Identification of Functional Positive and Negative Thyroid Hormone-Responsive Elements in the Rat Apolipoprotein AI Promoter†

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ABSTRACT: Transcription of the antiatherogenic protein apolipoprotein AI is regulated by the thyroid hormone, L-triiodothyronine. Transient transfection and electrophoretic mobility shift assays were used to identify the *cis*-acting elements involved. In transient transfection assays, hormone bound to either thyroid hormone receptor α or *β* exerts a positive effect through a thyroid hormone response element, site A (−208 to −193). In the absence of site A, liganded receptor α or *β* have a negative effect on promoter activity. This negative effect is mediated by a 40 bp fragment spanning nucleotides −46 to −7. Closer examination of this region of the gene shows there to be a negative thyroid hormone response element at position −25 to −20 which is fused to the 3′ end of the TATA element. Electrophoretic mobility shift assays show that bacterially expressed chicken or rat thyroid hormone receptor α1 binds to site A, either as a homodimer or as a heterodimer with the human 9-*cis*-retinoic acid receptor α. In contrast, the negative thyroid hormone responsive element binds chicken thyroid hormone receptor α exclusively as a monomer. Site-directed mutagenesis of the negative thyroid hormone response element abolished the inhibitory effects of the hormone and increased basal promoter activity by up to 40-fold. These data suggest that functional positive and negative thyroid hormone response elements coexist within the rat apolipoprotein AI promoter and both elements contribute to the control of apolipoprotein AI gene expression.

Apolipoprotein AI (apo-AI),[1] the major protein component of high density lipoprotein particles (HDL), is synthesized in the liver and small intestine (Mahley et al., 1984). HDL particles promote the efflux of cholesterol from peripheral tissues and transfer it to the liver in a process known as reverse cholesterol transport (Eisenberg, 1984). Hypercholesterolemia is a major risk-factor in the development of coronary arterial disease (Frohlich & Pritchard, 1989). Epidemiological studies have shown an inverse correlation between serum apo-AI concentrations and the incidence of coronary heart disease (Abbott et al., 1988; Stamler et al., 1986). Furthermore, transgenic mice that overexpress human apo-AI are protected from this disease (Rubin et al., 1991). Therefore, increased expression of apo-AI protein should raise HDL levels and prevent hypercholesterolemia-induced coronary heart disease (Forte & McCall, 1994).

Apo-AI gene expression is regulated by a variety of physical, developmental, dietary, and hormonal factors (Taylor & Ward, 1993; Haddad et al., 1986; Sorci-Thomas et al., 1989; Elshourbagy et al., 1985; Wong & Oppenheimer, 1986). Previous studies have shown that the thyroid hormone L-triiodothyronine (T3) increases apo-AI expression (Davidson et al., 1988), by enhancing transcription of the gene (Strobl et al., 1992; Romney et al., 1992) and synthesis of the protein (Wong & Oppenheimer, 1986; Staels et al., 1990). The actions of T3 are mediated by nuclear T3 receptors (TRs), which exist as two major isoforms, α and *β* (Lazar & Berrodin, 1990). TRs are members of a superfamily of nuclear ligand receptors that include those which bind the steroid hormones (Evans, 1988). The specific DNA sequences that mediate the effects of TR are known as thyroid hormone response elements, TREs (Shepard & Eberhardt, 1993). These elements may bind TR in the presence or absence of ligand (Evans, 1988; Brent et al., 1991). In addition, TRs bind to TREs as homodimers or heterodimers with other nuclear proteins (Yen & Chin, 1994).

Positive TREs have been identified to be *cis*-acting elements that consist of a pair of hexanucleotide repeats separated by an optimum spacing of four nucleotides (Yen & Chin, 1994; Umesono et al., 1991), but those having a space of zero to five nucleotides may also function as positive TREs (Miyamoto et al., 1994). Although positive TREs are relatively common and have been studied extensively, little is known about the rarer negative TREs. The only mammalian genes negatively regulated by chronic T3 administration are found in the pituitary or hypothalamus (Larsen, 1981; Fisher et al., 1976; Burger & Patel, 1977; Trainer & Howard, 1983). The expression of other genes, such as the lymphocytic *β*-adrenoceptor (Basso et al., 1991) and the epidermal growth factor receptor (Kesavan et al., 1991), are only transiently repressed by T3. More recent data suggest that T3 represses gene expression by binding TR monomer to a specific hexanucleotide sequence equivalent to one-half of a positive TRE (Carr & Wong, 1994; Suzuki et al., 1994).

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[1] Abbreviations: apo-AI, apolipoprotein AI; HDL, high density lipoprotein; T3, the thyroid hormone L-triiodothyronine; TR, T3 receptor; TRE, thyroid hormone response element; CAT, chloramphenicol acetyl transferase; RXRα, 9-*cis*-retinoic acid receptor α; R_f, relative migration; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction.

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In a previous study, we showed that liganded TRα increased the transcriptional activity of the rat apo-AI promoter (Romney et al., 1992). The stimulatory effect of T3 was mediated by a positive TRE, site A (−208 to −193) an element comprised of two hexanucleotide repeats separated by a two nucleotide spacing. Contrary to our expectations, deletional promoter constructs that lack site A were repressed by T3 (Romney et al., 1992). These observations suggested that both positive and negative TREs coexist in the rat apo-AI promoter. Although we know where T3 exerts a positive effect, our knowledge of rat apo-AI promoter function is incomplete without information on the element(s) that mediate the negative effects of T3. Therefore, we attempted to identify the putative negative TRE(s) in the rat apo-AI and examined the functional importance of this motif. The presence of both positive and negative TREs in the rat apo-AI promoter provides a unique model for investigating the nuclear effects of T3.

MATERIALS AND METHODS

Preparation of Plasmid DNA Used in Transient Transfection Assays. The deletional constructs pAI.474.CAT, pAI.235.CAT, pAI.186.CAT, pAI.144.CAT, and pAI.46.CAT, spanning rat apo-AI promoter nucleotides −474 to −7, −235 to −7, −186 to −7, −144 to −7, and −46 to −7, respectively, were previously described (Chan et al., 1993). The plasmids pΔA.CAT, which has an internal deletion of a DNA fragment (−232 to −187) containing site A, and p5′A.CAT, which has two copies of this fragment fused to the heterologous SV40 viral promoter, have been described (Romney et al., 1992). Human TRα1 or β1 cDNA were contained in pECE-hTRα and pECE-hTRβ, respectively (gifts from Dr. M. Pfahl, La Jolla Cancer Research Foundation, La Jolla, CA).

