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Mechanism of synergistic transcriptional transactivation by the human glucocorticoid receptor

(steroid hormone action/cooperative DNA binding/yeast)

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ABSTRACT **Induction of transcription from a promoter with two upstream glucocorticoid response elements is 10- to 20-fold greater than that from a similar promoter with only one response element. We have shown that interactions involving the major transactivation domain of the glucocorticoid receptor (τ_1) are the sole determinant of such synergistic transactivation by the receptor. The other transactivation domain of the receptor (τ_2) did not mediate synergistic transactivation, and therefore the ability to synergize is operationally distinct from the transactivation function *per se*. The level of synergistic transactivation observed *in vivo* can be accounted for by the level of cooperative DNA binding seen *in vitro* for a glucocorticoid receptor derivative containing only the τ_1 and DNA-binding domains. Cooperative DNA binding was also observed using a τ_1–DNA-binding domain protein, which was expressed in *Escherichia coli* and extensively purified. Therefore, it is likely that direct protein–protein interactions between τ_1 domains mediate the cooperative DNA binding. The role of cooperative DNA binding for synergistic transactivation *in vivo* is discussed in relation to other possible mechanisms.**

Regulation of gene expression in eukaryotic organisms involves interaction of regulatory proteins with DNA sequences that control gene expression. Such regulatory sequences often contain multiple binding sites for one or more regulatory proteins, and it is common that the transcriptional transactivation from the intact regulatory region exceeds the sum of that from the individual sites. This synergism can produce a large induction of transcription in response to a small change in the level of a regulatory factor, and it can also impose a requirement for the presence of more than one factor for the level of response to be significantly increased.

Two non-mutually exclusive models could explain synergistic transcriptional enhancement. In the first, cooperative binding of factors to DNA could account for synergistic transactivation by increasing the probability that a given site will be occupied. Such cooperative binding might result from direct protein–protein interactions between the factors (Fig. 1*A*). However, the promiscuity with which different factors synergize makes this unlikely to be a complete explanation, since it would require the presence of compatible interaction surfaces within the structures of a wide range of otherwise diverse transcription factors. A variation of this model has been proposed as part of the explanation for synergistic transactivation by the yeast transcription factor GAL4 (1, 2). Cooperative binding to multiple binding sites, in this case, appears to be mediated indirectly via protein–protein interactions between the transactivation domains of several GAL4 molecules and a second protein, which may be the target protein that is contacted in order to achieve transcriptional transactivation. Thus all factors that function via the same target protein would be able to synergize by a cooperative binding mechanism without the requirement for direct contacts between them (Fig. 1*B*). In the second model, synergism occurs at a post-DNA-binding level such that the transactivation activity of two or more bound factors is greater than the sum of that from the individual factors (Fig. 1*C*). Various mechanisms for how this could occur have been suggested. For GAL4, which shows synergistic transactivation of transcription *in vitro* when all the DNA-binding sites are occupied in addition to cooperative DNA binding, it has been suggested that the DNA-associated transactivation domains cooperate to synergistically induce transcription (1). However, for the GCN4, Jun, and Fos factors it has been argued that synergistic transactivation is a consequence of the number of DNA-bound molecules *per se* and that it works by a mechanism independently of the number of transactivation domains contained within the DNA-bound molecules (3).

