Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1209–1213, February 1992
Biochemistry

A negative retinoic acid response element in the rat oxytocin promoter restricts transcriptional stimulation by heterologous transactivation domains

(transcription factor/herpes simplex viral protein VP16/fusion protein)

STEVEN M. LIPKIN*†, CHARLES A. NELSON‡, CHRISTOPHER K. GLASS§, AND MICHAEL G. ROSENFELD*‡

‡Howard Hughes Medical Institute, *Eukaryotic Regulatory Biology Program, †Program in Neuroscience, §Department of Medicine, School of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0648

Communicated by Daniel E. Steinberg, October 14, 1991

ABSTRACT **Retinoic acid receptors are ligand-dependent transcription factors that stimulate gene transcription from promoters containing retinoic acid or thyroid hormone response elements. We describe a high-affinity binding site from the rat oxytocin promoter that mediates negative transcriptional regulation by the retinoic acid receptor. To examine whether strong, constitutive transactivation domains would be capable of stimulating gene transcription when bound to this DNA binding site that normally mediates transcriptional repression, we fused the transactivation domain of the herpes simplex viral protein VP16 to the amino terminus of the retinoic acid receptor and tested the activity of the chimeric protein on the negative retinoic acid response element. This chimeric retinoic acid receptor acted as a strong, constitutive transactivator when bound to promoters containing palindromic thyroid hormone/retinoic acid response elements but surprisingly it still repressed gene transcription when bound to promoters containing the oxytocin-negative retinoic acid response element. These results suggest that a negative DNA binding site itself can inhibit the function of even potent constitutive transactivation domains, and provide evidence that tethering of a constitutive transactivation domain to DNA is insufficient to activate gene transcription.**

Eukaryotic transcription factors possess two essential functional regions, a DNA-binding domain and a transcriptional activation domain. A remarkable feature of transactivation domains is that they are modular and transferable. For example, when amino acid sequences corresponding to the glucocorticoid receptor transactivation domains are fused to the minimal DNA-binding domains of either LexA or GAL4, the resultant fusion proteins are much more effective in stimulating transcription than the LexA or GAL4 DNA-binding domains alone (1–6). These experiments have been interpreted to imply that strong constitutive transactivation domains can function in any context once bound to DNA. In contrast, studies with several classes of transcription factors, including nuclear hormone receptors, have suggested that the nature of the DNA binding site itself can influence the functional activity of a transcription factor. Response elements mediating either positive or negative transcriptional regulation have been described for the glucocorticoid and thyroid hormone receptors (7–13) and the homeodomain protein Ultrabithorax (14, 15). This context-dependent activity of at least some types of transcription factors contrasts greatly with the stimulation of gene transcription by virtually all transactivation domains fused to the GAL4 or LexA DNA-binding domains.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Retinoic acid receptors (RARs) (15–20) are ligand-dependent transcription factors that stimulate gene transcription from promoters containing retinoic acid (RA) and thyroid hormone (triiodothyronine, T_3) response elements (21–23). Here we present evidence that an estrogen response element (ERE) from the rat oxytocin promoter (5′-CCTGAGGCGGTGACCTGACCCCA-3′) binds the RAR with high affinity and functions as a negative retinoic acid response element (oxytocin nRARE). Because numerous experiments have demonstrated that transcriptionally inactive DNA-binding proteins can be made active by the addition of heterologous transactivation domains (1–6), we examined whether a constitutive transactivation domain appended to the RAR could reverse transcriptional inhibition from the nRARE. We therefore fused the most potent known constitutive transcriptional stimulator, from the herpes simplex viral protein VP16 (4, 24), to the RAR amino terminus. Surprisingly, this chimeric receptor failed to activate transcription from promoters containing the oxytocin nRARE, despite binding to this site with high affinity. These results suggest that the nature of a DNA binding site can restrict the function of even potent, constitutive transactivation domains.

MATERIALS AND METHODS

Transfection of CV-1 Cells. CV-1 cells (African green monkey kidney cell line) were transfected as described (13).

Plasmids. To construct RAR–VP16τ, an in-frame ATG codon was ligated to the DNA sequence encoding VP16 residues 401–479, which was then fused to the human RARα *Kpn* I site. For the construction of the RD88 mutant, the cysteine at amino acid 88 was changed to aspartate by mutagenesis. Both the RAR–VP16τ and RD88 mutants were sequenced.