To construct the mutant rat apo-AI sequences containing the putative negative TRE, we performed PCR using one of the following templates; pAI.474.CAT, pAI.235.CAT, or pAI.186.CAT. In all reactions, we used a common 5′ oligonucleotide homologous to the M13 motif in the pUC vector and a common 3′ oligonucleotide (ACACACATATATAGGCTGGGGAAGAAGACCTGG) where the underlined trinucleotide differed from the wild-type (TCA). The product of each PCR reaction was cloned into pGEM-T (Promega Corporation, Madison, WI) before excision and cloning into the *Sma*I−*Xba*I site of pUC.CAT (Chan et al., 1993). All constructs were verified by nucleotide sequence analysis.

Cell Culture and Transfection. Human fetal hepatoma (HuH-7) cells were maintained in RPMI-ISE medium as previously described (Romney et al., 1992). For transient transfection, cells were subcultured to 25 cm^2 flasks at an initial plating density of $(2-4) \times 10^4$ cells per cm^2 and grown in a humidified atmosphere of 5% CO_2 in air for 72 h. Cells were transfected, via the calcium phosphate coprecipitation method (Cullen, 1987), with up to 5 μg of test plasmid DNA along with 5 μg of either pECE-hTRα or pECE-hTRβ and 2.5 μg of the bacterial plasmid pRSV-βgal (Edlund et al., 1985), to correct for DNA uptake by these cells. After 24 h of incubation, the medium was exchanged for one containing either 3×10^{-9} M T3 or vehicle. After a further 24 h of incubation, transfected cells were harvested, and cellular protein was assessed for β-galactosidase activity and chloramphenicol acetyltransferase activity (CAT), as described (Rosenthal, 1987; Gorman et al., 1982). Protein concentrations and the time of the CAT assay were adjusted so that all samples were within the linear range for the assay (5−27%). Results were corrected for background conversion, transfection efficiency, protein concentration, and length of assay and expressed as a percentage of total radioactivity.

Expression of Chicken TRα, Rat TRα, Rat TRβ, and Human RXRα. Chicken TRα1 (cTRα1; Sap et al., 1986) cDNA in pET-cT3R (a gift from H. H. Samuels, New York State University, New York, NY) or rat TRα1 (rTRα1) and rat TRβ (rTRβ1) in pT7-7 (a gift from S. Tabor, Harvard Medical School, Cambridge, MA) were grown in *Escherichia coli* strain BL21(DE3) and purified as described (Forman & Samuels, 1991). Control plasmid pT7-7 was used to transform the same *E. coli* strain. Production and isolation of human RXRα protein was performed as described by Chan et al. (1993), using vector (pECE) containing the cDNA encoding human RXRα (Mangelsdorf et al., 1992; a gift from R. M. Evans, Salk Institute, La Jolla, CA).

Electrophoretic Mobility Shift Assays (EMSA). Apo-AI sequences from −37 to −7 bp (apo-TATA) were synthesized with protruding 3′ *Eco*RI sites at either end of both sense and antisense strands. Annealed duplex DNA was radiolabeled by incubating with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. The sequence from −232 to −187 of the rat apo-AI gene was subcloned into the *Pst*1 site of pTZ18R, and a fragment containing site A (110 bp) excised with *Hin*dIII and *Bam*HI. This fragment and TREpal (Glass et al., 1989) were radiolabeled by incubating with [α-^{32}P]dATP and Klenow fragment. All probes were purified using Sephadex G-50 size-exclusion chromatography (Maniatis et al., 1983). Oligomers used for competition experiments include the mutated rat proximal negative TRE, described above, a mutant human apo-AI negative TRE which is identical to the rat mutant except that the underlined nucleotides were changed to CCC, the negative TRE from the TSHβ gene (AGAGTCTGGGTCATCACAGCATTAACTCGCCAGTGCAAAGTAAGGTAGTCTCTACC), rat malic enzyme positive TRE (AATTAGGACGTTGGGTTAGGGGAGGACAAATT), the synthetic positive TRE, DR+4 (AGCTTCAGGTCACAGGAGGTCAGAGAATT), the synthetic positive TRE, TREpal (AATTTCAGGTCATGACCTGA), the mouse albumin promoter site B (AATTTAAGTATGGTTAATGATCTACAGTT) or the mouse albumin promoter site D (AATTTGGTATGATTTTGTAATGGGGTAGGA). EMSA assays were performed using 4% polyacrylamide gels with 0.5× TBE buffer (1× TBE is 89 mM Tris base, 89 mM boric acid, and 1.25 mM EDTA). Gels were electrophoresed for 5 min prior to the loading of samples. Reactions contained up to 80 000 cpm of purified ^{32}P-labeled oligonucleotide, 2 μg of poly(dI·dC), 25 mM Tris base (pH 7.8), 0.5 mM EDTA (pH 8.0), 1 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100, 10 μg of BSA, and heparin-agarose column-purified bacterially expressed chicken or rat TRα1, rat TRβ1, or control bacterial extract. Either competing unlabeled oligonucleotide or COS-7 cell expressed RXRα was added to this basic mixture. The protein−DNA binding reaction was allowed to occur either within 25−30 min at 24 °C or for 1 h at 4 °C. Bromophenol blue/xylene cyanol dye was added and one quarter to one half of the reaction mixture loaded on the gel. The samples were electrophoresed at 0.4 V/cm^2 for 150−210 min at 4 °C. Gels were dried under a vacuum to Whatman 3MM paper and

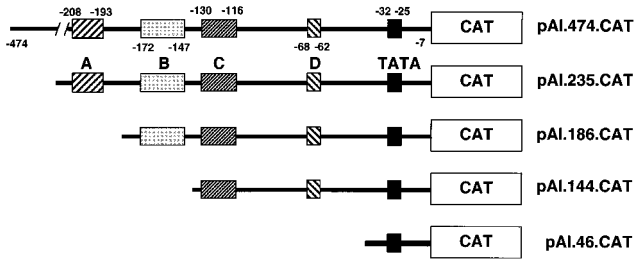

FIGURE 1: Rat apolipoprotein AI promoter-CAT constructs. The *cis*-acting elements of the rat apolipoprotein AI promoter are represented by the boxes. The numbering is relative to the transcription start site, according to the rat apo-AI numbering system of Widom et al. (1991).

exposed to X-ray film (Kodak X-OMAT AR5) for 18 h, at −80 °C, in the presence of intensifying screens.

Statistical Analyses. All results are expressed as mean ± SD, and statistical significance is determined by one-way ANOVA using Fisher's modified least significant difference test. Probability values of $p < 0.05$ were accepted as statistically significant.