The glucocorticoid receptor (GR) is a member of a family of conditional transcription factors that regulate gene expression only in the presence of their cognate ligands. The family mediates the effect of a wide range of compounds including steroid hormones, thyroid hormones, retinoic acid, and vitamin D_3. The hormone-bound GR binds to glucocorticoid responsive elements (GREs) as a homodimer (4, 5) due to protein–protein interactions, some of which lie within the DNA-binding domain (DBD) (6, 7). Two regions of the human GR, designated τ_1 (residues 77–262) and τ_2 (residues 526–556), are involved in transcriptional transactivation by the DNA-bound GR (8). The transactivation potential of the GR is increased about 10-fold when mediated via synthetic promoters containing two GREs compared with one (9, 10). A similar increase in binding of the GR to a DNA fragment containing two GREs compared with one has been reported (11), and therefore it is likely that cooperative DNA binding is responsible for at least part of the synergistic transactivation by the GR. With regard to the first model discussed above, it is not known whether cooperative DNA binding results from direct or indirect protein interactions between GR dimers or which parts of the GR are involved in the interactions. The τ_1 domain has been implicated in synergistic interaction with the estrogen receptor (12), but the level at which this occurs is not known. Indeed the mechanism of synergistic transactivation by the estrogen receptor from two estrogen response elements is unclear since both cooperative and noncooperative DNA binding have been reported in different systems (13, 14).

The aim of this study was to determine the extent to which mechanisms proposed for synergism by the extensively studied factor GAL4 (discussed above) also apply to the GR. For there to be a common mechanism, two conditions would have to be satisfied. First, the region of the GR mediating synergism must colocalize with one or both of the transactivation domains. Second, cooperative binding by the GR must be mediated indirectly by interaction of receptor dimers with a

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Abbreviations: GR, glucocorticoid receptor; DBD, DNA-binding domain; GRE, glucocorticoid response element.

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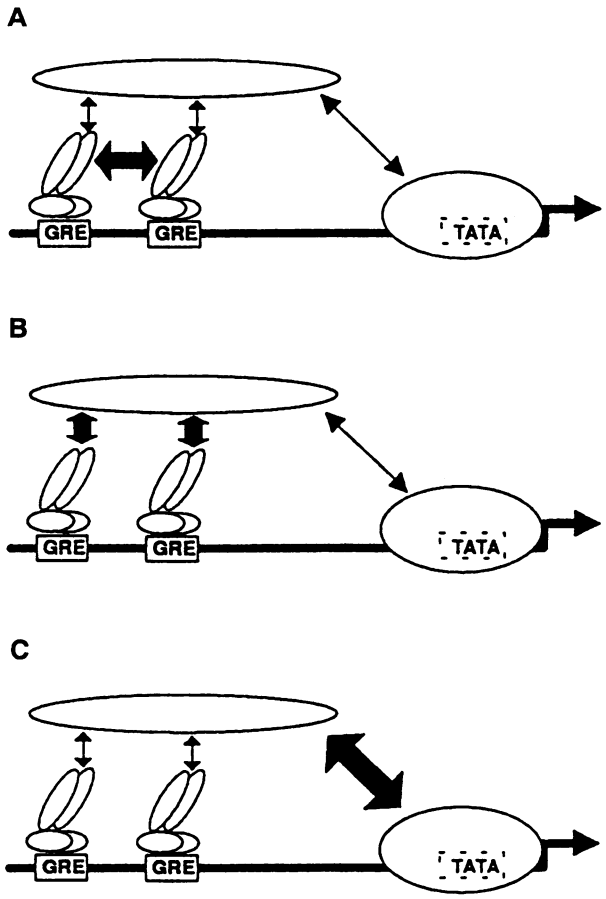


FIG. 1. Putative models for the mechanism of synergistic transactivation by the GR. The double arrows indicate hypothetical protein–protein interactions that stabilize the preinitiation complex, thereby causing an increase in transcription rate. The thick double arrows indicate interactions associated with synergism according to the models discussed in the text. (*A*) Cooperative DNA binding by the GR due to direct protein contacts between GR dimers. (*B*) Cooperative DNA binding by the GR resulting from indirect protein contacts with a common target protein. (*C*) Synergistic transactivation via a post-DNA-binding mechanism.

second protein and not by direct interactions between dimers. The results reported here show that only the first condition was fulfilled, and consequently the mechanism proposed for synergism by GAL4 does not apply to the GR. A mechanism to account for the known synergistic properties of the GR is proposed.