DNA-Binding and Crosslinking Assays. Binding of ^{35}S-labeled RAR was quantitated by the avidin–biotin complex DNA-binding assay (25). Biotinylated oligonucleotides used included the rat oxytocin nRARE (−170 to −158) (5′-CCTGAGGCGGTGACCTTGACCCCA-3′), palindromic T_3/RA response element (5′-CTTTGATCAGGTCAGGTCATGACCTGAC-3′) (13), or RARβ promoter RARE (5′-CTTTGTTCACCGAAAGTTCACCC-3′) (22). Each point represents the mean of triplicate data points. Crosslinking experiments (25) used 1 mM disuccinyl suberimidate. Gel shift experiments used 8 fmol of *in vitro* translated receptor in 24 μl of buffer H (13) plus 500 ng of poly(dI-dC), 250 ng of salmon sperm DNA, 0.5% nonfat dried milk, and 1 μl of CV-1 nuclear extract (25). This mixture was incubated with 0.5 ng of

Abbreviations: RA, retinoic acid; RAR, RA receptor; RARE, RA response element; nRARE, negative RARE; ER, estrogen receptor; ERE, estrogen response element; TK, thymidine kinase; T_3, triiodothyronine.

^{32}P-labeled oxytocin nRARE and 0, 1.5, 5, or 50 ng of unlabeled competitor.

RESULTS

Functional Activity of the RAR and RAR Chimeras on the Oxytocin nRARE. In transient transfections, we have observed that a 1.2-kilobase region of the rat oxytocin promoter directs low-level expression in CV-1 cells. Cotransfection of estrogen receptor (ER) expression construct causes a strong (50- to 80-fold) stimulation of this promoter upon estrogen treatment, consistent with previous studies in other cell lines (26). Interestingly, we observed that RA treatment antagonizes the estrogen-dependent transcriptional stimulation of the oxytocin promoter (data not shown). To determine whether this functional antagonism was mediated through a common DNA binding site, we linked a previously characterized oxytocin ERE (26) to the herpes simplex virus thymidine kinase (TK) promoter in front of a luciferase cDNA and tested functional activity in CV-1 cells by transient transfection assays. When tested on the TK promoter containing one or two copies of the oxytocin nRARE, cotransfected RAR decreased basal promoter activity in an element-dependent manner. Treatment with RA further decreased this basal transcription (Fig. 1). Transfection of ER and addition of estrogen increased transcription from the oxytocin ERE/nRARE constructs, in accord with studies showing this element to be a positive ERE (26). In experiments where both the RAR and the ER were expressed, treatment with RA antagonized the estrogen-dependent transcriptional stimulation (Fig. 1). These results, in concert with studies of the intact oxytocin promoter, indicate that this positive ERE also functions as a nRARE. In the same experiments RAR pos-