RESULTS

Rat Apo-AI Promoter Is Responsive to Both TRα and TRβ. Four *cis*-acting elements, designated sites A, B, C, and D (Figure 1), have been identified within the −235 to −7 region of the rat apo-AI gene (Widom et al., 1991). In a previous study using TRα (Romney et al., 1992), we showed that T3 induction of apo-AI gene activity was mediated by an element residing between −208 and −193 of the rat apo-AI promoter, designated site A. The transient transfection procedure used in the present studies has been improved since the studies of Romney et al. Therefore, we have repeated those studies using the new procedure to ensure that the results are not significantly different and to simplify the comparison of experiments with TRβ to those with TRα. In the repeated studies, cotransfection of human TRα and either the wild-type promoter construct pAI.474.CAT or pAI.235.CAT (Figure 2A) reduced promoter activity to 43% and 44%, respectively, when compared to control (i.e., cells transfected with pAI.474.CAT or pAI.235.CAT alone). Exposure of similar cells to T3 produced a 3-fold increase in pAI.474.CAT promoter activity to 131% of control. Similarly, pAI.235.CAT promoter activity increased 4-fold (from 44% to 160%) following treatment with T3. These data show that a positive thyroid hormone-responsive element resides within the −235 to −7 region of the apo-AI and are qualitatively similar to those observed previously (Romney et al., 1992).

To test whether TRβ had the same effect as TRα on pAI.474.CAT and pAI.235.CAT, we performed similar experiments but replaced TRβ for TRα (Figure 2B). TRβ alone reduced both pAI.474.CAT and pAI.235.CAT promoter activities to 67% and 73%, respectively, of control. T3 treatment of cells containing TRβ increased the promoter activities of both pAI.474.CAT and pAI.235.CAT constructs. These data are similar to the TRα results, but T3 induction is only 2−3-fold as compared to the 3−4-fold for TRα. These data show that α and β isoforms of TR act similarly; both inhibit promoter activity in the absence of T3 and both stimulate promoter activity in the presence of T3.

Site A Mediates Positive Effects of TRα and TRβ. To show that site A contains a positive TRE, we measured the activities of two constructs, p5′A.CAT and pΔA.CAT. The p5′A.CAT construct has two copies of site A fused to the heterologous SV40 viral promoter (Romney et al., 1992). The experiments with p5′A.CAT showed that site A conferred T3 responsiveness to a nonresponsive viral promoter (Figure 2A,B). Similar to pAI.474.CAT and pAI.235.CAT, the activity of p5′A.CAT when cotransfected with TRα or TRβ was repressed to 38% and 72%, respectively, relative to p5′A.CAT alone. In the presence of T3 and TRα or TRβ, activity of the reporter template increased roughly 4-fold to 180% and 262% relative to control cells that contained p5′A.CAT alone.

The pΔA.CAT construct is identical to pAI.474.CAT except for an internal deletion of the −232 to −187 sequence, a fragment containing site A (Romney et al., 1992). TRα alone inhibited pΔA.CAT promoter activity to 63% of control, and T3 unexpectedly reduced promoter activity further to 22% of control (Figure 2A). In contrast to the suppressing effects of TRα alone, TRβ on its own had no effect on the activity of pΔA.CAT (Figure 2B). The addition of T3 to these HuH-7 cells, however, reduced pΔA.CAT

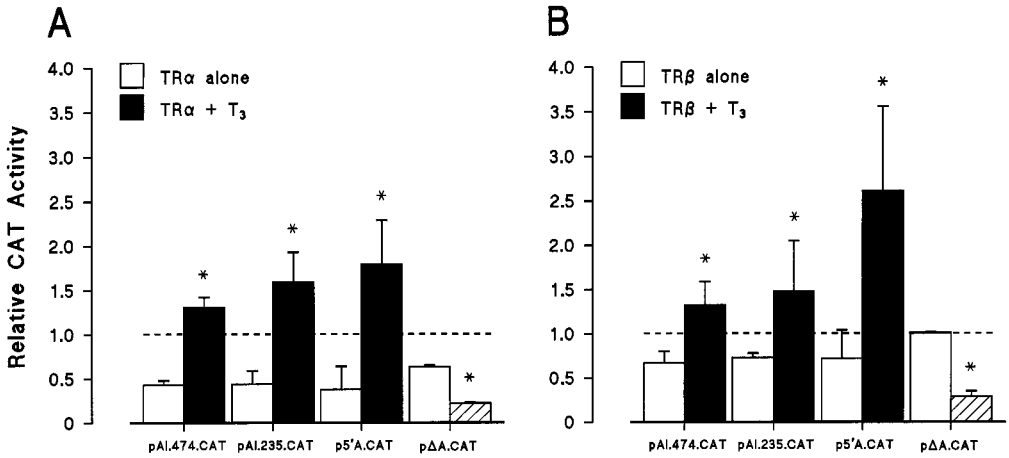

FIGURE 2: Site A contains a positive TRE. HuH-7 cells cotransfected with the indicated deletional constructs and either TRα (A) or TRβ (B) were treated with T3 (solid bars). Cellular proteins were then assayed for CAT activity as described in Materials and Methods. Data are expressed as CAT activity relative to construct alone and are means ± SD of at least six independent experiments performed in duplicate. The dashed line indicates the control value for each construct. *$p < 0.05$; one-way ANOVA with Fisher's modified least significance difference test.

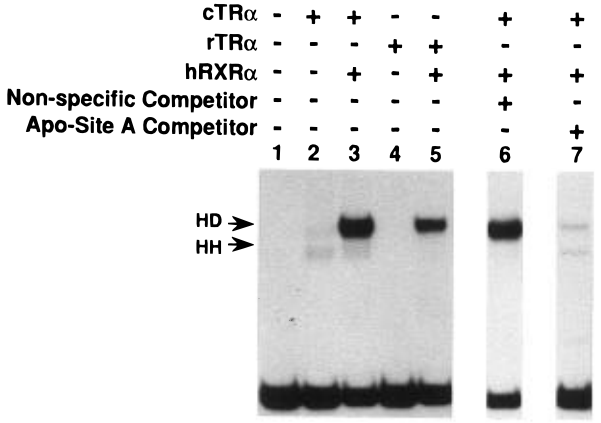

FIGURE 3: Binding of cTRα1 and rTRα1 to site A DNA. Bacterially expressed cTRα1 (lanes 2, 3, 6, and 7) and rTRα1 (lanes 4 and 5) were incubated with radiolabeled site A. Reactions were complemented with human RXRα (lanes 3, 5, 6, and 7) and a 400-fold molar excess of either albumin site B DNA (lane 6) or apo-AI site A DNA (lane 7). Bound complexes were separated from free probe as described under Materials and Methods. HD = heterodimer; HH = homodimer. Data are representative of experiments performed at least three times.