MATERIALS AND METHODS

Plasmid Constructions. The yeast expression vector, pKV50, used for the expression of all the GR derivatives has been described (15). Proteins were expressed from the yeast *PGK* promoter, the upstream activation sequences of which have been replaced with those from the *GAL1,10* promoter; thus expression is tightly repressed during growth on glucose and induced on galactose-containing media. Derivatives of the human GR expressed from the pKV50 expression vector are described below (see Fig. 2). pKV-NX expresses the intact human GR from a truncated cDNA from which the 5′ untranslated sequences have been removed (generously provided by Brian West, Metabolic Research Unit, University of California, San Francisco) and which has been described (7). pKV-NX-$\Delta\tau_1$ expresses the same protein as pKV-NX except that the region encoding the τ_1 domain (residues 77–261 inclusive) has been deleted. pKV-AX encodes residues 415–777 preceded by Met-Val and has been described (15). pKV-τ_1-AX encodes residues 77–262 fused to residues 415–777 preceded by Met-Val-Asn-Ser-Ser-Ser-Pro-Gly and with Pro-Leu between the receptor domains. pKV-BE encodes residues 262–500 preceded by Met and followed by Asp-His-Gly. pKV-XE encodes residues 370–500 preceded by Met-Val-Asn-Ser-Ser-Ser-Val-Pro-Gly-Asp-Arg and followed by Asp-His-Gly. pKV-AE encodes residues 416–500 preceded by Met-Val-Asn-Ser-Ser-Ser-Val-Pro-Gly-Asp-Arg-Pro and followed by Asp-His-Gly. pKV-τ_1-XE encodes residues 77–262 fused to residues 369–500 preceded by Met-Val-Asn-Ser-Ser-Ser-Val-Pro-Gly, with Pro-Leu between the domains and followed by Asp-His-Gly. pKV-τ_1-AE encodes residues 77–262 fused to residues 415–500 and includes the same polylinker-encoded residues described for pKV-τ_1-XE.

GR-responsive reporter plasmids contained the β-galactosidase gene from *Escherichia coli* fused to a basal yeast promoter derived from the *CYC1* gene in which the normal upstream activation sequences were replaced by GREs. The reporter plasmid pLGZ-TAT, containing a single copy of the TAT_{II} GRE from the rat tyrosine aminotransferase gene, has been described (15). pLGZ-2TAT is identical to pLGZ-TAT except that it contains two adjacent TAT_{II} GREs, the centers of which are separated by 32 base pairs.

The τ_1-DBD protein (as encoded by pKV-τ_1-AE) was expressed in *E. coli* as a fusion protein with protein A of *Staphylococcus aureus*, from the plasmid pHGR-τ_1-AE. This was done by using a modified version of the pRIT33 vector (16), pRIT33(chym), which contains a hypersensitive chymotrypsin site between the protein A sequences and the polylinker and was generously provided by Karin Dahlman-Wright (Center for Biotechnology, Karolinska Institute, Stockholm). The chymotrypsin site in pRIT33(chym) is a sequence from the human GR used previously to release the GR DBD from protein A (16). This allows release of essentially the same protein as was expressed in yeast by cleavage of the fusion protein with chymotrypsin.

Transcriptional Transactivation Assays. These were carried out in yeast strain W303-1A/K396-11B (15) containing combinations of each expression plasmid with each reporter plasmid. For strains expressing GR derivatives including the steroid-binding domain, the cells were grown and induced as described (15). Corticosterone was added to the induced cells at a level sufficient to induce a low but significant level of expression from a single GRE. The same level was then used for the equivalent expression strain containing reporter plasmids with two GREs. The levels of corticosterone used to activate different derivatives were 0.4 μM (pKV-NX and pKV-τ_1-AX), 2.0 μM (pKV-NX-$\Delta\tau_1$), and 10 μM (pKV-AX). Strains expressing GR derivatives lacking the steroid-binding domain were treated in the same way except that the incubation with steroid hormone was omitted. Extracts of proteins from the cells were prepared and assayed for β-galactosidase activity as described (15).

