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THE USE OF A DNA-BINDING DOMAIN REPLACEMENT METHOD FOR THE DETECTION OF A POTENTIAL TR3 ORPHAN RECEPTOR RESPONSE ELEMENT IN THE MOUSE MAMMARY TUMOR VIRUS LONG TERMINAL REPEAT

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Summary. TR3 orphan receptor is a human homologue of the mouse nur77, N10 and rat NGFI-B, TIS1 genes which may represent an early response gene involved in the control of cell proliferation. We have studied potential target genes for TR3 orphan receptor using the DNA-binding domain replacement method. We found that mouse mammary tumor virus long terminal repeat-linked chloramphenicol acetyltransferase expression can be activated in transfected cells by a chimeric androgen receptor/TR3 orphan receptor/androgen receptor construct (AR/TR3/AR) in the presence of androgen. By deletion analysis, a region with 20 nucleotides in length between positions -1178 and -1159 of the mouse mammary tumor virus long terminal repeat was confirmed as a potential TR3 orphan receptor response element. These results suggest the feasibility of using the DNA-binding domain replacement method to detect target sequences of orphan receptors.

Both TR2 and TR3 orphan receptors are proteins of unknown function which possess sequence homology to the family of nuclear receptor transcriptional factors (1-6). TR3 orphan receptor is homologous to several immediate early genes such as the mouse nur77 (7), N10 (8) and rat NGFI-B (9), TIS1 (10) genes which are rapidly and transiently induced in cultured cells by the addition of serum growth factors. Recently, Nakai *et al.* have isolated a cDNA clone, NAK1, from human fetal muscle library which corresponds to TR3 orphan receptor (11). By constructing expression vectors containing chimeric human androgen receptor (AR) cDNAs (12,13) with the AR DNA-binding domain replaced by the DNA-binding domain of either TR2 or TR3 orphan receptor (designated as AR/TR2/AR and AR/TR3/AR), a search can be conducted the specificity of hormone response element (HRE) to each receptor using androgen as the ligand to activate transcription. This approach bypasses the need for knowledge of the natural ligands for each receptor. In initial experiments reported here, we examined whether TR2 or TR3 orphan receptor

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Abbreviations: AR, androgen receptor; HRE, hormone response element; MMTV LTR, mouse mammary tumor virus long terminal repeat; CAT, chloramphenicol acetyltransferase; TR3RE, TR3 response element; DHT, 5α-dihydrotestosterone; R1881, 17α-methyl-17β-hydroxy-estra-4,9,11-trien-3-one.

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response elements were present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) by measuring androgen-induced chloramphenicol acetyltransferase (CAT) expression in cells co-transfected with MMTV-CAT reporter plasmids and chimeric AR expression vectors.

MATERIALS AND METHODS

Materials. [^{14}C]-labeled compound was purchased from New England Nuclear. Restriction and other enzymes were from Boehringer Mannheim, New England BioLabs and BRL. pLC1 and pMPC1 plasmids were generously provided by Drs. Peterson and Kuo.

Site-directed mutagenesis and plasmid construction. Human AR, TR2-11 orphan receptor and TR3 orphan receptor were inserted into M13mp19 for site-directed mutagenesis (14,15). The DNA-binding domains of mutagenized TR2 and TR3 orphan receptors were ligated into AR to produce the AR/TR2/AR and AR/TR3/AR chimeric receptors, respectively. Chimeric constructs were excised from M13mp19 and inserted into pSG5 expression vector (16). Site-directed mutagenesis methods were similarly used to produce deletion mutants in the MMTV LTR. All chimeric constructs and deletion mutants were sequenced by the dideoxynucleotide chain termination method (17) as a final verification.

A pair of complimentary oligonucleotides was synthesized according to the search of the HRE consensus sequence between positions -1178 and -1159 in the MMTV LTR. They were annealed and cloned into the *Bgl* II site of the pCAT-Promoter plasmid (Promega). Several plasmids containing one to four copies of potential TR3 response element (TR3RE) were confirmed by sequencing.