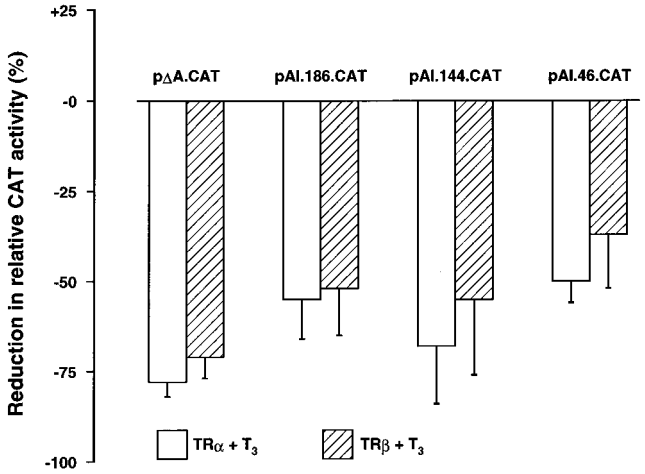

FIGURE 4: Negative TRE is confined to the −46 to −7 bp sequence. Cellular proteins from HuH-7 cells cotransfected with the indicated deletional constructs and TRα (open bars) or TRβ (hatched bars) treated with T3 were assayed for CAT activity as described in Materials and Methods. Data are expressed as the percent reduction in CAT activity relative to construct alone and are means ± SD of at least six independent experiments performed in duplicate. All values are statistically different from their respective controls but not from each other (one-way ANOVA with Fisher's modified least significance difference test).

activity to 29% of control. These observations provide additional evidence that site A contains a positive TRE and that it interacts with either TRα/β. Furthermore, the data also suggest that removal of the positive TRE, site A, has unmasked the activity of a negative TRE in the apo-AI promoter.

Bacterially Expressed Chicken TRα and Rat TRα Bind to Site A. To test whether bacterially expressed c/rTRα1 could bind to radiolabeled site A, we used the EMSA (Figure 3). The binding of either c/rTRα1 was weak (lanes 2 and 4). Mobility of the single complex detected in reactions containing cTRα1 and site A was comparable to that of cTRα1 homodimer (HD) bound to the positive palindromic TRE, TREpal (Miyamoto et al., 1994). The R_f values for the site A and TREpal homodimer complexes were 0.34 and 0.37, respectively. Binding of either cTRα1 or rTRα1 to site A was greatly enhanced by the presence of hRXRα (lanes 3 and 5). RXRα by itself did not bind to site A (data not shown). The slightly slower mobility of the c/rTRα1/hRXRα/site A (R_f = 0.23) compared to that of c/rTRα1/site A indicates heterodimerization of TR with hRXRα and is consistent with the known higher M_r (50.8 kDa) of hRXRα relative to that of c/rTRα1 (46 and 49 kDa, respectively) (Sap et al., 1986; Ichikawa & DeGroot, 1986). Additionally, the R_f value compares well with other heterodimer mobilities, for example, the hTRα1/hRXRα/DR+2 complex of Miyamoto et al. (1994) has a R_f value of 0.25. The binding of TR monomer to site A was not detected.

Competition studies using a nonspecific oligomer (albumin site B) which is similar in length to site A were used to show specific binding. A 400-fold molar excess of this sequence failed to displace cTRα1 and RXRα binding to site A (lane 6). As expected, a 400-fold molar excess of unlabeled site A displaced cTRα1 binding to the labeled probe (lane 7). Together these data show specific binding of cTRα1 to labeled site A and that the binding is enhanced by RXRα.

A Negative TRE Is Fused to the TATA Element. Under the section entitled *Site A Mediates Positive Effects of TRα and TRβ* (Figure 2A,B), we showed that removal of site A from the promoter unmasked a negative response to T3. Hormone reduced the promoter activity of pΔA.CAT by 78% and 71% in presence of TRα and TRβ, respectively (Figure 4, note that the pΔA.CAT data are identical to those shown in Figure 2, except that they are shown in an inverse form so that comparison with the following negative TRE constructs is simplified). To identify the *cis*-acting site(s) in the promoter that mediate the inhibitory effects of T3, we tested the activity of the following deletional constructs pAI.186.CAT, pAI.144.CAT, and pAI.46.CAT (Figure 1). The promoter activities of each construct showed a significant reduction in the presence of TRα and T3 (Figure 4). The deletional construct pAI.186.CAT was reduced by 55%, pAI.144.CAT by 68%, and pAI.46.CAT by 50% relative to control (i.e., activity in cells transfected with the same construct without TRα or T3). Similarly, TRβ and T3 inhibited the activities of pAI.186.CAT, pAI.144.CAT, and pAI.46.CAT by 52%, 55%, and 37%, respectively relative to control. These reductions were not significantly different from those obtained with pΔA.CAT (Figure 4).

These results show that progressive deletion of the promoter had no significant outcome on T3 reduction of promoter activity and localized the negative T3 effect to a 40 bp DNA fragment (−46 to −7). Inspection of the DNA fragment showed a potential negative TRE sequence (AGGTCA; −25 to −20) fused to the 3′ end of the TATA element. To show clearly that the preceding motif was indeed a functional negative TRE, two additional pieces of information were required. First, does TR bind to this fragment, and, secondly, would site-directed mutagenesis of this element abolish the negative effect of T3?

TR Binds to the Negative TRE as a Monomer. An oligomer spanning nucleotides −37 to −7, designated apo-TATA, was used in EMSA to determine whether TR binds to the putative negative TRE (Figure 5). cTRα1 bound to radiolabeled apo-TATA and formed a single complex. The formation of this complex was disrupted by oligomers containing negative TREs (lanes 4 and 5). In contrast, nonspecific DNA containing the mouse albumin site B (lane

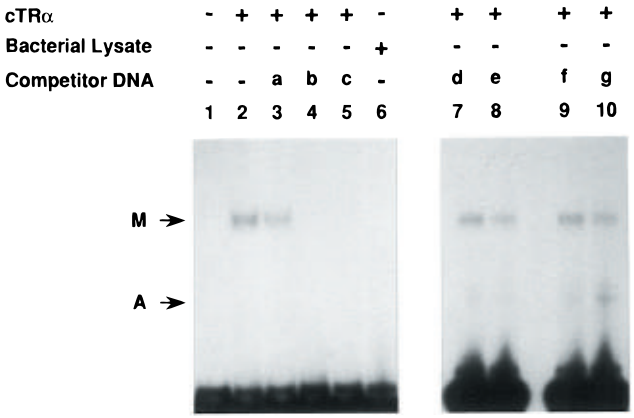

FIGURE 5: Specific binding of cTRα1 to apo-TATA. Bacterially expressed cTRα1 (lanes 2−10) was incubated with radiolabeled apo-TATA DNA, except for lane 6, which contains an equivalent quantity of T7 *E. coli* bacterial proteins. A 400-fold molar excess of the specific and nonspecific oligomer DNA fragments was added to reactions as follows: (a) albumin site B; (b) apo-TATA; (c) rat TSHβ nTRE; (d) rat mutant apo-TATA; (e) human mutant apo-TATA; (f) DR+4; (g) ME positive TRE. Bound receptor complexes were separated from free probe and visualized as described under Materials and Methods. M = monomer binding; A = artifact. Results are representative of experiments performed twice.