Gel Mobility Shift Assays with Yeast Extracts. Yeast extracts were prepared essentially as described (15) except that the extraction buffer was 20 mM Hepes, pH 8.0/20% glycerol/5 mM $MgCl_2$/100 mM KCl/0.2 mM EDTA/2 mM dithiothreitol. For DNA-binding assays, 5 μl of extract was incubated with an equal volume of DNA containing 10 pmol of the tyrosine aminotransferase GRE-binding sites end-labeled with ^{32}P to a specific activity of about 10^8 cpm per μg (each double GRE fragment represents two binding sites), 1.5 nmol of a palindromic estrogen response element (ERE1) described previously (17), and 100 ng of poly(dI-dC)·poly(dI-dC) (Pharmacia). Protein–DNA complexes were separated from free DNA by nondenaturing electrophoresis as described (6). Cooperative DNA binding was detected as an increase in the total amount of binding to the double GRE probe compared with the single GRE. To quantitate DNA binding, all specific complexes were measured by densitometry.

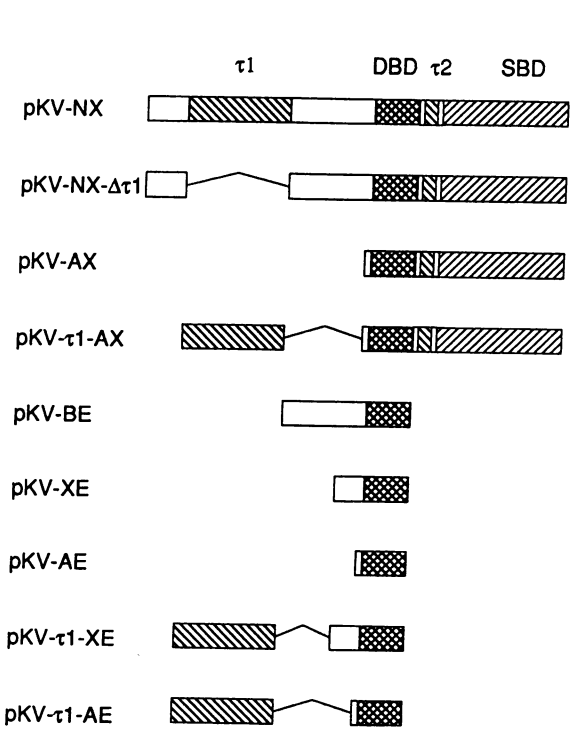
Purification of τ_1-DBD Protein. The *E. coli* strain was the same as reported previously for expression of the GR DBD (6) except that it contained the pHGR-τ_1-AE plasmid. Protein expression was induced, lysates were prepared and loaded onto IgG-Sepharose columns, and the columns were washed as described in ref. 16 and modified in ref. 6 for the DBD purification. The column containing almost pure immobilized protein A–τ_1-DBD protein was incubated with one column volume of buffer B (6, 16) containing chymotrypsin (2 μg/ml) at 4°C for 30 min. The column was eluted with one column volume of buffer B. One millimolar phenylmethylsulfonyl fluoride and 100 μM $ZnSO_4$ were added to the eluate to inhibit chymotrypsin activity. The τ_1-DBD protein was purified further on an FPLC Superose 12 gel-filtration column (Pharmacia) by using buffer B that contained 1 mM phenylmethylsulfonyl fluoride and 100 μM $ZnSO_4$ as the elution buffer. Peak fractions containing the intact τ_1-DBD protein were identified by gel electrophoresis (18) and Western blotting (19) using antibodies directed against the GR DBD (generously provided by Ann-Charlotte Wikström and Marika Rönnholm, Department of Medical Nutrition, Karolinska Institute, Stockholm). These fractions were pooled and used in gel mobility shift assays as described above, except that 5 μl of protein in buffer B was added to 5 μl of the labeled DNA in the absence of unlabeled competitor DNA.