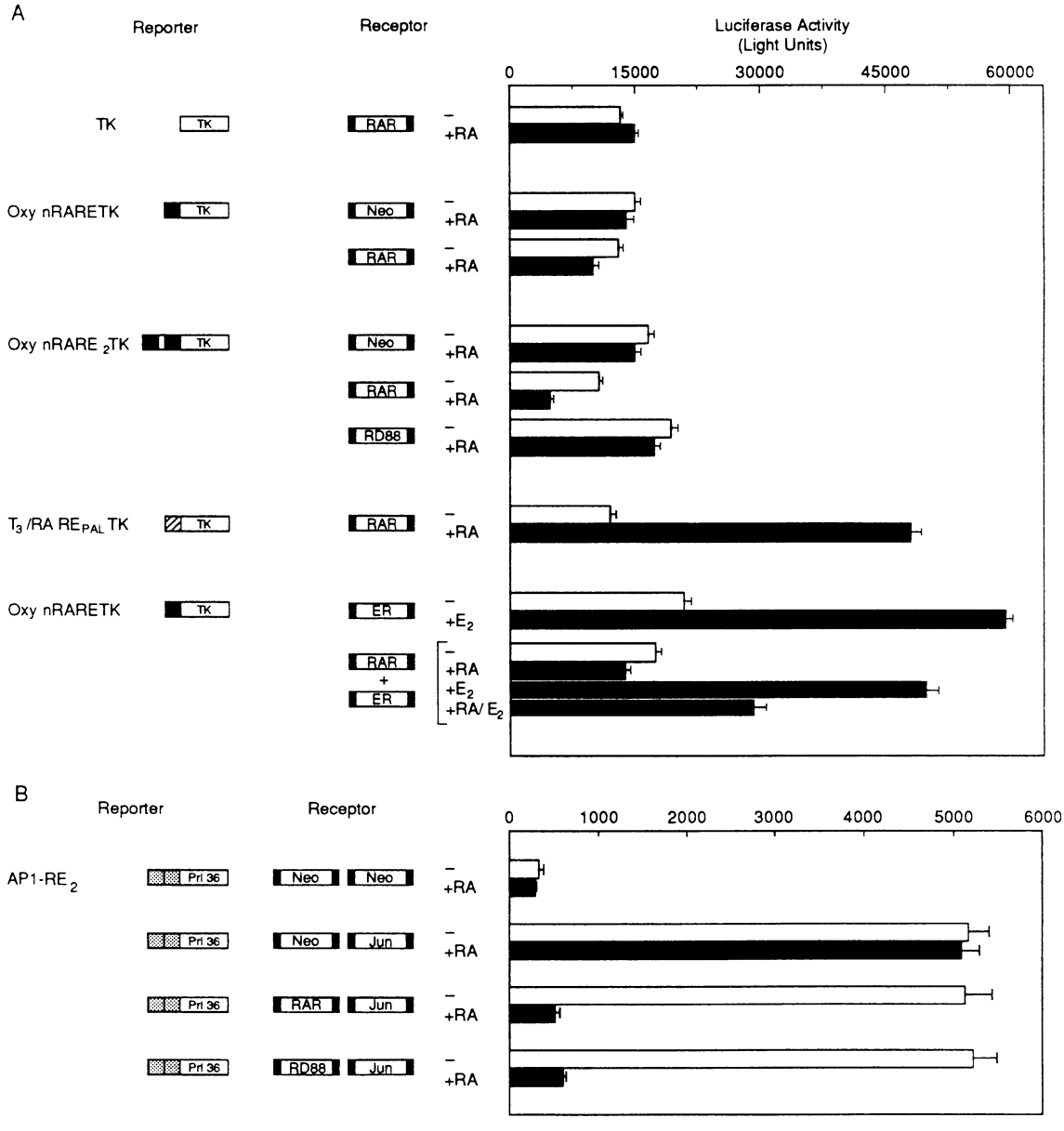

FIG. 1. (*A*) Transcriptional properties of RAR on promoters containing the oxytocin nRARE and the palindromic T_3/RA response element. Cotransfection assays were performed with CV-1 cells. Plasmids containing the Rous sarcoma virus long terminal repeat to direct expression of either the RARα, ER, or neomycin-resistance (Neo) gene were cotransfected with reporter plasmids containing firefly luciferase cDNA under the control of the TK promoter "linkered" to one or two copies of the oxytocin (Oxy) nRARE or the palindromic T_3/RA response element (T_3/RA RE_{PAL}). Luciferase activity was assayed in lysates of transfectants that had been incubated with or without RA (1 μM) and/or estradiol (E_2, 0.1 μM); activity is expressed as arbitrary light units. Each bar represents the average of five separate experiments, with SEM indicated. RD88, RAR with the first cysteine of the first zinc finger (amino acid 88) mutated to aspartate; this mutant is incapable of DNA binding. (*B*) Transcriptional properties of RAR and RD88 on promoters containing AP-1 response elements. Prl, minimal prolactin promoter.

itively regulated the TK promoter linked to palindromic T_3/RA response elements (Fig. 1). Mutation of the first conserved cysteine of the DNA-binding domain to aspartate (RD88) abolished the ability of the RAR to act positively on a T_3/RA response element and negatively on the oxytocin nRARE (Fig. 1 and data not shown), indicating that both effects required DNA binding.