3; Wuarin et al., 1990) mutated rat or human negative TREs (lanes 7 and 8) failed to displace cTRα1 binding to apo-TATA. Bacterial lysates that did not contain cTRα1 showed no binding to apo-TATA (lane 6). These data indicate specific binding of cTRα1 to apo-TATA. The positive TRE sequences, direct repeat plus four bases (DR+4) and malic enzyme positive TRE (ME), were poor competitors compared to sequences containing negative TREs (lanes 9 and 10). This observation suggests that cTRα1 binds to negative TREs with a higher affinity than to positive TREs.

To determine whether cTRα1 binds to apo-TATA as a monomer or as a homodimer, we used a longer probe (101-mer) to enhance TR binding to the motif and compared the mobility of the TR/apo-TATA complex with those of TR bound to the positive TREs, site A (110-mer), and TREpal (121-mer). The 30 bp apo-TATA (−37 to −7) sequence was ligated into the multiple cloning site of KS+ Bluescript and a 101 bp insert excised using *Xba*I and *Xho*I. DNA fragments of comparable length, created using the same approach containing site B or site D from the albumin promoter (Wuarin et al., 1990), served as negative controls. cTRα1 bound to apo-TATA 101-mer and formed a single band on EMSA (Figure 6A). Only unlabeled apo-TATA 101-mer was an efficient competitor for cTRα1 binding (Figure 6A; lanes 2−10). hRXRα failed to produce a slower migrating heterodimer band (lane 7). These observations suggest that cTRα1 binds specifically to apo-TATA negative TRE as a monomer and its binding is not enhanced by RXRα.

To be certain that cTRα1 bound apo-TATA as a monomer, we compared the mobility of this complex with that of cTRα1/site A and cTRα1/TREpal (Figure 6B). The cTRα1/apo-TATA complex (lane 2) migrated faster than either cTRα1 homodimer or cTRα1 plus RXRα heterodimer bound to site A (lanes 4 and 5). Similarly, rTRα1 or rTRβ1 dimerized with RXRα when bound to site A (lanes 6 and 7). A 400-fold molar excess of unlabeled apo-TATA 101-mer displaced cTRα1/RXRα heterodimer binding to site A (lane 8). Since TR is known to bind TREpal in the form of

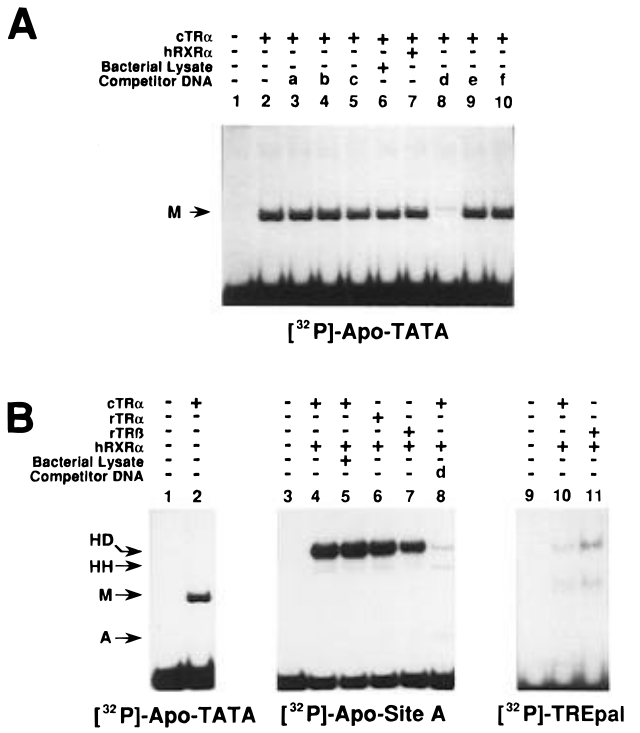

FIGURE 6: Binding of TR to apo-TATA, site A, and TREpal. (A) Bacterially expressed cTRα1 was incubated with apo-TATA 101-mer DNA (lanes 1−10). Reactions were complemented with T7 *E. coli* bacterial proteins (lane 6), COS-7 cell expressed human RXRα (lane 7), or a 400-fold molar excess of the following DNA fragments: (a) albumin site B, (b) albumin site D, (c) KS+ Bluescript *Xba*I−*Xho*I fragment, (d) apo-TATA 101-mer, (e) TREpal, (or f) apo-AI site A 110-mer. (B) Bacterially expressed cTRα1, rTRα1, or rTRβ1 were incubated with either ^{32}P-labeled apo-TATA 101-mer DNA (lanes 1 and 2), apo-AI site A 110-mer (lanes 3−8) or TREpal 121-mer (lanes 9−11). Human RXRα was added to reactions containing positive TREs (lanes 4−11). In other reactions, bacterially expressed rTRα1 (lane 6) and rTRβ1 (lanes 7 and 11) were used instead of cTRα1. The specific DNA fragment apo-TATA 101-mer was used to show DNA binding competition for cTRα1. Bound receptor complexes were separated from free probe and visualized as described under Materials and Methods. HD = Heterodimer binding; HH = homodimer binding; M = monomer binding.

a monomer or heterodimer with RXRα (Zhang et al., 1992), we examined binding of cTRα1 and RXRα to this element. Results showed the expected presence of two complexes. The complex with the slower mobility is comparable to the known migration position of heterodimer bound to TREpal and the faster migrating species of cTRα1 monomer bound to labeled probe. The comparable mobility of the cTRα1/apo-TATA complex in lane 2 with that of the monomer cTRα1/TREpal (R_f values are 0.505 for cTRα1/apo-TATA complex and 0.511 for monomer cTRα1/TREpal) suggest that the predominant TR species binding to apo-TATA is a monomer.