RESULTS

To determine if the function of the GR that mediates synergistic transactivation could be located to a specific domain, various deletion mutants were tested for their ability to transactivate synergistically expression of a reporter gene in yeast cells. The amount of expression from reporter genes with either one or two GREs placed upstream is shown in Fig. 2. Significant levels of synergism were only seen for constructs containing the τ_1 transactivation domain. Other constructs, containing the DBD and τ_2 domains (e.g., pKV-NX-$\Delta\tau_1$ and pKV-AX), transactivate well but show additive rather than synergistic increases in transactivation from the double GRE reporter construct. Therefore, the synergistic transactivation function of the GR colocalizes with the τ_1 transactivation domain, and thus it is specific for this domain and is not simply correlated with transactivation potential *per se*.

The colocalization of synergistic and transactivation functions in the GR is compatible with both mechanisms for synergistic transactivation discussed in the introduction. To test directly whether the τ_1 domain of the GR could mediate cooperative binding to DNA, protein extracts were prepared from a yeast strain expressing the τ_1 domain fused to the DBD (from plasmid pKV-τ_1-XE). Binding to DNA fragments containing the same single and double GREs used in the transactivation assays was assessed by using a gel retardation assay (Fig. 3*A*). When the same number of binding sites was added to the reaction mixtures, the binding to the double GRE was greatly enhanced compared with binding to a single GRE. Quantitation indicates that about 14-fold more τ_1-DBD protein (as encoded by pKV-τ_1-XE) binds to the double GRE compared with a single site. Similar extracts containing the DBD protein alone (expressed from pKV-XE) were equally effective in binding to both the single and double sites (Fig. 3*B*). Therefore, the τ_1 domain appears to mediate cooperative DNA binding to adjacent GREs.

To determine whether τ_1-mediated cooperative binding resulted from a direct protein–protein interaction involving the τ_1 domain or from an indirect interaction via, for example, a transactivation target protein, extensively purified τ_1-DBD protein was used. The protein was expressed in *E. coli* as a fusion protein with protein A and separated from most *E. coli* proteins by immobilization on an IgG-Sepharose column. The τ_1-DBD protein was released from the immobilized fusion protein by cleavage at an artificial chymotrypsin site C-terminal of the protein A sequences. Fig. 4*A* is a Coomassie-stained gel showing the expression lysate (Fig. 4*A*, lane 1) and the material eluted after chymotrypsin



	Reporter Gene Activity (units β-Gal/min/mg)		Relative Activation
	1GRE	2GRE	(2GRE/1GRE)
pKV-NX	19.6	385.7	19.7
pKV-NX-Δτ1	15.2	32.4	2.2
pKV-AX	10.6	18.4	1.7
pKV-τ1-AX	60.0	842.6	14.0
pKV-BE	10.0	25.7	2.6
pKV-XE	4.3	7.3	1.7
pKV-AE	3.9	11.7	3.0
pKV-τ1-XE	269.2	4025.0	15.0
pKV-τ1-AE	260.2	4095.7	15.7

FIG. 2. The synergism function of the GR colocalizes with the τ_1 transactivation domain. The levels of β-galactosidase expression induced by different derivatives of the GR from reporter plasmids containing one (pLGZ-TAT) or two (pLGZ-2TAT) GREs upstream of the reporter gene are indicated. The relative activation is the fold increase in transactivation from two GREs (2GRE) compared with a single site (1GRE). The τ_1 transactivation domain, DBD, τ_2 transactivation domain, and steroid-binding domain (SBD) are indicated.