Cell transfection. PC-3 human prostatic carcinoma cells (18) were used for the transfection assay as described previously (19). pMSG-CAT plasmid (Pharmacia) contains MMTV LTR-linked CAT gene. All CAT assays were normalized for the transfection efficiency by β-galactosidase activity. The quantitation of CAT assay was performed by PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

Trans-activation studies: Activation of MMTV LTR-linked CAT expression by chimeric AR/TR3/AR protein. Chimeric AR cDNAs were constructed by introducing unique *Sac* II or *Hpa* I and *Xho* I restriction sites at the termini of the DNA-binding domains by site-directed mutagenesis and replacing the AR DNA-binding domain with either TR2 or TR3 orphan receptor DNA-binding domain (Fig. 1a). pSG5 constructs expressing intact AR, AR/TR2/AR, or AR/TR3/AR were co-transfected into PC-3 cells with pMSG-CAT, and the transfectants were treated with increasing concentrations of 5α-dihydrotestosterone (DHT). The PC-3 cell line, derived from a human prostatic carcinoma (18), is androgen-independent and expresses little endogenous AR. Both intact AR and AR/TR3/AR, but not AR/TR2/AR, were able to activate CAT expression in the presence of DHT in a dose-dependent manner (Fig. 1b). The level of AR/TR3/AR activation was lower than that of intact AR, however. Based on the percent conversion of chloramphenicol to the monoacetylated products, AR/TR3/AR had 17% and 28% of the activity of intact AR at 0.1 and 1 nM DHT, respectively. Higher concentrations of DHT did not significantly increase the percent conversion in cells transfected with AR/TR3/AR or intact AR (data not shown). Cells co-transfected with the parent plasmid pSG5 and pMSG-CAT were not sensitive to DHT treatment (lanes 1-4, Fig. 1b), indicating that endogenous AR activity was absent or negligible. CAT activity was not stimulated in cells co-transfected with plasmids expressing intact TR2-11 or TR3 orphan receptor under control of the SV40 early promoter in the presence or absence of DHT treatment (lanes 17,18). This implicates that the *trans*-activation capability of TR3

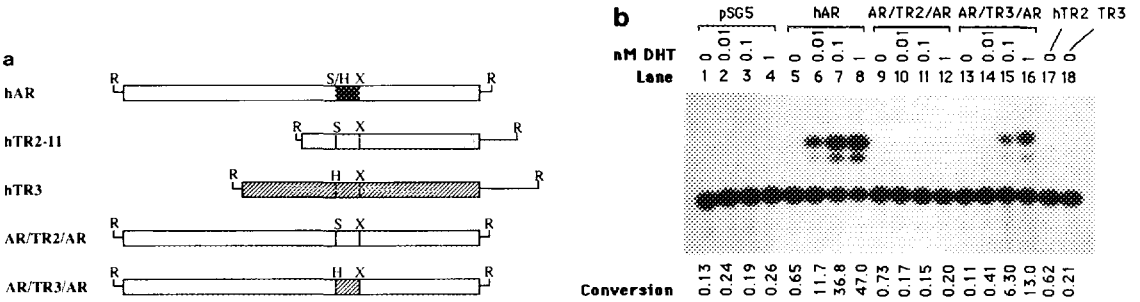

Fig. 1. (a) Schematic structure of the human androgen receptor (AR), TR2 orphan receptor, TR3 orphan receptor and chimeric cDNA constructs. The rectangles represent the coding regions for the intact receptors in the cDNA clones. The DNA-binding domain of the AR (solid box) is replaced with the DNA-binding domain of either the TR2 or TR3 orphan receptor (stippled boxes) in the chimera. H = *Hpa* I, R = *Eco* RI, S = *Sac* II, X = *Xho* I. (b) Induction of CAT expression by DHT in PC-3 cells co-transfected with either pSG5 (lanes 1-4), pSG5-hAR (lanes 5-8), pSG5-AR/TR2/AR (lanes 9-12), pSG5-AR/TR3/AR (lanes 13-16), pSG5-hTR2-11 (lane 17), or pSG5-TR3 (lane 18). Concentrations of DHT and percent conversion of [^{14}C]-chloramphenicol to monacetylated products are indicated.

orphan receptor at this site was too weak to be detected. In addition, mutagenized AR constructs which encoded proteins with two additional amino acid residues (Pro-Val or Val-Asp) and three additional residues (Ala-Arg-Glu) at the N- and C-termini of the DNA-binding domains did not differ significantly in *trans*-activation from non-mutagenized AR (data not shown).