Mutation of the Negative TRE Increases Promoter Activity. To determine the role of the putative negative TRE fused to the TATA box in apo-AI promoter activity, we used PCR to construct mutant templates. The three mutant constructs were identical to pAI.474.CAT, pAI.235.CAT, or pAI.186.CAT except for a trinucleotide change from TCA to CTG at position −23 to −20. The activities of the mutant templates were compared to their wild-type counterpart in transient transfection studies. HuH-7 cells cotransfected with TRα/β plus a mutant or wild-type reporter were treated with

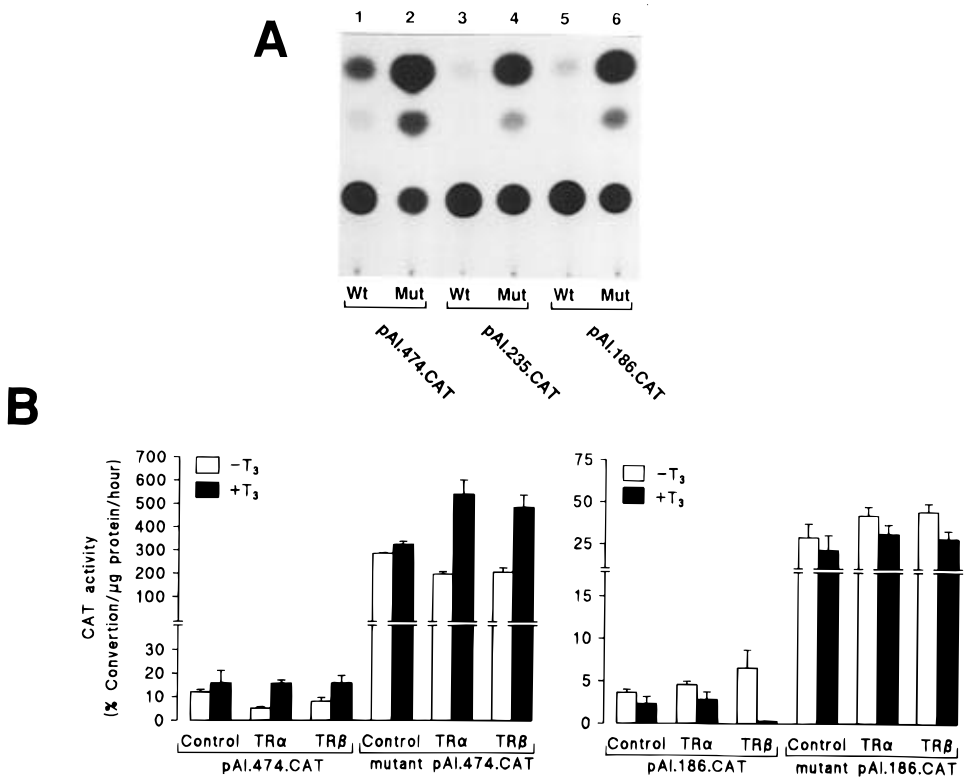

FIGURE 7: Mutation of the nTRE increases promoter activity and affects only the inhibitory effect of T3. (A) Basal CAT-activity of protein extracts (10 μg) from HuH-7 cells transfected with either wild-type (Wt) or mutant (Mut) pAI.474.CAT, pAI.235.CAT, or pAI.186.CAT. (B) The left panel shows the relative CAT-activity of wild-type pAI.474.CAT, which contains both intact positive and negative TREs, compared a similar construct containing an intact positive TRE but a mutated negative TRE. Both sets of cells were cotransfected with TRα or TRβ and either untreated (open bars) or treated with T3 (solid bars). The right panel shows a similar set of experiments using wild-type pAI.186.CAT, which lacks the positive TRE but contains an intact negative TRE, and mutant pAI.186.CAT which is an identical construct containing a mutated negative TRE. Data are the means ± SD for two independent experiments performed in duplicate.

or without T3. Results showed that the basal CAT activity associated with the mutant pAI.474.CAT, pAI.235.CAT, or pAI.186.CAT constructs in the absence of T3 was higher by 24-, 40-, and 8-fold, respectively, compared to their wild-type counterparts (Figure 7A). Further studies of the wild-type and mutant pAI.474.CAT (constructs containing an intact positive TRE) showed that T3-induced activity of both the wild-type and mutant constructs (Figure 7B, left panel). T3 significantly increased CAT-activity in cells containing the mutant pAI.474.CAT cotransfected with TRα or TRβ by 2.7- and 2.3-fold, respectively. Similar results were obtained for the mutant pAI.235.CAT construct (data not shown).

In contrast, studies with wild-type and mutant pAI.186.CAT constructs, which lack the positive TRE, showed that T3 repressed the activity of the wild-type construct but not that of the mutant template (Figure 7B, right panel). Together these observations indicate three significant findings: (A) mutation of the negative TRE markedly increases the basal activity of the promoter, (B) mutation of the negative TRE abrogates the repressive effects of T3, and (C) that the motif fused to the TATA box mediates negative effects of T3. Additional studies showed that insertion of the wild-type negative TRE in front of the viral thymidine kinase promoter (Sudhof et al., 1987) abolished its activity (data not shown). Insertion of the mutant negative TRE in front of the viral thymidine kinase promoter increased thymidine kinase promoter activity to within the linear range of the CAT assay. However, neither liganded nor unliganded TR down-regulated this construct (data not shown).

DISCUSSION

In this report, we have examined the effect of T3 on the rat apo-AI promoter. Previous studies showed that the positive effect of T3 on the wild-type promoter is mediated by the A element. The site A motif differs from other positive TREs because it is comprised of two hexanucleotide repeats separated by two rather than the expected four bp (Umesono et al., 1991). Although we have previously documented the induction of this motif by TRα, its response to TRβ remained unknown. Results of the present studies summarized above show clearly that both receptor isoforms repress the activity of site A in the absence of T3 and induce a 2−4-fold rise in activity of the promoter following exposure to T3. Unliganded TR repression and liganded TR induction of gene transcription appears to be a common feature for this nuclear factor (Brent et al., 1989; Graupner et al., 1989).

TR binds to the A motif and RXRα enhances its binding. This finding is consistent with the known role of RXRα to enhance TR binding to positive TREs (Miyamoto et al., 1994; Kliewer et al., 1992). TRα or TRβ alone are weakly associated with positive TREs and the DNA−protein interaction is stabilized by heterodimerization with RXRα (Wahlström et al., 1992; Ikeda et al., 1994). However, unlike other positive TREs that may bind TR as a monomer (Suzuki et al., 1994), TR monomer binding to site A was undetectable (Figure 3). Although the reason for this is unclear, one possibility may be that the apo-AI site A monomer TR−TRE interaction is weak and falls below our detection threshold. The increased abundance of heterodimer signal suggests that RXRα facilitates TR binding. RXRα has been

shown to cause a conformational change in the DNA of a number of genes such as the human growth hormone-related gene (Leidig et al., 1992). However, since RXRα on its own does not bind to the rat apo-AI site A motif, RXRα must enhance TRα binding via a different mechanism. These observations also suggest that RXRα requires the presence of other steroid/thyroid hormone receptor superfamily members for optimum binding. The mechanism of RXRα enhancement of TR binding is the subject of intense research (Zhang et al., 1992; Kliewer et al., 1992) and beyond the scope of this article.