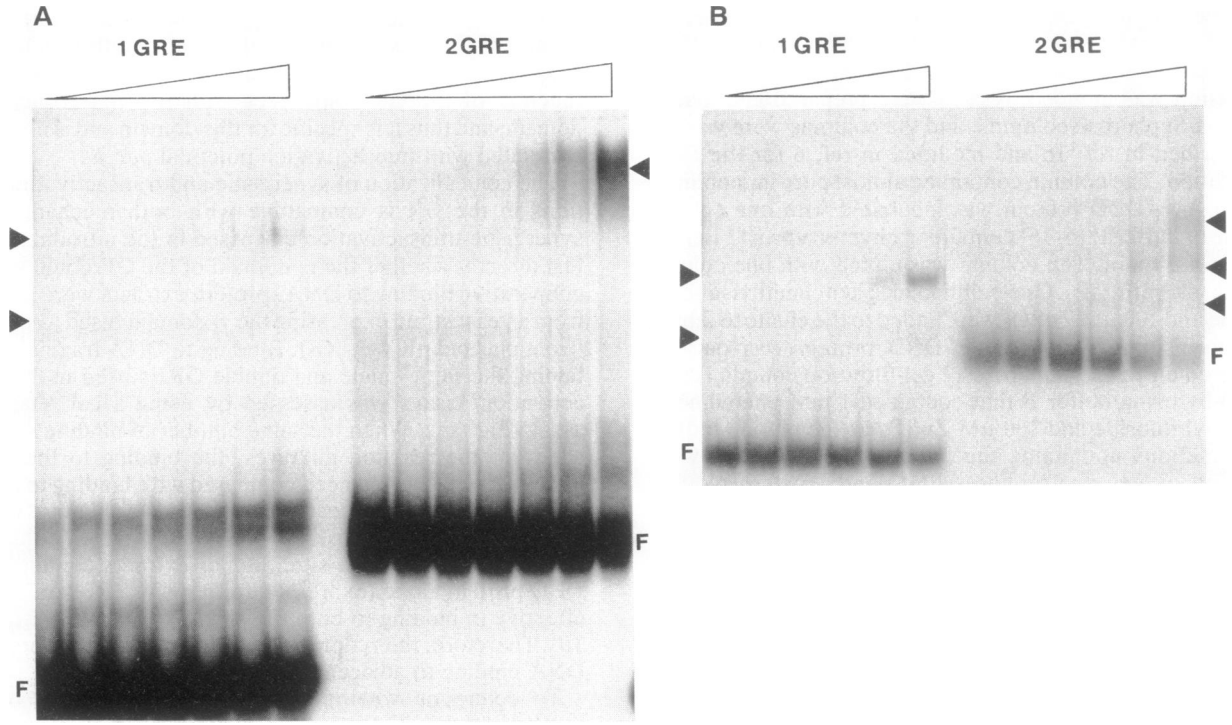


FIG. 3. Cooperative binding of the GR to a double GRE is mediated via the τ_1 domain. (*A*) Binding of a serially diluted yeast extract containing τ_1-DBD protein (expressed from the plasmid pKV-τ_1-XE) to DNA fragments containing one or two GREs. Specific complexes not found in extracts not expressing the τ_1-DBD protein (data not shown) are indicated by arrowheads. Based on their migration, the specific complexes appear to represent one and two molecules, respectively, of protein binding to the single GRE and a poorly resolved mixture of three and four molecules, respectively, binding to the double GRE. F, free DNA probe. (*B*) Binding of a serially diluted yeast extract containing DBD protein expressed from the plasmid pKV-XE to the same DNA fragments as in *A*. Annotations are the same as for *A*. The migration of the specific complexes suggests that the two fastest migrating complexes contain one and two protein molecules bound, respectively, whereas the slowest migrating complex is likely to contain four bound DBD molecules.

