Signal Transduction and Transcriptional Regulation by Glucocorticoid Receptor–LexA Fusion Proteins

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The glucocorticoid receptor regulates transcriptional initiation upon binding to its cognate hormone. A series of fusion genes was constructed to examine the mechanism of hormone-regulated transcriptional enhancement. The DNA binding domain of the bacterial LexA repressor was fused to receptor derivatives lacking the region that is necessary and sufficient for specific DNA binding and transcriptional enhancement at glucocorticoid response elements (GRE's). The resultant hybrid proteins activated transcription from promoters linked to the lex operator. Enhancement still required hormone binding by the hybrid receptor regardless of the exact positioning of the LexA binding domain within the protein. Thus, the unliganded hormone binding domain of the receptor acts as a strong but reversible inhibitor of receptor activity in a manner that is independent of the means by which the receptor recognizes DNA. The results also show directly that the receptor contains at least one "enhancement domain" other than that overlapping the GRE binding region; the second domain, enh2, occupies a region near the receptor amino terminus.

THE GLUCOCORTICOID RECEPTOR IS AN INTRACELLULAR protein that, upon interaction with functional hormonal ligands, binds to specific DNA sequences termed glucocorticoid response elements (GRE's) and enhances transcription from linked promoters (*1*). Receptor complementary DNA's have been cloned from rat (*2–4*), and in vitro studies of the 795–amino acid gene product have localized the steroid binding domain to the carboxyl-terminal third of the protein, amino acid residues 540 to 795, whereas the DNA binding domain is between amino acids 440 and 525 (*5, 6*). It has been suggested that the DNA binding regions of all members of the "nuclear receptor gene family" contain two "zinc finger" structural motifs (*7*); indeed, this region of the rat glucocorticoid receptor associates with two metal ions, and metal coordination is necessary for GRE binding (*8*).

The DNA binding domain is itself sufficient to confer transcriptional enhancement in transfected cells, albeit at low levels (*9, 10*). The overlap of the DNA binding and enhancement (denoted enh1) regions has compromised attempts to assess whether additional enhancement regions might lie elsewhere in the receptor, since any added segment that increased enhancement activity might simply be facilitating the proper folding of the enh1 domain (*10*).

Receptor derivatives that lack the hormone binding domain enhance transcription constitutively from GRE-linked promoters (*5, 9*). Thus, the interaction between hormone and receptor derepresses one or more functional activities outside of the hormone binding region. Data from early investigations implied that the DNA binding activity is subject to such derepression (*11*) and, more recently, nuclear localization of the receptor has also been shown to be hormone-dependent (*12*). The precise mechanism of this ligand-mediated derepression, and the possibility that additional receptor activities might also be subject to derepression, remain open questions.

Activation regions of several yeast transcriptional regulators have been mapped by fusing segments of those proteins to the DNA binding domain from a heterologous protein, such as the *Escherichia coli* repressor, LexA (*13–15*). It seemed conceivable that this strategy could be exploited to determine, first, whether additional enhancement domains reside within the receptor at positions other than the DNA binding domain and, second, whether hormonal regulation of receptor function is maintained in receptor species that contain a nonreceptor DNA binding region. We describe here the construction and functional analysis of a series of receptor-LexA fusions to approach novel issues concerning transcriptional regulation and signal transduction.

Lex operator functions as a transcriptional enhancer. We constructed a chimeric gene, NLxC, in which the sequences encoding receptor amino acids 1 to 449 (denoted N) and 524 to 795 (denoted C) are fused amino and carboxyl terminal, respectively, to those coding for the DNA binding domain of the *E. coli* LexA repressor (amino acids 1 to 87) (*16*); thus, this manipulation replaces the receptor DNA binding domain with that of LexA. In transfected CV-1 cells (which lack endogenous receptor), the NLxC protein had no effect on expression from a cotransfected reporter gene GMCO, in which the murine mammary tumor virus (MTV) GRE and promoter direct transcription of bacterial chloramphenicol acetyltransferase (CAT) (Fig. 1); as expected (*3, 5*), the intact receptor, N795, strongly enhances GMCO transcription upon hormone treatment (*17*) (Fig. 1). However, NLxC strongly induced transcription from XMCO, a GMCO derivative containing a 26-bp lex operator (*18*) inserted between the MTV GRE and promoter; moreover, induction by NLxC is fully hormone-dependent (Fig. 1). As was expected, the intact receptor also activated XMCO transcription, since the MTV GRE is retained in XMCO. In control experiments, the LexA DNA binding domain alone, Lx87, did not affect expression from either XMCO or GMCO (Fig. 1). Thus, NLxC selectively stimulates transcription in a hormone-dependent