During the course of studies on site A, we examined a construct which lacks the positive TRE (pΔA.CAT). As predicted, pΔA.CAT was not induced by T3. However, to our surprise, the CAT activity of cells transfected with pΔA.CAT were inhibited by liganded TR, indicating the presence of a negative TRE in part of the promoter other than site A. The present studies were initiated to identify the position and function of this negative TRE. The results show it to be localized to a minimal segment of the gene spanning nucleotides −46 to −7 and showed that this 40 bp fragment retains the repressive effects in response to hormone (Figure 4). Additional evidence that pointed to the presence of a TRE in this gene fragment included its ability to bind TR (Figures 5 and 6). The increased signal intensity arising from the binding of cTRα1 to the negative TRE contained within a longer oligomer (Figure 6A versus Figure 5) seems to suggest that neighboring *cis*-acting elements might be involved in TRα binding to the negative TRE. Alternatively, the increased signal intensity may simply reflect an increase in the stability of the DNA−protein interaction inherent to longer oligonucleotides due to more native configurations of the DNA or increased specific activity of the radiolabeled oligonucleotide. Since negative TREs are postulated to be a hexamer with the sequence AGGTCA (Carr & Wong, 1994; Suzuki et al., 1994), we examined the −46 to −7 sequence for this motif and located an exact match (−25 to −20) that was fused to the 3′ end of the TATA-element. Site-directed mutagenesis of the trinucleotide at the 3′ end of the hexamer motif increased activity of the entire promoter by 8−40-fold and abolished the negative but not the positive effects of T3. This observation confirms that the mutated site mediates the negative effects of T3 and that it is separate from the apo-AI site A motif that responds to hormone. Similarly, the negative effects of T3 on the wild-type but not the mutant negative TREs fused to the thymidine kinase promoter show this effect to be transferable from one promoter to another. The reduction in thymidine kinase promoter activity by wild-type negative TRE suggests that tissue- and promoter-specific factors are both involved in T3 negative TRE regulation of the apo-AI promoter.

Increased activity of constructs containing a mutant negative TRE indicates a potential role of this motif in basal promoter activity. For example, the activity of the wild-type pAI.474.CAT is only 4.2% of its mutated counterpart. This finding suggests that the removal of the negative TRE releases the promoter from the restrictive effects of this element. Additionally, the similar ratios of T3-dependent increases in the CAT-activities of wild-type and mutant pAI.474.CAT (constructs that contain site A) suggest that mutation of the negative TRE has not only removed the T3 repression of this site but may have altered the characteristics of the TATA element to cause the observed superinduction of basal activity. This possibility is the topic for future investigation. To determine whether the information from our studies have added to the knowledge of thyroid hormone action, we compared our results with those obtained from the study of other model genes that are also induced by T3, e.g., the rat S14, rat malic enzyme, and rat growth hormone genes. When the positive TREs are removed from the S14 and malic enzyme promoters, the activity of the deletional mutants is not inhibited by T3 (Zilz et al., 1990; Petty et al., 1990). However, removal of the positive TRE from the rat growth hormone gene uncovered a transient inhibitory element (TIE) that represses the activity of the deletional mutant in response to T3 (Wright et al., 1987). The inhibitory effect of the TIE is active in pituitary GC cells but is transient and present only between 4 and 12 h following exposure to T3 (Wright et al., 1987). By comparison, our observations that the inhibitory effect of the apo-AI negative TRE and the inductive effect of the positive TRE are still discernible 24 h after T3 exposure appear to be unique among T3-regulated genes. The presence of the negative TRE probably dampens the enhancing effects of T3 on the promoter.

The induction of the chicken ovalbumin gene expression by estrogen shows some similarities to the induction of rat apo-AI by T3. In common with the rat apo-AI promoter, the ovalbumin promoter contains both positive and negative responsive elements (Sanders & McKnight, 1988). Additionally, when a motif that mediated the positive effects of estrogen was removed, a negative regulatory element was exposed (Schweers et al., 1990). However, unlike the negative TRE in the rat apo-AI promoter, the ovalbumin negative regulatory element is not activated by hormone.

In summary, both liganded TRα and TRβ when bound to site A exert a positive effect on the rat apo-AI promoter. The A-site TRE is comprised of duplicate hexanucleotides arranged in head to tail orientation separated by 2 bp. TR as a homodimer or heterodimer (TR/RXR) binds to this motif in a specific manner. Removal of site A from the wild-type promoter exposes an element that imparts a negative response to T3. The repressive element is a hexanucleotide motif that shares 67% homology with the hexanucleotide sequence of the positive site A but is identical to the hexanucleotide consensus TRE sequence. TRα binds to the apo-AI negative TRE as a monomer. As expected, mutation of the negative TRE abolishes the repressive effects of T3, but the positive response mediated by the separate A element remains intact, indicating the presence of both functional positive and negative elements in the rat apo-AI promoter. Based on preceding results, rat apo-AI appears to be a useful model for studying positive and negative TREs.

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REFERENCES

Abbott, R. D., Wilson, P. W., Kannel, W. B., & Castelli, W. P. (1988) *Arteriosclerosis 8*, 207−211.

Basso, A., Piantanelli, L., Rossolini, G., Piloni, S., Vitali, C., & Masera, N. (1991) *J. Clin. Endocrinol. Metab. 73*, 1340−1344.