Localization of the potential TR3 orphan receptor response element in the MMTV LTR. To determine whether the chimeric AR/TR3/AR protein binds to MMTV LTR, MMTV-CAT mutants lacking successively larger regions of the MMTV LTR from the 5' terminus were used in co-transfection experiments (Fig. 2a). Transfected AR activated CAT expression in the presence of 1 nM DHT from pLC1 and every deletion mutant except pMPC1 and pMPC1-HR, as expected, because of the presence of the HRE (Fig. 2b, lanes 1-8). CAT expression from pLC1-450 (lane 5) was over 5 folds greater than that from the parent pLC1 plasmid, suggesting the presence of a negative control element in the -600 to -450 bp segment of the MMTV LTR. Increased expression, however, was not obtained by the pLC1-225 mutant, perhaps the result of the deletion of a positively-acting element in the -450 to -225 bp region of the LTR. Higher basal CAT activity was reproducibly obtained from pLC1-450 when transfected alone or with parent pSG5 plasmid (data not shown). Transfected AR/TR3/AR activated CAT expression only from the full-length MMTV LTR present in pLC1 (lane 9, Fig. 2b), but not from deletion mutants (lanes 10-15). This result suggests that the MMTV LTR site at which the AR/TR3/AR protein binds is located within the -1200 to -1000 bp segment, and is distinct from the HRE where glucocorticoid, progesterone, mineralocorticoid and androgen receptors bind in the -192 to -71 bp region (20-22).

The use of the MMTV LTR deletion mutants also revealed the presence of what appear to be negatively and positively acting elements in the segments from -600 to -450 and -450 to -225, respectively. Previously, Morley *et al.* (23) had identified a negative element in a 91 bp *Sau* 3A/*Rsa* I fragment which is present in the -450 to -225 segment in plasmid pLC1-450. The reason for the discrepancy between the present results and those of Morley *et al.* is unclear so far. Morely

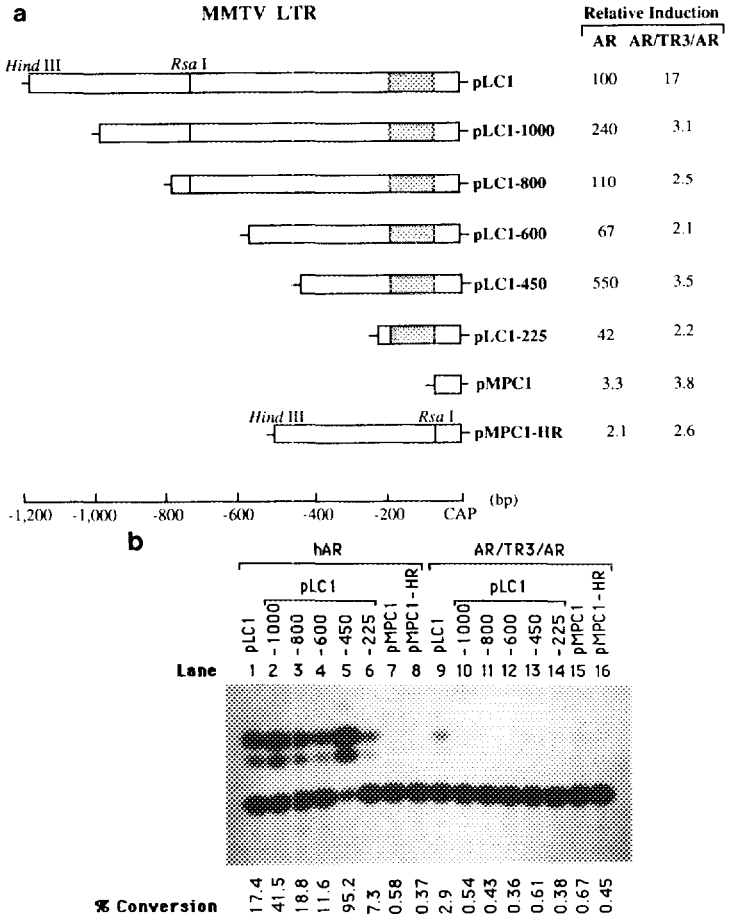

Fig. 2. (a) pLC1 MMTV LTR deletion mutants. The stippled box represents the position of the HRE. Relative induction data were derived from percent conversion data from (b), with the value for pLC1 co-transfected with pSG5-hAR = 100. (b) Induction of CAT expression in PC-3 cells co-transfected with 10 μg of either pSG5-hAR (lanes 1-8) or pSG5-AR/TR3/AR (lanes 9-16) and 10 μg of pLC1 MMTV LTR deletion mutants. Percent conversion of [^{14}C]-chloramphenicol to monacetylated products is indicated.