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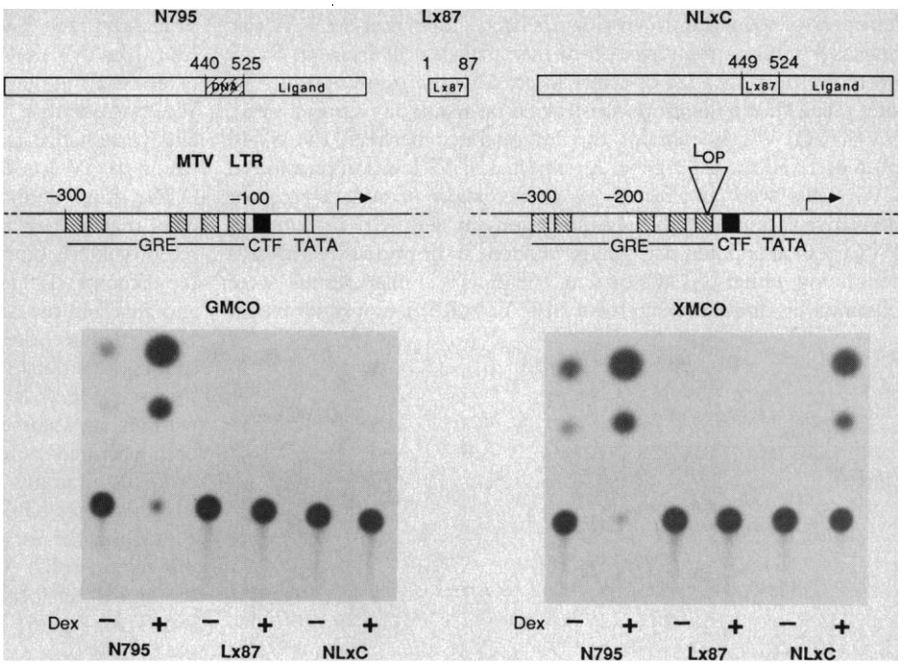

Fig. 1. Transcriptional regulation by receptor-LexA fusion proteins. (**Top**) Schematic representation of receptor, the LexA DNA binding domain Lx87, the receptor-LexA fusion protein NLxC, and the regulatory region of reporter genes (*32, 33*). N795 is the wild-type receptor. Lx87 contains amino acids 1 to 87 of the LexA protein; to express Lx87 alone, it is fused in frame via a Bam HI linker to sequences encoding the first five amino acids of HSV (herpes simplex virus) thymidine kinase, which provide a eukaryotic translation start. Accumulation of Lx87 in transfected cells has not been measured. In NLxC, Lx87 replaces receptor amino acids 450 to 523. Expression of the chloramphenicol acetyltransferase (CAT) gene is directed by the MTV promoter in GMCO. Binding sites for the glucocorticoid receptor (GRE) and the CAAT transcription factor (CTF) and the TATAA box are indicated. XMCO was derived from GMCO by insertion of a lex operator (Lop) (*18*) at the Sac I site at −109 relative to the transcription start site. (**Bottom**) CAT assays correspond to transient transfection (*3*) of duplicate cultures of receptor-deficient CV-1 cells with 5 μg of a receptor (or Lx87) expression plasmid and 0.5 μg of either GMCO or XMCO per 10-cm culture dish. Duplicate transfected cultures were incubated for 30 hours with (+) or without (−) 1 μ*M* dexamethasone (Dex). Extracts were prepared, and CAT activities were determined by a thin-layer chromatographic assay (*34*); the two upper spots in the appropriate lanes are the reaction products.

fashion from a lex operator–MTV promoter construct; furthermore, these results suggest that at least one segment of the receptor in addition to the DNA binding–enh1 region is sufficient for transcriptional enhancement (see below).