Brent, G. A., Dunn, M. K., Harney, J. W., Gulick, T., Larsen, P. R., & Moore, D. D. (1989) *New Biol. 1*, 329−336.
Brent, G. A., Moore, D. D., & Larsen, P. R. (1991) *Annu. Rev. Physiol. 53*, 17−35.
Burger, H. G., & Patel, Y. C. (1977) *Clinics Endocrinol. Metab. 6*, 83−100.
Carr, F. E., & Wong, N. C. (1994) *J. Biol. Chem. 269*, 4175−4179.
Chan, J., Nakabayashi, H., & Wong, N. C. (1993) *Nucleic Acids Res. 21*, 1205−1211.
Cullen, B. R. (1987) *Methods Enzymol. 152*, 684−704.
Davidson, N. O., Carlos, R. C., Drewek, M. J., & Parmer, T. G. (1988) *J. Lipid Res. 29*, 1511−1522.
Edlund, T., Walker, M. D., Barr, P. J., & Rutter, W. J. (1985) *Science 230*, 912−916.
Eisenberg, S. (1984) *J. Lipid Res. 25*, 1017−1058.
Elshourbagy, N. A., Boguski, M. S., Liao, W. S., Jefferson, L. S., Gordon, J. I., & Taylor, J. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8242−8246.
Evans, R. M. (1988) *Science 240*, 889−895.
Fisher, D. A., Dussault, J. H., Sack, J., & Chopra, I. J. (1976) *Recent Prog. Horm. Res. 33*, 59−116.
Forman, B. M., & Samuels, H. H. (1991) *Gene 105*, 9−15.
Forte, T. M., & McCall, M. R. (1994) *Curr. Opin. Lipidol. 5*, 354−364.
Frohlich, J. J., & Pritchard, P. H. (1989) *Clin. Biochem. 22*, 417−423.
Glass, C. K., Lipkin, S. N., Devary, O. V., & Rosenfeld, M. G. (1989) *Cell 59*, 697−708.
Gorman, C. M., Moffat. L. F., & Howard, B. H. (1982) *Mol. Cell. Biol. 2*, 1044−1051.
Graupner, G., Wills, K. N., Tzukerman, M., Zhang, X.-K., & Pfahl, M. (1989) *Nature 340*, 653−656.
Haddad, I. A., Ordovas, J. M., Fitzpatrick, T., & Karanthanasis, S. K. (1986) *J. Biol. Chem. 261*, 13268−13277.
Ichikawa, K., & DeGroot, L. J. (1986) *J. Biol. Chem. 261*, 16540−16546.
Ikeda, M., Rhee, M., & Chin W. W. (1994) *Endocrinology 135*, 1628−1638.
Kesavan, P., Mukhopadhayay, S., Murphy, S., Rengaraju, M., Lazar, M. A., & Das, M. (1991) *J. Biol. Chem. 266*, 10282−10286.
Kliewer, S. A., Umesono, K., Manglesdorf, D. J., & Evans, R. M. (1992) *Nature 355*, 446−449.
Larsen, P. R. (1981) *Prog. Clin. Biol. Res. 74*, 81−83.
Lazar, M. A., & Berrodin, T. J. (1990) *Mol. Endocrinol. 4*, 1627−1635.
Leidig, F., Shepard, A. R., Zhang, W. G., Steller, A., Cattini, P. A., Baxter, J. D. & Eberhardt, N. L. (1992) *J. Biol. Chem. 267*, 913−921.
Mahley, R. W., Innerarity, T. L., Rall, S. C., Jr., & Weisgraber, K. H. (1984) *J. Lipid Res. 25*, 1277−1294.
Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A., & Evans, R. M. (1992) *Genes Dev. 6*, 329−344.
Maniatis, T., Fritsch, E. F., & Sambrooke, J. (1983) *Molecular cloning: A Laboratory Manual*, pp 464−465, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miyamoto, T., Suzuki, S., DeGroot, L. J. (1994) *Mol. Cell. Endocrinol. 102*, 111−117.
Petty, K. J., Desvergne, B., Mitsuhashi, T., & Nikodem, V. M. (1990)) *J. Biol. Chem. 265*, 7395−7400.
Romney, J. S., Chan, J., Carr, F. E., Mooradian, A. D., & Wong, N. C. (1992) *Mol. Endocrinol. 6*, 943−950.
Rosenthal, N. (1987) *Methods Enzymol. 152*, 704−720.
Rubin, E. M., Krauss, R. M., Spangler, E. A., Verstuyft, J. G., & Clift, S. M. (1991) *Nature 353*, 265−267.
Sanders, M. M., & McKnight, G. S. (1988) *Biochemistry 27*, 6550−6557.
Sap, J., Muñoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz., A, Beug, H., & Vennström, B. (1986) *Nature 324*, 635−640.
Schweers, L. A., Frank, D. E., Weigel, N. L., & Sanders, M. M. (1990) *J. Biol. Chem. 265*, 7590−7595.
Shepard, A. R., & Eberhardt, N. L. (1993) *Clin. Lab. Med. 13*, 531−541.
Sorci-Thomas, M., Prack, M. M., Dashti, N., Johnson, F., Rudel, L. L., & Williams, D. L. (1989) *J. Lipid Res. 30*, 1397−1403.
Staels, B., van Tol, A., Chan, L., Will, H., Verhoeven, G., & Auwerx, J. (1990) *Endocrinology 127*, 1144−1152.
Stamler, J., Wentworth, D., & Neaton, J. D. (1986) *J. Am. Med. Assoc. 256*, 2823−2828.
Strobl, W., Chan, L., & Patsch, W. (1992) *Atherosclerosis 97*, 161−170.
Sudhof, T. C., Russell, D. W., Brown, M. S., & Goldstein, J. L. (1987) *Cell 48*, 1061−1069.
Suzuki, S., Miyamoto, T., Opsahl, A., Sakarai, A., & DeGroot, L. J. (1994) *Mol. Endocrinol. 8*, 305−314.
Taylor, P. A., & Ward, A. (1993) *Arch. Intern. Med. 153*, 1178−1184.
Trainer, T. D., & Howard, P. L. (1983) *Crit. Rev. Clin. Lab. Sci. 19*, 135−171.
Umesono, K., Murakami, K. K., Thomson, C. C., & Evans, R. M. (1991) *Cell 65*, 1255−1266.
Wahlström, G. H., Sjöberg, M., Andersson, M., Nordström, K., & Vennström, B. (1992*) Mol. Endocrinol. 6*, 1013−1022.
Widom, R. L., Ladias, J. A. A., Kouidou, S., & Karathanasis, S. K. (1991) *Mol. Cell. Endocrinol. 11*, 677−687.
Wong, N. C., & Oppenheimer, J. H. (1986) *J. Biol. Chem. 261*, 10387−10393.
Wright, P. A., Crew, M. D., & Spindler, S. R. (1987)) *J. Biol. Chem. 262*, 5659−5663.
Wuarin, J., Mueller, C., & Schibler, U. (1990) *J. Mol. Biol. 214*, 865−874.
Yen, P. M., & Chin, W. W. (1994) *Trends Endocrinol. Metab. 5*, 65−72.
Zhang, X.-k., Hoffmann, B., Tran, P. B., Graupner, G., & Pfahl, M. (1992) *Nature 355*, 441−446.
Zilz, N. D., Murray, M. B., & Towle, H. C. (1990) *J. Biol. Chem. 265*, 8136−8143.

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