cleavage of the IgG-Sepharose-immobilized fusion protein (Fig. 4*A*, lane 2). After this step, the τ_1-DBD protein was highly purified. Two contaminating proteins of about 70 kDa probably correspond to uncleaved fusion protein (see Fig. 4*B*, lane 1) and the 69-kDa heat shock protein of *E. coli*, which has been shown to bind protein A with high affinity (20). Other bands migrating faster than the τ_1-DBD protein correspond to degradation products of the protein (see Fig. 4*B*, lane 1). The τ_1-DBD protein was further purified by gel-filtration chromatography. The Western blot in Fig. 4*B* shows the eluate from the IgG-Sepharose column (lane 1) and the peak fractions from the gel-filtration column (lanes 2–7) probed with an antibody directed against the DBD of the GR. After gel filtration the τ_1-DBD protein appears as a double band on SDS/PAGE gels, which is also seen when the protein is expressed in yeast cells (data not shown). Importantly, the gel-filtration step separated the τ_1-DBD protein from smaller, DBD-containing degradation products. The peak fractions (Fig. 4*B*, lanes 2–7) were pooled and used in gel shift assays. Fig. 4*C* shows that the purified τ_1-DBD protein shows enhanced binding to a double GRE: about 7-fold more protein is bound than with a single site. Therefore, direct protein contacts between GR dimers are likely to mediate cooperative binding to the double GRE site. As expected, isolated DBD protein purified from *E. coli* showed no enhanced binding to the double GRE fragment (data not shown), providing further confirmation that the interactions involve the τ_1 domain.

DISCUSSION

This study shows that interactions involving the τ_1 transactivation domain are the sole determinant of synergistic transactivation of gene expression by the human GR from a double GRE binding site. Furthermore, the capacity for transactivation *per se* is not sufficient to promote synergism since other constructs containing the DNA and τ_2 transactivation domains are also functional in transactivation but do not synergize. This suggests that the functions of transactivation and synergism may be operationally distinct.

This result was consistent with mechanisms for synergistic transactivation involving cooperative DNA binding, via direct or indirect protein contacts, and/or post-DNA-binding events mediated by the τ_1 domain. By using yeast extracts containing the τ_1 domain fused to the DBD of the GR, we have shown cooperative binding to a double GRE similar in magnitude to that reported for the intact receptor (11). As predicted from the transactivation results, extracts expressing the DBD alone showed no cooperative binding to the double GRE DNA fragment. Therefore, we confirm the previous report (11) that dimers of the GR bind to a double GRE fragment cooperatively, and in addition we show that interactions involving the τ_1 domain mediate this cooperative DNA binding. The cooperative binding was also observed by using τ_1-DBD protein expressed in, and extensively purified from, *E. coli*. Therefore, unlike the mechanism proposed for synergism by GAL4, cooperative DNA binding by GR dimers appears to be mediated by direct protein contacts involving the τ_1 domain. It is possible that this interaction is formed between the τ_1 domain and the DBD, whose net charge is opposite to that of τ_1. However, the lack of a requirement for a fixed spatial geometry between the two domains suggests that each functions independently of the other and that the cooperative interactions are likely to occur between the τ_1 domains of adjacent GR dimers. Consistent with structural constraints, likely to be imposed by such direct interactions, it has been shown that synergistic transactivation by the related receptor for progesterone does not increase signifi-