We next tested whether NLxC could regulate transcription from promoters not normally subject to glucocorticoid control. We constructed reporter plasmids that contained a β-globin promoter-CAT fusion with the lex operator fragment inserted either at −125 bp (pXBCO) or at +1.65 kb (pOBCX) relative to the transcription start site. In hormone-treated cultures, NLxC stimulated XBCO expression more than 50-fold (Fig. 2C), similar to the extent of induction by the intact receptor of an analogous GRE-β-globin promoter construct (*19*); transcripts induced by NLxC displayed the correct start sites (see below). The expression of OBCX was also induced by NLxC in the presence of hormone, albeit at lower levels, as would be expected for constructs in which the promoter and regulatory elements are widely separated (Fig. 2C). Similarly, NLxC also induced transcription from thymidine kinase promoter constructs bearing upstream or downstream operator sequences (*19*). Thus, the lex operator is a transcriptional enhancer under these conditions, and the NLxC fusion protein functions as a bona fide enhancer activating protein.

Hormonal regulation of receptor-LexA hybrid proteins. It was striking that hormonal regulation persisted in the NLxC derivative in which the zinc finger DNA binding structure of the receptor has been substituted by a helix-turn-helix motif. This demonstrates that receptor activity is controlled by ligand binding regardless of the conformation of the DNA binding domain. To examine the effect of altering the relative position of the DNA binding region within the receptor, we substituted the NH_2-terminal 150 amino acids of receptor with Lx87. The receptor derivative used for this construct was ΔZ, which lacks receptor amino acids 450 to 505, and therefore lacks the receptor DNA binding domain. This chimeric receptor, LxΔZ, enhanced XBCO and OBCX expression in a hormone-dependent manner, whereas ΔZ itself had no effect (Fig. 2). Thus, hormonal regulation of receptor activity is maintained even when a heterologous DNA binding region is introduced into the protein at a heterologous position.

Picard and Yamamoto (*12*) mapped two nuclear localization signals in the glucocorticoid receptor and showed that accumulation of receptor within the nucleus is hormone-dependent. Therefore, hormonal regulation of transcriptional regulation by the receptor-Lx87 hybrid proteins might simply reflect control of their intracellular location. However, when we visualized NLxC and LxΔZ by immunofluorescence, a substantial fraction of each was detected in nuclei even in the absence of hormone (Fig. 3); after the hormone was added, the residual cytoplasmic receptor migrated stably into nuclei. These results suggest that regulation of nuclear localization is relaxed in the receptor-Lx87 chimeras, and therefore that control of nuclear localization cannot fully account for the hormone dependence of enhancement by these fusion proteins.

Deletion of the hormone binding domain from the receptor yields derivatives that constitutively enhance transcription from appropriate GRE's (*5, 9*). To determine whether analogous receptor-Lx87 fusion proteins might function similarly in association with the lex operator, we deleted sequences encoding 272 COOH-terminal amino acids from NLxC. The resultant species, NLx, lacks the hormone binding domain, and strongly activates XBCO expression even in the absence of hormone (Fig. 4C). Moreover, both NLxC and NLx stimulate transcription from the normal β-globin transcription initiation sites, as assessed by ribonuclease (RNase) protection analysis (Fig. 4D).

Multiple enhancement domains. Receptor derivatives lacking amino acids 1 through 439 retain DNA and hormone binding activities and mediate hormone-dependent transcriptional enhancement, albeit at reduced levels (*10*). To test directly whether the hormone binding region itself is a competent enhancement domain, we fused receptor amino acids 524 to 795 to Lx87. This derivative, LxC, had no effect on XBCO expression, although it was produced at levels similar to NLxC and NLx, as judged by protein blotting (Fig. 4B), and was localized to nuclei as shown by immunofluorescence (Fig. 3).

We next determined whether LxC protein was actually binding to

lex operator sequences in vivo. It has been shown that the *E. coli* lac repressor inhibits transcription in mammalian cells from an SV40 promoter bearing a lac operator insert near the transcription start sites (*20*, *21*). We therefore constructed an analogous reporter gene, VV(X)CO, which contains the lex operator between the SV40 promoter and the CAT gene. As expected, CAT levels in transfected CV-1 cells were unaffected by coexpression of ΔZ, a receptor derivative lacking a DNA binding domain (Fig. 5). In contrast, VV(X)CO expression was greatly reduced in hormone-treated cells containing either NLxC or LxC (Fig. 5); similar results were obtained by direct measurement of CAT mRNA by primer extension assays (*19*). These species failed to inhibit expression of VVCO (*21*) [the VV(X)CO parent, which lacks operator sequences], demonstrating that the LexA binding site is essential for repression. We conclude that LxC binds to the lex operator in vivo, but that it fails to enhance transcription of an operator–β-globin promoter fusion in CV-1 cells (*22*).