et al. used endogenous glucocorticoid receptor in mouse Ltk$^-$ cells to induce MMTV-CAT expression. Therefore, it is possible that transcription factors which bind to positive or negative elements in the MMTV LTR interact with nuclear receptors such as AR and glucocorticoid receptor in ways specific to each receptor. In addition, the levels or ratios of accessory transcription factors may differ between human PC-3 and mouse L cells.

To further localize TR3RE in the MMTV LTR precisely, we constructed the pTR3RE-CAT plasmid which contains the potential TR3RE and performed CAT assay. Three amino acid residues in the DNA-binding domain of TR3 orphan receptor, EGckG, which may determine target DNA-binding specificity, and place TR3 orphan receptor in the group II class of receptors (24). The DNA target sequence may be similar to the HRE consensus of this class: AGGTCA---TGACCT (24). Recently, Wilson *et al.* have identified the DNA binding site (5'-AAAGGTCA-3')

for the rat NGFI-B protein which contains a half-site of the HRE (25,26). A search of the MMTV LTR sequence for related HRE consensus sequences revealed that, although no perfect matches are present, a region with 20 nucleotides in length between positions -1178 and -1159 matches 8 out of 12 nucleotides (5'-AGcagAaatggtTGAaCT-3') in pLC1. This site was deleted in all deletion mutants which did not support CAT expression in AR/TR3/AR-transfected cells. We, therefore, synthesized a pair of complimentary oligonucleotides (-1159 to -1178) covered this potential TR3RE and cloned into pCAT-Promoter plasmid (Fig. 3a). These pTR3RE-CAT plasmids were confirmed to contain one to four copies of potential TR3RE by sequencing.

The chimeric AR/TR3/AR plasmid was transiently expressed in PC-3 cell line together with p(TR3RE)4-CAT or parent pCAT-Promoter plasmid in the presence or absence of 1 nM R1881(a synthetic androgen). Androgen can trigger near 2 folds induction in CAT activity when cells were co-transfected with chimeric AR/TR3/AR and p(TR3RE)4-CAT (Fig. 3b). By contrast, androgen stimulation of CAT activity was slightly detected while PC-3 cells were co-transfected with