The identification of an element on which the RAR was a negative transcriptional regulator permitted us to investigate whether fusion of a strong, constitutive transactivation domain to the RAR would create a chimeric receptor that was capable of stimulating gene transcription when bound to the nRARE. Accordingly, we created a fusion protein in which the potent transactivation domain (amino acids 401–479) from herpes simplex viral protein VP16 was joined to the amino terminus of the RAR (see Fig. 4). The transcriptional activity of this chimeric receptor (RAR–VP16τ) was tested functionally in transient transfections (Fig. 2). When tested on the positive T_3/RA palindromic response element, the heterologous VP16 transactivation domain fused to the RAR amino terminus stimulated transcription even in the absence of RA, increasing basal activity almost 20-fold from the TK promoter containing two copies of this element. This result suggests that the RAR can bind to response-element sequences *in vivo* in the absence of ligand. The RAR–VP16τ chimera retained the ability to be induced by hormone even in the presence of the constitutive VP16 transactivation domain, as treatment with RA further stimulated RAR-VP16τ activity. Thus, the constitutive VP16 transactivation domain and the RAR endogenous ligand-inducible transactivation domain(s) appeared to stimulate luciferase expression independently of each other, in a nonsynergistic manner. These results are in accord with previous studies demonstrating that fusion of the VP16 transactivation domain to a DNA-binding protein augments the ability of that protein to stimulate transcription from positive response elements (1–6). When tested on promoters containing the oxytocin nRARE, transfection of RAR–VP16τ in the absence of ligand surprisingly repressed transcription to below basal levels (Fig. 2). Treatment with RA further decreased transcription from this element. These transcriptional effects were more pronounced on promoters containing two copies of the oxytocin nRARE (Fig. 2). Thus, in all cases examined with the oxytocin nRARE, the repressive activity of the RAR was able to manifest itself despite the presence of the VP16 transactivation domain, in marked contrast to the dramatic positive activity of the VP16 transactivation domain on the palindromic T_3/RA response element (Fig. 2).

***In Vitro* Binding of RAR and RAR–VP16τ to the Oxytocin nRARE.** The DNA-binding properties of the RAR and the RAR–VP16τ fusion protein on the oxytocin nRARE were defined and compared. Because the RAR can interact with other nuclear factors on response elements (25), binding experiments were performed in the presence of CV-1 nuclear extract to mimic *in vivo* conditions more faithfully. In a gel shift DNA-binding assay, *in vitro* translated RAR or RAR–VP16τ mixed with CV-1 nuclear extract caused the appearance of two major DNA–protein complexes when incubated with ^{32}P-labeled oxytocin nRARE (Fig. 3) or palindromic T_3/RA response element (data not shown) DNA probes. Addition of an antiserum raised against a peptide corresponding to the carboxyl-terminal 20 amino acids of the RAR decreased the mobility of both major bands, indicating that both contained RAR (data not shown). Competition experiments demonstrated that both RAR and RAR–VP16τ bound to the oxytocin nRARE with a higher affinity than to the positive palindromic T_3/RA response element (Fig. 3). The relative binding affinity for the oxytocin nRARE was comparable with that for the RARβ promoter RARE. Unrelated elements, such as a cis-active element from the adenovirus 5 genome that does not bind the RAR, did not compete (data not shown). In all cases examined, RAR and RAR–VP16τ exhibited identical binding characteristics.

The binding affinities of RAR and RAR–VP16τ for the oxytocin nRARE, T_3/RA palindromic response element, and RARβ promoter RARE were quantitated by an avidin–biotin complex DNA-binding assay. ^{35}S-labeled *in vitro* translated RAR and RAR–VP16τ were incubated with biotinylated oligonucleotide probes in the presence of CV-1 nuclear proteins, and the resultant DNA–protein complexes were assayed by streptavidin-agarose precipitation (13). Scatchard analysis indicated that both receptors bound with high affinity to the oxytocin nRARE (Fig. 4). In all cases examined