The observation that NLx stimulates XBCO transcription indicates that at least one "enhancement domain" resides outside the receptor DNA binding region and within the NH_2-terminal half of the receptor. To delimit further the active region, we constructed and analyzed the activity and subcellular distribution of a series of NLxC deletion derivatives. Protein blotting established that these species accumulate to similar levels in transfected cells (*19*). As shown in Fig. 6, large portions of the NH_2-terminus can be deleted without apparent effect on enhancement; analogous results have been obtained with the bona fide receptor (*9*, *10*). A LexA fusion protein containing 213 amino acids of the receptor, residues 106 to 318, enhances XBCO transcription nearly as well as the derivative containing the intact NH_2-terminal portion of receptor; we term this region enh2. Moreover, a significant fraction of enh2 activity can be further localized to an 82–amino acid region between 237 and 318 (Fig. 6). Consistent with these results, NΔ150-300Lx,

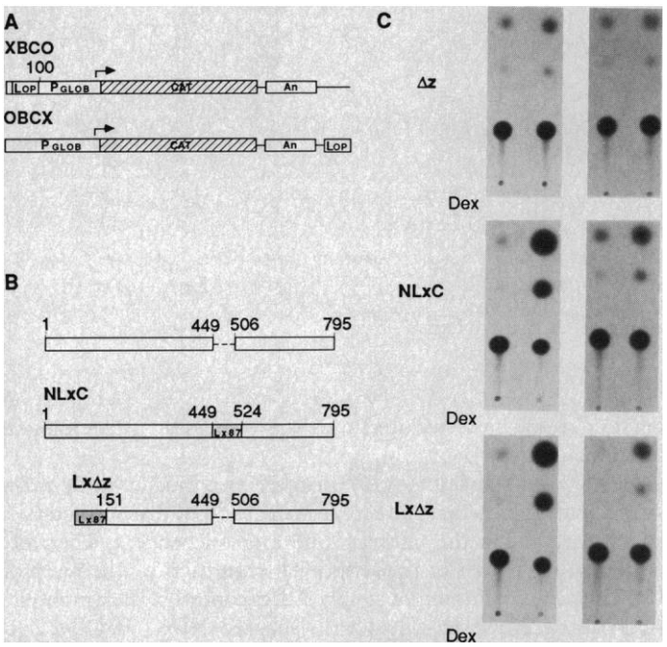

Fig. 2. Receptor-Lx87 fusion proteins enhance transcription of the β-globin promoter. (**A**) Structure of reporter genes. XBCO contains a lex operator at −125 relative to the transcription initiation site of the rabbit β-globin promoter; in OBCX, the lex operator (Lop) is at +1.65 kb, positioned downstream of the SV40 polyadenylation site (An). (**B**) Structure of receptor derivatives. ΔZ contains a 56–amino acid deletion in the receptor DNA binding domain; NLxC is as described in Fig. 1; LxΔZ contains Lx87 fused via an Xho I linker to ΔZ receptor sequences at amino acid 151 (*32*). (**C**) CAT assays of transiently transfected CV1 cells, as described in Fig. 1. Expression of OBCO, a β-globin CAT construct lacking a lex operator, was unaffected by NLxC or LxΔZ (*19*).

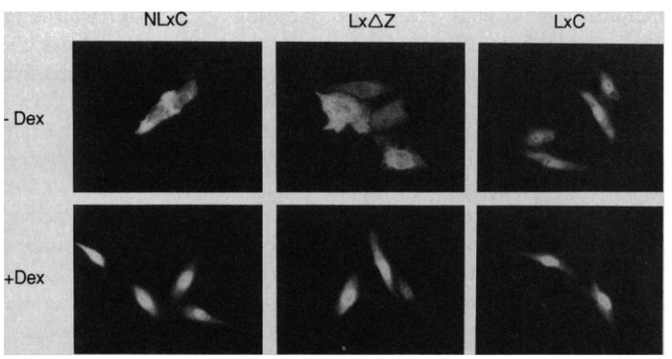

Fig. 3. Subcellular distribution of receptor-LexA fusion proteins. Immunofluorescence micrographs of fusion proteins NLxC, LxΔZ, or LxC expressed transiently in CV1 cells in the absence (−Dex) or presence (+Dex) of 1 μ*M* dexamethasone. Transfected cells were fixed (*12*) and reacted with rabbit anti-serum to LexA (*34*) and then with a rhodamine-labeled goat antiserum to rabbit antibody.