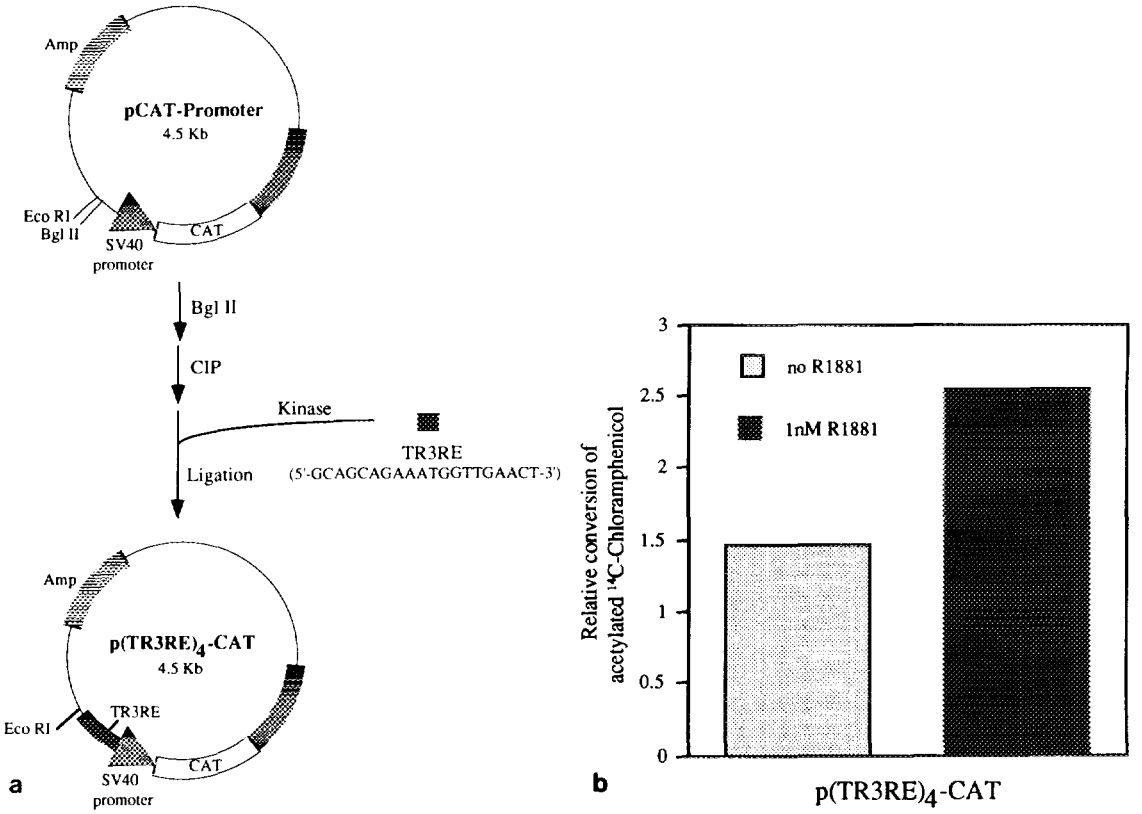

Fig. 3. (a) Schematic structure of the reporter plasmid containing the potential TR3RE in pCAT-Promoter plasmid. One to four copies of the potential TR3RE were cloned into pCAT-Promoter plasmid at *Bgl* II site before SV40 promoter. p(TR3RE)4-CAT plasmid contains 4 copies of TR3RE in the parent pCAT-Promoter vector. (b) Induction of CAT activity in PC-3 cells co-transfected with chimeric AR/TR3/AR and p(TR3RE)4-CAT plasmids in the presence or absence of 1 nM R1881. All CAT assays were normalized for the level of β-galactosidase activity. Each value represents the mean of four independent experiments.

chimeric AR/TR3/AR as well as parent pCAT-Promoter plasmid in the presence or absence of androgen (data not shown). These results demonstrated that the sequence with 20 nucleotides between positions -1178 and -1159 of the MMTV LTR is a potential TR3 orphan receptor response element. The imperfect match of TR3RE may account for the weakness of the induction observed when compared with entire MMTV LTR fragment (1-1,200 bp) induced by AR/TR3/AR chimeric receptor. More recently, the study of androgen response element (ARE) in the probasin gene indicated that other sequences may be required for the fulfillment of full CAT function (27). In addition, steroid receptors such as AR may bind at multiple sites within the MMTV HRE, thus increasing the *trans*-activation effect. When we expand this region (-1,200 to -1,000 bp) to (-1,200 to-750 bp)into plasmid pMPC1-HR androgen, however, was not able to induce the CAT activity (Fig 2b, lane 16). This may suggest that there may have a negative control element in the -1,000 to -750 bp segment of the MMTV LTR.

Whether the TR3RE is functional *in vivo* in the MMTV LTR or its incidental presence is, therefore, unclear. The present results, however, point out the feasibility of using the DNA-binding domain replacement method (28) to detect target sequences of DNA-binding proteins, and identify ligands for unknown receptors such as COUP-TF (29). Currently we are using these chimeric plasmids AR/TR3/AR and AR/TR2/AR as well as subtractive library technique (30) to identify the target genes of TR2 and TR3 orphan receptors. We are also using chimeric constructions with the DNA-binding domains of TR2 and TR3 orphan receptors replaced by AR DNA-binding domain (designated as TR2/AR/TR2 and TR3/AR/TR3) to detect the existence of natural ligands of TR2 and TR3 orphan receptors. The success of these projects could shed a great deal of light on the regulatory mechanisms which operate, possibly in a cascade-like fashion, to control cell growth in the androgen-sensitive prostate and perhaps cell growth in general.

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