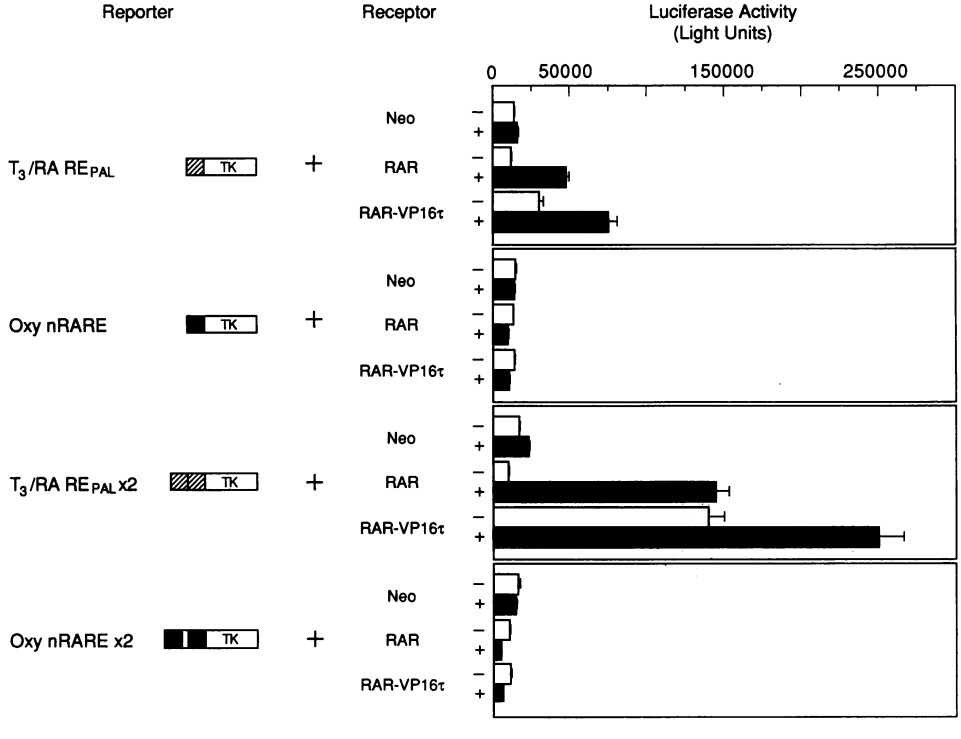

FIG. 2. Transcriptional properties of RAR and RAR–VP16τ chimera on promoters containing the oxytocin nRARE and the palindromic T_3/RA response element. Transfection experiments were performed as described in Fig. 1. Cells were incubated without (−, open bars) or with (±, filled bars) 1 μM RA. RAR–VP16τ, VP16 transactivation domain/RAR fusion protein. Results represent the average ± SEM of triplicate determinations in four separate experiments.

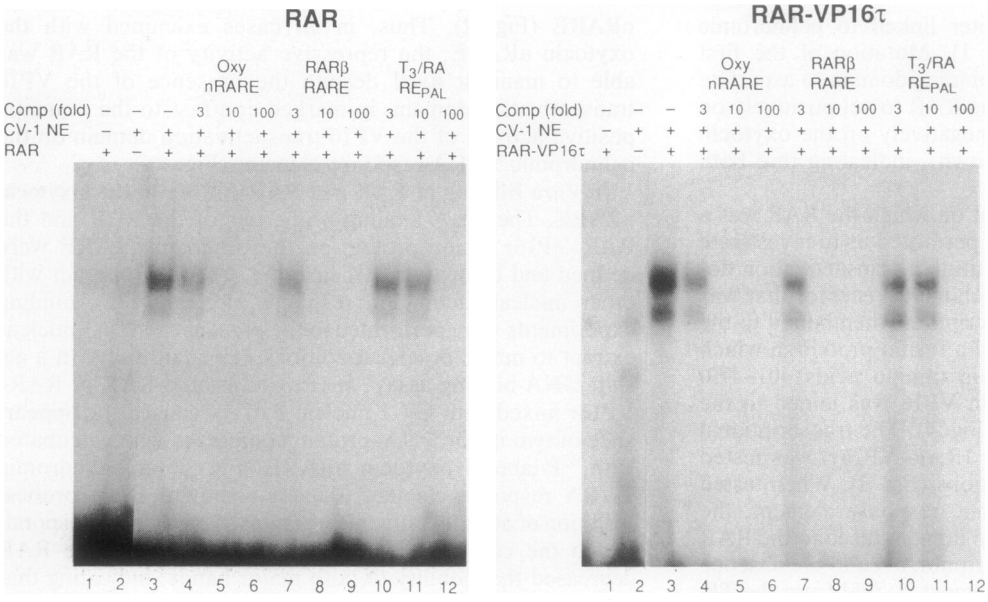

FIG. 3. Electrophoretic mobility-shift analysis of the RAR and the chimeric VP16 transactivation domain/RAR fusion protein on the oxytocin nRARE. *In vitro* translated RAR or RAR–VP16τ protein alone (lane 1) or mixed with CV-1 nuclear extract (lanes 3–12) was bound to a radioactive DNA probe containing the oxytocin nRARE. The ability of a 3-, 10-, or 100-fold excess of unlabeled oxytocin (Oxy) nRARE, RARβ RARE, or palindromic T_3/RA response element (T_3/RA RE_{PAL}) to compete was tested.

with this binding assay, RAR and RAR–VP16τ exhibited identical binding characteristics.