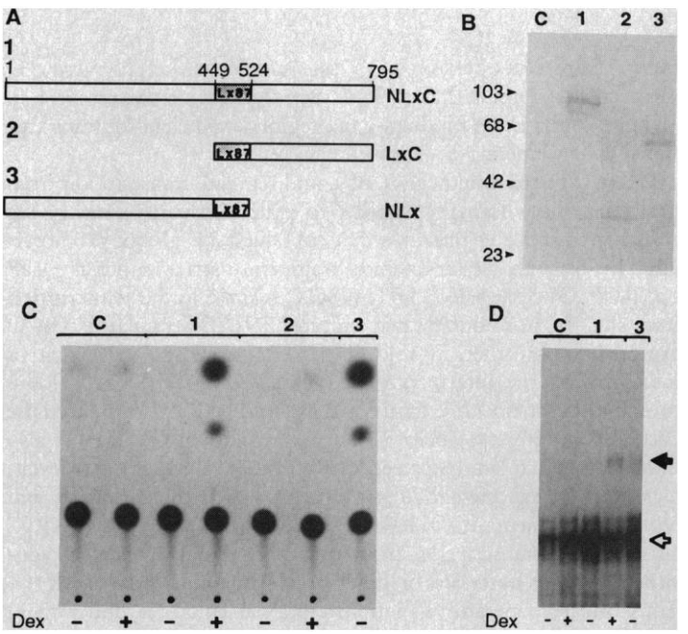

Fig. 4. Analysis of receptor-LexA derivatives with NH_2-terminal or COOH-terminal deletions. (**A**) Structure of receptor-LexA fusion proteins (*32*). (**B**) Accumulation of fusion proteins in transfected cells. Whole cell extracts were prepared 36 hours after transfection with receptor-LexA expression plasmids encoding the following fusion proteins: lane c, mock; lane 1, NLxC; lane 2, LxC; and lane 3, NLx. Equivalent amounts of protein were fractionated on a 9.25 percent polyacrylamide gel and transferred to nitrocellulose. The blots were reacted with a rabbit antiserum to LexA and then with a mouse antiserum to rabbit antibody coupled to horseradish peroxidase. Positions of molecular markers are indicated. (**C**) CAT assays from cells cotransfected with receptor-LexA expression plasmids and the reporter plasmid pXBCO. Lane designations are as in (B). (**D**) Quantitative RNase protection analysis (*35*) of transcripts induced by receptor-LexA fusion proteins. Total cellular RNA was prepared 36 hours after cells were transfected with receptor-LexA expression plasmids and the reporter plasmid pXBBO, which contains a lex operator at −125 of the β-globin gene. The ΔREF plasmid (*36*) was included as an internal control; assays utilized 30 μg of total RNA, lane designations are as in (B). The protected bands for XBBO (179 nucleotides; solid arrow) and ΔREF (151 nucleotides; open arrow) are indicated.

which lacks most of the enh2 region, retains little enhancement activity. However, fusion of the hormone binding domain to NΔ150-300Lx yields competent hormone-regulated enhancement (NΔ150-300LxC) (Fig. 6), despite the fact that neither NΔ150-300Lx nor LxC displays substantial activity. This result may imply the existence of yet another enhancement region (as discussed below).

Our studies have defined two discrete segments of the glucocorticoid receptor, enh1 and enh2, that can function independently as enhancement domains. Consistent with our direct assays of enh2 function, mutations within this region of the bona fide receptor result in reduced enhancement activity [10 to 50 percent, depending on the context of the remainder of the molecule (*9*, *10*, *23*, *24*)]; taken together, these results support the idea that enh2 is functional within the intact receptor. Similarly, we have isolated a mutant in enh1 that virtually abolishes enhancement without apparent effect on DNA binding (*25*); this suggests that enh1 also functions within the intact receptor, and that enh1 and enh2 probably communicate.

