–292.
Krust,A., Green,S., Argos,P., Kumar,V., Walter,P., Bornert,J.M. and Chambon,P. (1986) *EMBO J.*, **5**, 891–897.
Krust,A., Kastner,P.H., Petkovich,M., Zelent,A. and Chambon,P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5310–5314.
Kumar,V. and Chambon,P. (1988) *Cell*, **55**, 145–156.
Kumar,V., Green,S., Staub,A. and Chambon,P. (1986) *EMBO J.*, **5**, 2231–2236.
Kumar,V., Green,S., Stack,G., Berry,M., Jin,J.R. and Chambon,P. (1987) *Cell*, **51**, 941–951.
Lanford,R.E. and Butel,J.S. (1984) *Cell*, **37**, 801–813.
Lang,I., Scholz,M. and Peters,R. (1986) *J. Cell Biol.*, **102**, 1183–1190.
Leid,M., Kastner,P., Lyons,R., Nakshatri,H., Saunders,M., Zacharewski, T., Chen,J.Y., Staub,A., Garnier,J.M., Mader,S. and Chambon,P. (1992) *Cell*, **68**, 377–395.
Levin,A.A., Sturzenbecker,L.J., Kazmer,S., Busakowski,T., Huselton,C., Allenby,G., Speck,J., Kratzeisen,C., Rosenberger,M., Lovey,A. and Grippo,J.F. (1992) *Nature*, **355**, 359–361.
Loosfelt,H., Atger,M., Misrahi,M., Guiochon-Mantel,A., Meriel,C., Logeat,F., Benarous,R. and Milgrom,E. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9045–9049.
Mader,S., Kumar,V., de Verneuil,H. and Chambon,P. (1989) *Nature*, **338**, 271–274.
Mangelsdorf,D.J., Ong,E.S., Dyck,J.A. and Evans,R.M. (1990) *Nature*, **345**, 224–229.
Maxwell,B.L., McDonnell,D.P., Conneely,O.M., Schulz,T.Z., Greene,G.L. and O'Malley,B.W. (1987) *Mol. Endocrinol.*, **1**, 25–35.
Meyer,M.E., Pornon,A., Ji,J., Bocquel,M.T., Chambon,P. and Gronemeyer,H. (1990) *EMBO J.*, **9**, 3923–3932.
Misrahi,M., Atger,M., d'Auriol,L., Loosfelt,H., Meriel,C., Friedlanski,F., Guiochon-Mantel,A., Galibert,A. and Milgrom,E. (1987) *Biochem. Biophys. Res. Commun.*, **143**, 740–748.
Morin,N., Delsert,C. and Klessig,D.F. (1989) *Mol. Cell. Biol.*, **9**, 4372–4380.
Nehrbass,U., Kern,H., Mutvei,A., Horstmann,H., Marshallsay,B. and Hurt,E.C. (1990) *Cell*, **61**, 979–989.
Newmeyer,D.D. and Forbes,D.J. (1988) *Cell*, **52**, 641–653.
Notides,A.C., Lerner,N. and Hamilton,D. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4926–4930.
Paine,P.L., Moore,L.C. and Horowitz,S.B. (1975) *Nature*, **254**, 109–114.
Petkovitch,M., Brand,N.J., Krust,A. and Chambon,P. (1987) *Nature*, **330**, 444–450.
Picard,D. and Yamamoto,K.R. (1987) *EMBO J.*, **6**, 3333–3340.
Picard,D., Kumar,V., Chambon,P. and Yamamoto,K.R. (1990) *Cell Regulation*, **1**, 291–299.
Ponglikitmongkol,M., Whie,J.H. and Chambon,P. (1990) *EMBO J.*, **9**, 2221–2231.
Pratt,W.B., Jolly,D.J., Pratt,D.V., Hollenberg,S.M., Guigere,V., Cadepond,F.M., Schweizer-Groyer,G., Catelli,M.G., Evans,R.M. and Baulieu,E.E. (1988) *J. Biol. Chem.*, **263**, 267–273.