Complex Formation with Nuclear Factors on the Oxytocin nRARE and Palindromic T_3/RA Response Element. The RAR can form heterodimers with other nuclear factors (13). Crosslinking experiments were therefore performed to determine whether complexes with different nuclear factors were being formed on the palindromic T_3/RA response element and the oxytocin nRARE that might account for the qualitative difference in RAR transcriptional activity. After incubation of radiolabeled RAR or RAR–VP16τ with CV-1 nuclear extract and either the palindromic T_3/RA response element or the oxytocin nRARE, the bifunctional crosslinker disuccinyl suberimidate was added (Fig. 5). Based on the migration of crosslinked species at 95 and 107 kDa for the RAR (51 kDa), and at 108 and 119 kDa for RAR–VP16τ (64 kDa) on both the palindromic T_3/RA response element and the oxytocin nRARE, it appeared that similar or identical nuclear factors interacted with both RAR and RAR–VP16τ, irrespective of binding to a positive (palindromic T_3/RA response element) or negative (nRARE) site. The observation of two crosslinked products is consistent with the appearance of two complexes containing the RAR in gel shift experiments. We interpret this result to indicate that the RAR can form heterodimers with two distinct CV-1 nuclear proteins, of ≈44 and ≈56 kDa. These results suggest that the same complexes containing RAR and CV-1 nuclear factors form on both the palindromic T_3/RA response element and the oxytocin nRARE and that RAR–VP16τ (64 kDa) can complex with the same CV-1 nuclear factors in an identical manner.

CONCLUSIONS

This paper describes a high-affinity nRARE in the rat oxytocin promoter. This element contains in its sequence a previously described positive ERE (26), and indeed, transfection experiments have shown that cotransfection of RAR antagonizes estrogen-dependent stimulation of transcription from both the native oxytocin promoter and heterologous promoters containing the oxytocin element (Fig. 1 and data not shown). The observation that the RAR exhibited qualitatively different transcriptional activities on the palindromic T_3/RA response element and the oxytocin nRARE permitted us to examine a basic issue concerning the actions of transactivation domains: the activity of a constitutive, acidic

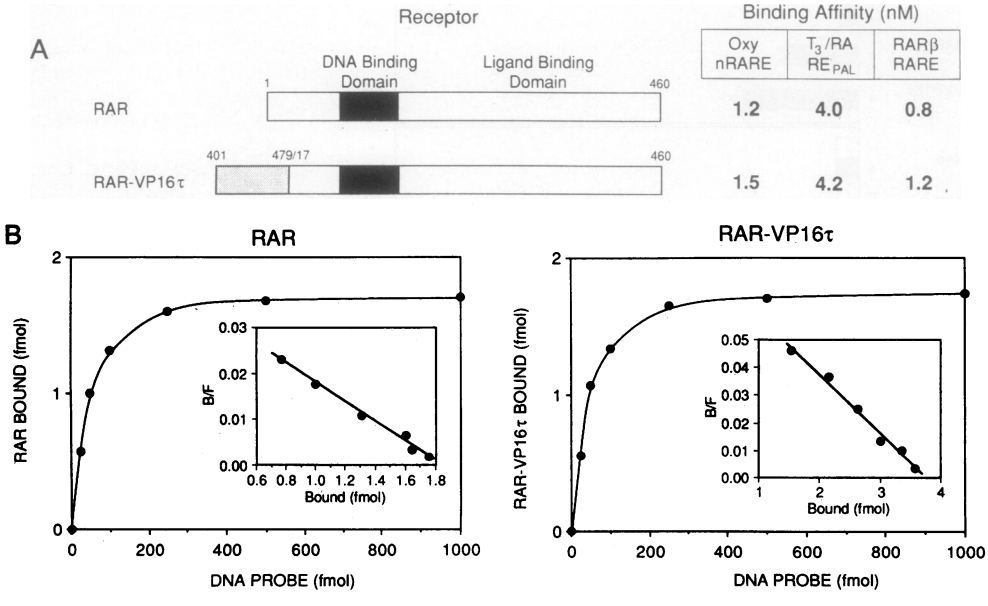

FIG. 4. Determination of binding affinity constants for the RAR and the chimeric VP16 transactivation domain/RAR fusion protein on positive and negative response elements. (*A*) Summary of binding affinity constants of RAR and RAR–VP16τ for the oxytocin (Oxy) nRARE, palindromic T_3/RA response elements (T_3/RA RE_{PAL}), and RARβ RARE as determined by Scatchard analyses using the avidin–biotin complex DNA-binding assay. (*B*) Binding of RAR and RAR–VP16τ to biotinylated DNA probes at various concentrations. DNA–protein complexes were precipitated and the quantity of bound receptor was calculated from the specific activity of [^{35}S]methionine. Each point represents the mean of triplicate determinations. (*Insets*) Scatchard plots of the same experiments. B/F, bound/free. Affinity constants are shown in *A*.