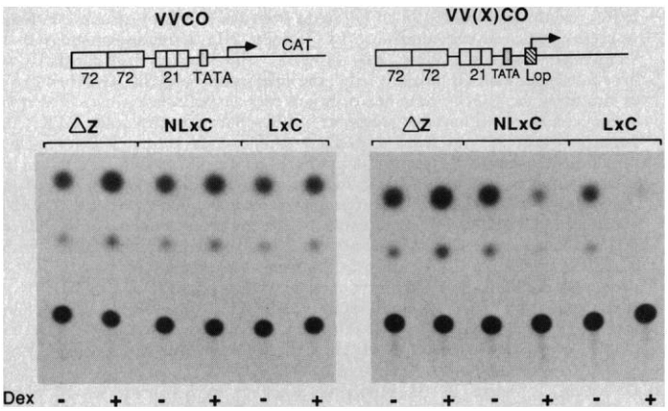

Fig. 5. Occupancy of lex operator sequences in vivo by receptor-LexA fusion proteins. (**Top**) Schematic representation of reporter genes. In VVCO [initially denoted pX8CAT (*21*)], the CAT gene is transcribed from the SV40 early promoter and enhancer element; the 21- and 72-bp repeats and the position of the TATA element are shown. VV(X)CO was constructed by insertion of a lex operator (Lop) at an Xho I site in VVCO just downstream of the TATA element. (**Bottom**) CAT assays are from duplicate cultures transfected with 5 μg of the appropriate receptor expression plasmid and 0.5 μg of the indicated reporter plasmid, and incubated for 30 hours in the absence (−) or presence (+) of 1 μ*M* dexamethasone. Similar results were obtained in five independent transfections.

Interestingly, NΔ150-300LxC, which lacks both enh1 and enh2, enhances XBCO transcription by 25- to 35-fold when hormone is present; this implies that the receptor may harbor a third enhancement domain. We have not directly identified such a region, as neither NΔ150-300Lx nor LxC is strongly active. Conceivably, the proper conformation of a putative "enh3" domain may be achieved only in the presence of both the NH_2-terminal and COOH-terminal portions of the receptor; whether such a region forms or functions in the context of the intact receptor is unknown.

Multiple enhancement domains within a single regulator have been observed elsewhere. For example, the yeast transcriptional activator GAL4 contains two separable activation regions (*26*). It has been suggested that activation regions carry a net negative charge (*15*, *26*, *27*). Interestingly, enh2 contains an abundance of negatively charged amino acids: 17 acidic and only 8 basic residues are found between amino acids 237 and 318, and the charge distribution is nearly identical to that of "activation region 1" of GAL4 (*27*). In striking contrast, however, the enh1 region of the receptor is positively charged, containing 6 acidic and 17 basic residues between amino acids 440 and 525. Conceivably, different enhancement regions might display distinct activities in different cell or molecular contexts (*1*), or they might even operate by entirely different mechanisms. It would be useful to test individually the activities of enh1 and enh2 upon different promoters or in different cell types.

Implications for signal transduction and receptor evolution. Previous studies of the hormone and the hormone binding domain as determinants of glucocorticoid receptor activity suggested that, in the absence of ligand, the hormone binding region assumes a structure that specifically precludes function of the DNA binding (*5*, *9*) and nuclear localization activities (*12*) of the receptor, and that the ligand-receptor interaction specifically derepresses those activities. Here we show that transcriptional enhancement remained fully hormone-dependent when the DNA binding domain was replaced with a structurally distinct DNA binding motif at either of two positions within the receptor. Thus, it appears that the unliganded hormone binding region can inhibit all receptor activities other than hormone binding itself without strict regard to the structure of the protein. This view is consistent with the results of a recent study in which the hormone binding region has been moved to other positions within the receptor, or fused to a nonreceptor protein (*28*). It has been reported that a heat shock protein, hsp90, interacts with steroid receptors in the absence but not in the presence of hormone (*29*). Perhaps the unliganded hormone binding domain is