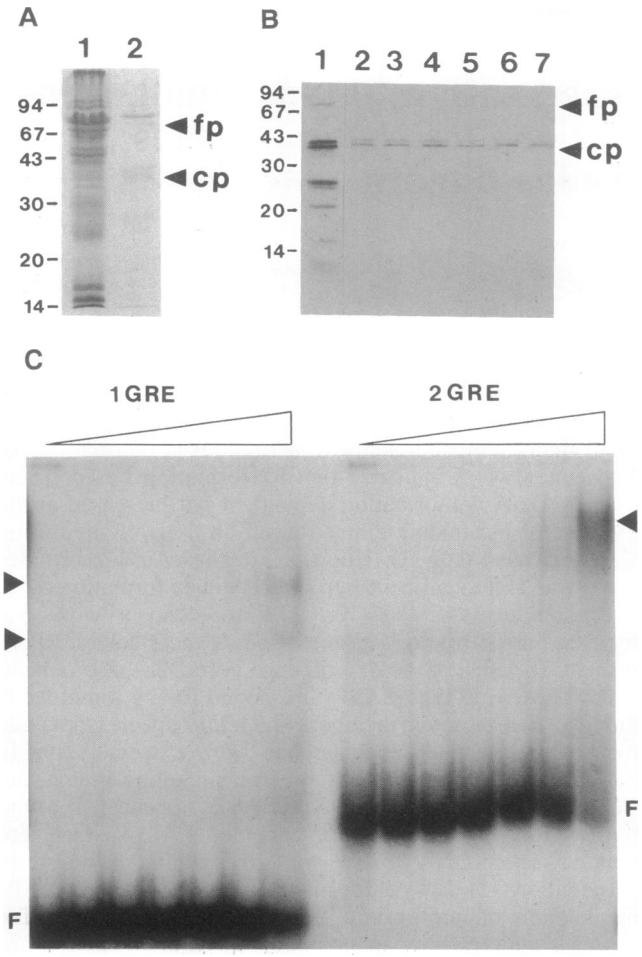


FIG. 4. Cooperative binding by the GR to a double GRE involves direct protein contacts. (*A*) Coomassie blue-stained gel showing the *E. coli* lysate expressing the protein A–τ_1-DBD fusion protein (fp; lane 1) and the eluate from the IgG-Sepharose column containing the cleaved τ_1-DBD protein (cp; lane 2). The migration of molecular size markers (in kDa) is indicated. (*B*) Western blot, probed with antibodies directed against the GR DBD, showing τ_1-derived proteins in the eluate from the IgG-Sepharose column (lane 1) and in the peak τ_1-DBD fractions from the Superose 12 column (lanes 2–7). The arrowheads indicate the protein A–τ_1-DBD fusion protein (fp) and the cleaved τ_1-DBD protein (cp). The migration of molecular size markers (in kDa) is shown. (*C*) Binding by serial dilutions of the purified τ_1-DBD protein to DNA fragments containing one or two GREs. The arrowheads indicate the complexes formed as described for Fig. 2. F, free DNA probe.

cantly when the number of DNA-binding sites is increased to more than two (21). Conversely, GAL4 shows increased synergism with the progressive addition of more than five sites (1).

Although the level of cooperative DNA binding measured *in vitro* is sufficient to account for the level of synergistic transactivation observed *in vivo*, we cannot be sure that the same levels of cooperative DNA binding occur in intact cells. It is possible that interactions between τ_1 transactivation domains cause the formation of a large transactivation surface, containing four τ_1 domains (two × two dimers), which transactivates synergistically via a post-DNA-binding mechanism (as in Fig. 1*C*). Therefore, we do not exclude the possibility that the τ_1 domain may mediate synergistic transactivation at a post-DNA-binding level in addition to the level of cooperative DNA binding.

Most, if not all, the cooperative DNA binding observed here appears to result from direct protein contacts. However, the enhancement of binding to a double GRE was about 2-fold lower for the purified τ_1-DBD protein than for the yeast expression extract. Thus we cannot exclude the possibility that a yeast protein plays a minor role in cooperative binding as a mediator of indirect τ_1 domain interactions. This would be consistent with other observations that the τ_1 domain interacts with yeast proteins, as demonstrated by an *in vivo* squelching assay (22) and by its specific association with some yeast proteins *in vitro* (I. J. McEwan, personal communication). However, addition of yeast proteins to the purified τ_1-DBD protein did not increase the enhancement of DNA binding (A.P.H.W., unpublished results), and therefore we believe that other explanations are more likely. It is possible that such indirect interactions via a second protein (as in Fig. 1*B*) may play a more important role for synergism between the GR and other transcription factors with unrelated structures.

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