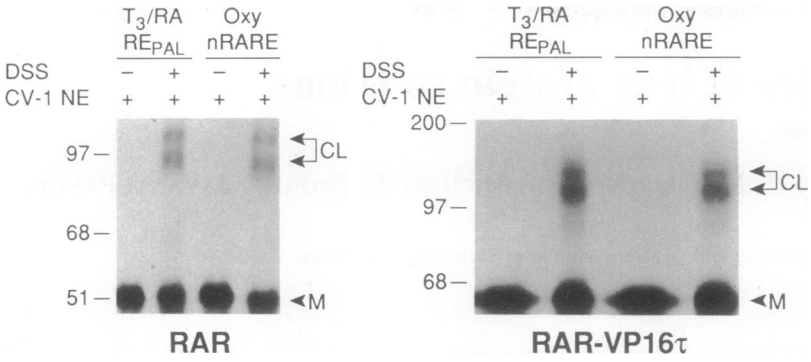

FIG. 5. Crosslinking of RAR and RAR–VP16τ to CV-1 coregulators. Radiolabeled RAR (*Left*) or RAR–VP16τ (*Right*) was incubated with CV-1 nuclear extract. Biotinylated palindromic T_3/RA response element (RE_{PAL}) or oxytocin (Oxy) nRARE probe was added, and DNA–protein complexes were precipitated with streptavidin-agarose. DNA–protein complexes were incubated with or without the crosslinker disuccinyl suberimidate (DSS). Crosslinked products were analyzed by SDS/PAGE. Numbers indicate molecular size markers (kDa). The migration positions of receptor monomers (M) and crosslinked products (CL) are indicated. Similar results were obtained in numerous additional experiments of similar design.

transactivation domain bound to a site conferring negative transcriptional control. We therefore fused the strongest known constitutive transactivation domain, from VP16, to the amino terminus of the RAR and tested its activity on a nRARE. In two independent DNA-binding assays, a gel shift assay and the avidin–biotin complex DNA-binding assay, the RAR–VP16τ chimera exhibited higher-affinity binding on the oxytocin nRARE than on the palindromic T_3/RA response element (Figs. 3 and 4). When tested functionally, the VP16 transactivation domain fused to the RAR displayed considerable specificity in its ability to stimulate transcription, functioning potently on the palindromic T_3/RA response element but not on the oxytocin nRARE. When tested functionally on the RARβ promoter RARE, RAR–VP16τ also increased basal transcription, although not as dramatically as observed on the palindromic T_3/RA response element (data not shown). Recently, it has been shown that Jun/Fos can antagonize RA-mediated transcriptional stimulation (27). However, for several reasons an interaction between RAR and Jun/Fos appears unlikely to explain the RA-mediated repression through the oxytocin nRARE. (*i*) In transient transfection, a RAR mutant incapable of DNA binding (RD88) can antagonize a Jun/Fos-dependent induction of a reporter construct containing multiple AP-1 response elements, while having no repressive effect on the oxytocin nRARE (Fig. 1*B* and unpublished observations). (*ii*) Under our transfection conditions, the oxytocin nRARE/ERE is not a positive AP-1 response element. (*iii*) In gel shift analyses, addition of c-Jun to RAR/CV-1 nuclear protein complexes abolishes binding to both the palindromic T_3/RA response element and the oxytocin nRARE (data not shown). Since we find no evidence for differences in the composition of RAR/CV-1 nuclear protein complexes on positively vs. negatively regulated binding sites, interactions with c-Jun cannot account for the differential regulation by RAR on the palindromic T_3/RA response element and the oxytocin nRARE. Our results are consistent with the possibility that the DNA recognition element can dictate whether bound transactivation domains assume active or inactive conformations, as has been suggested to occur for the yeast pheromone receptor transcription factor and T_3 receptor (13, 28). The most surprising aspect of our studies is that even constitutive transactivation domains can be constrained by the DNA binding sites to which the receptor is bound. Recent studies using mutagenesis suggest that, in addition to overall negative charge, specific structural elements are crucial to the ability of the VP16 transactivation domain to stimulate transcription (29). We propose that different protein–protein and protein–DNA interactions impose differences in the configurations of the RAR on the palindromic T_3/RA response element or oxytocin nRARE, respectively. This conformational alteration is proposed to differentially dictate activity of endogenous RAR and exogenously fused VP16, with resultant positive or negative regulation of transcription. A precise understanding of these conformational alterations should provide further insights into the molecular mechanisms by which transactivation domains exert their functions.