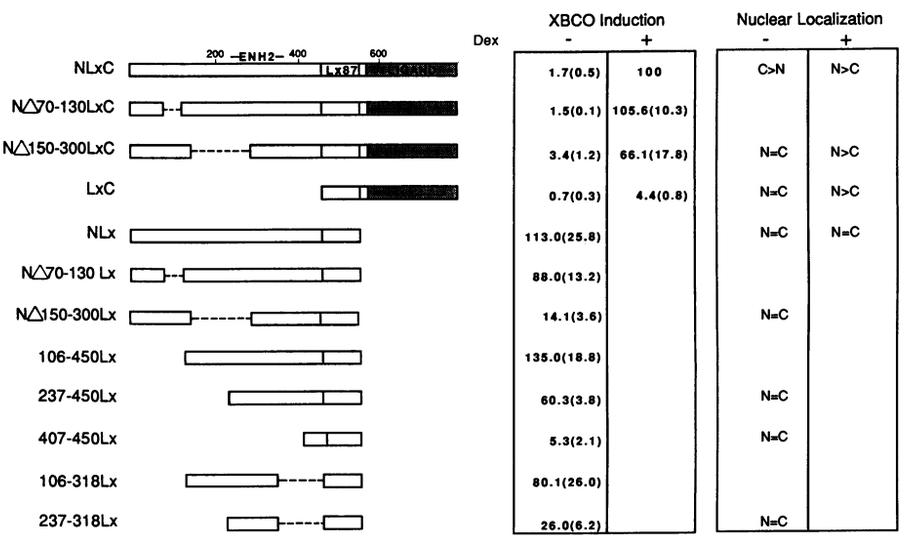

Fig. 6 Transcriptional activation and subcellular distribution of receptor-LexA fusion proteins. The structure of the fusion proteins are diagramed, and their subcellular distribution in transfected cells in the absence (−) or presence (+) of 1 μ*M* dexamethasone are indicated. Immunofluorescence was carried out as described (*12*). XBCO expression is presented as a percentage of that observed in cells cotransfected with NLxC and incubated with 1 μ*M* dexamethasone. The results represent the average of at least three independent transfections; standard deviations are shown in parentheses. Control transfections ΔZ were 1.5 or 3 percent of NLxC in the absence or presence of dexamethasone, respectively.

sufficient for the formation of such a complex, which in turn might inhibit receptor function (*28*). By this view, hormone binding would derepress receptor activity by altering the conformation of the hormone binding region and disrupting the receptor-hsp90 complex.

It is now apparent that different segments of steroid receptors comprise functional domains that can operate independently of one another, or when placed into different molecular contexts. Thus, Green and Chambon (*30*) showed that replacement of the estrogen receptor DNA binding domain with that from the glucocorticoid receptor yields a chimeric protein that induces transcription from GRE's in response to estrogen; similar "finger-swap" experiments involving other receptor proteins have been reported (*31*). Picard and Yamamoto (*12*) demonstrated that the hormone binding domain of the glucocorticoid receptor functions as a ligand-dependent nuclear localization cassette when fused to β-galactosidase. Finally, we establish here that hormone-dependent transcriptional regulation is retained in chimeric receptor derivatives that contain a structurally distinct DNA binding domain and, conversely, that the enh2 region functions independently as an enhancement region in association with a heterologous DNA binding domain. Taken together, these results underscore the modular structure of steroid receptors, and suggest that these and other regulatory proteins might be evolving by independent reassortment of functional units, perhaps via exon shuffling.

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32. Receptor-Lx87 protein constructs are denoted according to the following nomenclature: constructs denoted N or C extend from the amino or carboxyl termini, respectively. Constructs containing Lx87 at the NH_2-terminus initiate with a "translation initiation cassette" (*6*)—MASWG (*33*)—derived from the HSV tk gene fused via a Bam HI linker to Lx87; the linker contributes SRGG. When Lx87 is located at the COOH-terminus of fusion proteins, translation ends at a stop codon contained within the polylinker, adding linker amino acids RPR. An "initiation cassette" was fused to receptor amino acids 106 or 237 to initiate proteins containing NH_2-terminal deletions. Lx87 was linked COOH-terminal to receptor amino acids 318 or 449 via linkers containing GSRGG and RSRGG (*33*), respectively. Lx87 was linked NH_2-terminal ro receptor amino acids 151 and 525 via amino acids RG or RP, respectively. ΔZ deletes receptor amino acids 450 to 505 and joins amino acids 449 and 506 via a Bgl II linker, encoding linker amino acids RSV. Receptor expression plasmids were constructed with p6R, a pSP65 derivative containing an RSV promoter and SV40 polyadenylation signals (*19*).
33. Abbreviations for amino acid residues are A, Ala; G, Gly; M, Met; R, Arg; S, Ser; and W, Trp.
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