Richardson,W.D., Mills,A.D., Dilworth,S.M., Laskey,R.A. and Dingwall,C. (1988) *Cell*, **52**, 655–664.
Robbins,J., Dilworth,S.M., Laskey,R.A. and Dingwall,C. (1991) *Cell*, **64**, 615–623.
Roberts,B. (1989) *Biochim. Biophys. Acta*, **1008**, 263–280.
Roberts,B.L., Richardson,W.D. and Smith,A.E. (1987) *Cell*, **50**, 465–475.
Rusconi,S. and Yamamoto,K.R. (1987) *EMBO J.*, **6**, 1309–1315.
Sabbah,M., Redeuilh,G. and Baulieu,E.E. (1989) *J. Biol. Chem.*, **264**, 2397–2400.
Sabbah,M., Gouilleux,F., Sola,B., Redeuilh,G. and Baulieu,E.E. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 390–394.
Sanchez,E.R., Hirst,M., Scherrer,L.C., Tang,H.Y., Welsh,M.J., Harmon,J.M., Simons,S.S., Ringold,G.M. and Pratt,W.B. (1990) *J. Biol. Chem.*, **265**, 20123–20130.
Schneider,J., Schindewolf,C., Van Zee,K. and Fanning,E. (1988) *Cell*, **54**, 117–125.
Skafar,D.F. (1991) *Biochemistry*, **30**, 6148–6154.
Tasset,D., Tora,L., Fromental,C., Scheer,E. and Chambon,P. (1990) *Cell*, **62**, 1177–1187.
Tora,L., Mullick,A., Metzger,D., Ponglikitmongkol,M., Park,I. and Chambon,P. (1989) *EMBO J.*, **8**, 1981–1986.
Tratner,I. and Verma,I.M. (1991) *Oncogene*, **6**, 2049–2053.
Tsai,S.Y., Carlstedt-Duke,J., Weigel,N.L., Dahlman,K., Gustafsson,J.A., Tsai,M.J. and O'Malley,B.W. (1989) *Cell*, **55**, 361–369.
Tuohimaa,P., Renoir,J.M., Radanyi,C., Mester,J., Joab,I., Buchou,T. and Baulieu,E.E. (1984) *Biochem. Biophys. Res. Commun.*, **119**, 433–439.
Turcotte,B., Meyer,M.E., Bocquel,M.T., Bélanger,L. and Chambon,P. (1990) *Mol. Cell. Biol.*, **10**, 5002–5006.
Umesono,K. and Evans,R.M. (1989) *Cell*, **57**, 1139–1146.
Weiler,I.J., Lew,D. and Shapiro,D.J. (1987) *Mol. Endocrinol.*, **1**, 335–362.
Weinberger,C., Thompson,C.C., Ong,E.S., Lebo,R., Gruol,D.J. and Evans,R.M. (1986) *Nature*, **324**, 641–646.
Welshons,W.V., Liebermann,M.E. and Gorski,J. (1984) *Nature*, **307**, 747–749.
White,R., Lees,J.A., Needham,M., Ham,J. and Parker,M. (1987) *Mol. Endocrinol.*, **1**, 735–744.
Wikstrom,A.C., Bakke,O., Okret,S., Bronnegard,M. and Gustafsson,J.A. (1987) *Endocrinology*, **120**, 1232–1242.
Wrenn,C.K. and Katzenellenbogen,B. (1990) *Mol. Endocrinol.*, **4**, 1647–1654.
Yamasaki,L., Kanda,P. and Landford,R.E. (1989) *Mol. Cell. Biol.*, **9**, 3028–3036.
Ylikomi,T., Gasc,J.M., Isola,J., Baulieu,E.E. and Tuohimaa,P. (1985) *Endocrinology*, **117**, 155–160.
Yu,V.C., Delsert,C., Andersen,B., Holloway,J.M., Devary,O.V., Näär,A.M., Kim,S.Y., Boutin,J.M., Glass,C.K. and Roenfeld,M.G. (1992) *Cell*, **67**, 1251–1266.

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