S.M.L. is supported by a predoctoral training grant to the Medical Scientist Training Program, University of California, San Diego. C.K.G. is the recipient of a Lucille Markey Scholarship for Biomedical Science. M.G.R. is an investigator with the Howard Hughes Medical Institute. These studies were supported by grants from the American Cancer Society and the National Institutes of Health.

1. Brent, R. & Ptashne, M. (1985) *Cell* **43**, 729–733.
2. Godowski, P., Picard, D. & Yamamoto, K. (1988) *Science* **241**, 812–816.
3. Hollenberg, S. & Evans, R. M. (1989) *Cell* **55**, 899–903.
4. Ptashne, M. (1988) *Nature (London)* **333**, 683–689.
5. Sadowski, I., Ma, J., Triezenberg, S. & Ptashne, M. (1988) *Nature (London)* **335**, 563–564.
6. Struhl, K. (1989) *Trends Biochem. Sci.* **6**, 137–140.
7. Akerblom, I., Slater, F., Beato, M., Baxter, J. D. & Mellon, P. L. (1988) *Science* **241**, 350–353.
8. Charron, J. & Drouin, J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8903–8907.
9. Diamond, M., Miner, J., Yoshinaga, S. & Yamamoto, K. (1990) *Science* **249**, 1266–1272.
10. Schule, R., Rangarajan, P., Kliewer, S., Ransone, L., Bolada, J., Yang, N., Verma, I. & Evans, R. (1990) *Cell* **62**, 1217–1226.
11. Yang-Yen, H., Chambard, J., Sun, Y., Smeal, T., Schmidt, T., Drouin, J. & Karin, M. (1990) *Cell* **62**, 1205–1215.
12. Jonat, C., Rahmsdorf, H., Park, K., Cato, A., Gebel, S., Ponta, H. & Herrlich, P. (1990) *Cell* **62**, 1189–1204.
13. Glass, C. K., Holloway, J. M., Devary, O. V. & Rosenfeld, M. G. (1988) *Cell* **54**, 313–323.
14. Jaynes, J. B. & O'Farrell, P. H. (1988) *Nature (London)* **336**, 744–749.
15. Robertson, M. (1987) *Nature (London)* **327**, 556–557.
16. Giguere, V., Ong, E. S., Segui, P. & Evans, R. M. (1987) *Nature (London)* **330**, 624–629.
17. Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. (1987) *Nature (London)* **330**, 444–450.
18. Brand, N., Petkovich, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, P. & Dejean, A. (1988) *Nature (London)* **333**, 669–672.
19. Benbrook, D., Lernhardt, E. & Pfahl, M. (1988) *Nature (London)* **333**, 669–671.
20. Zelent, A., Krust, A., Petkovich, M., Kastner, Ph. & Chambon, P. (1989) *Nature (London)* **339**, 414–417.
21. Umesono, K., Giguere, V., Glass, C. K., Rosenfeld, M. G. & Evans, R. (1988) *Nature (London)* **335**, 262–265.
22. de The, H., Cicanco-Ruiz, M., Tiollais, P., Stunnenber, H. & Dejean, A. (1990) *Nature (London)* **343**, 177–180.
23. Vasios, G., Gold, M., Petkovich, M., Chambon, P. & Gudas, L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9099–9103.
24. Pellett, P., McKnight, J., Jenkins, F. & Roizman, B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5870–5874.
25. Glass, C. K., Lipkin, S. M., Devary, O. D. & Rosenfeld, M. G. (1989) *Cell* **59**, 697–708.
26. Richard, P. & Zingg, H. (1990) *J. Biol. Chem.* **265**, 6098–6103.
27. Schule, R., Rangarajan, P., Yang, N., Kliewer, S., Ransone, L. J., Bolado, J., Verma, I. M. & Evans, R. M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6092–6096.
28. Tan, S. & Richmond, T. (1990) *Cell* **62**, 367–377.
29. Cress, W. & Triezenberg, S. (1991) *Science* **251**, 87–90.