Ultraspiracle, a *Drosophila* Retinoic X Receptor α Homologue, Can Mobilize the Human Thyroid Hormone Receptor To Transactivate a Human Promoter†

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ABSTRACT: We have analyzed the functional domains of the *Drosophila* orphan receptor Ultraspiracle (usp), a homologue of the vertebrate retinoic X receptor α, as well as the ability of heterodimers between usp and the thyroid hormone receptor *β* ($T_3R\beta$) to transactivate the human apolipoprotein A-II (apoA-II) promoter. DNA binding assays demonstrated that heterodimers of usp and the human $T_3R\beta$ can bind to the hormone response element (HRE) of the regulatory element AIIJ (−734 to −716) of the human apoA-II promoter. Cotransfection experiments have shown that the combination of usp and $T_3R\beta$ can transactivate the human apoA-II promoter in COS-1 cells 7−8-fold in the presence of thyroid hormone (T_3). The observed transactivation was not affected by the deletion of the amino-terminal residues 1−85 of usp, which represent a putative transactivation domain, suggesting that the function of usp is to recruit $T_3R\beta$. Furthermore, a mutant usp, with impaired DNA binding properties, can form heterodimers with $T_3R\beta$ *in vitro* but has reduced ability to transactivate the human apoA-II promoter. A minimal thymidine kinase (tk) promoter driven by four AIIJ regulatory elements is repressed to 20% of its original activity by $T_3R\beta$ and the repression is relieved by usp/$T_3R\beta$ heterodimers. Deletion analysis demonstrated that factors bound to the regulatory elements AIIJ, AIIAB, and AIIH participate in the usp/$T_3R\beta$-mediated transactivation of the human apoA-II promoter. Similarly to element AIIJ, element AIIAB binds usp/$T_3R\beta$ heterodimers, whereas element AIIH binds a COS-1 nuclear activity that is supershifted with anti-hepatic nuclear factor 1 antibodies. The findings suggest that optimal transactivation of the apoA-II promoter by usp/$T_3R\beta$ heterodimers requires complex interactions between these heterodimers and factors bound to other regulatory elements. The observed transcriptional activation through heterodimer formation between nuclear receptors from species as divergent in the evolutionary scale as insects and mammals indicates that the functional domains of these proteins have been highly conserved.

Nuclear hormone receptors represent a superfamily of transcription factors. A few members can be activated by hormones such as steroids, retinoids, T_3,[1] vitamin D, or ecdysone, whereas the ligand for the majority of them remains unknown (Chambon, 1996; Thummel, 1995). All members of the family contain five distinct domains, designated A−E. Domain C is involved in DNA binding and is highly conserved throughout evolution and among different members (Luisi et al., 1991). Domain E is involved in ligand binding, dimerization, and ligand-dependent transcriptional activation (Thummel, 1995; Luisi et al., 1991; Qi et al., 1995; Zhang et al., 1992a; Froman & Samuels, 1990), whereas domain A/B is involved in ligand-independent transcriptional activation. Domains A/B and the E are the least conserved (Thomson & Evans, 1989; Nagpal et al., 1992, 1993). Homo- or heterodimers of hormone nuclear receptors recognize hormone response elements (HRE), which contain a consensus AGG/TTCA half-site motif. The recognition sequence of the dimers may consist of direct, inverted, or palindromic half-site motifs (Umesono et al., 1991; Ladias et al., 1992; Giguere, 1994; Mangelsdorf et al., 1994; Glass, 1994). It has been proposed that the spacing between the half-sites contributes to the selection of specific sets of homo- or heterodimers (Umesono et al., 1991). Several members of the nuclear hormone receptor superfamily have been identified in *Drosophila*. Some of them appear to have vertebrate homologues. This includes the seven-up (svp) (Mlodzik et al., 1990), E75 (Segraves & Hogness, 1990), and ultraspiracle (usp) (Shea et al., 1990; Oro et al., 1990; Henrich et al., 1990) genes, which have homologies to human COUP-TF, EAR-1, and RXR, respectively. We have shown previously that the *Drosophila* usp and the mammalian $T_3R\beta$ can form heterodimers *in vitro* that can recognize the regulatory element AIIJ of the human apoA-II promoter (Khoury Christianson et al., 1992). In the present study, cotransfection experiments showed that heterodimers of usp/$T_3R\beta$ can transactivate the human apoA-II

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[1] Abbreviations: apoA-II, apolipoprotein A-II; EcR, ecdysone receptor; HNF-1, hepatic nuclear factor 1; HNF-4, hepatic nuclear factor 4; HRE, hormone response element; usp, ultraspiracle; RXRα, retinoic X receptor α; RARα, retinoic acid receptor α; T_3, triiodothyronine; $T_3R\beta$, thyroid hormone receptor *β*; ARP-1, apoA-I regulatory protein 1; DMS, dimethylsulfate; tk, thymidine kinase; SDS, sodium dodecyl sulfate; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; wt, wild type.

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promoter. Transactivation is reduced by alteration of the DNA binding domain of usp but not by the deletion of the amino-terminal residues 1−85 residues. The findings show that transactivation requires heterodimer formation between usp and $T_3R\beta$, which bind to their cognate sites on the apoA-II, and indicate that the transactivation function is provided by the $T_3R\beta$ moiety of the usp/$T_3R\beta$ heterodimer.

EXPERIMENTAL PROCEDURES

Materials

T_4 DNA ligase, polynucleotide kinase, and restriction enzymes were purchased from New England Biolabs. Transformation-competent bacterial cells DH5α were purchased from Bethesda Research Laboratories. [γ-^{32}P]ATP (5000 Ci/mmol), [α-^{32}P]dNTPs, [^{3}H]acetyl coenzyme A (200 mCi/mmol), and Econofluor scintillation fluid were purchased from DuPont−New England Nuclear. Reagents for automated DNA synthesis were purchased from Applied Biosystems, Inc. The sequencing kit was purchased from U.S. Biochemical Corp. Bactotryptone and bacto yeast extract were purchased from Difco. Chloramphenicol *O*-nitrophenyl-β-D-galactopyranoside, T_3, retinoic acid, Hepes free acid, tetramethylethylenediamine (TEMED), and mercaptoethanol were purchased from Sigma. The plasmid pCH110, double-stranded poly(dI-dC), chloramphenicol acetyltransferase (CAT), β-galactosidase, and dNTPs were purchased from Pharmacia LKB Biotechnology Inc. The anti-FLAG antibody M2, acrylamide, sodium dodecyl sulfate (SDS), Tris, and urea were purchased from International Biotechnologies, Inc. Taq polymerase, the 5× reporter lysis buffer, and the *in vitro* transcription−translation coupled reticulocyte lysate system were purchased from Promega. The plasmid pBluescript KS± was purchased from Stratagene. Immobilon-P membranes were purchased from Millipore.

Methods

Oligonucleotide Synthesis. Oligonucleotides were synthesized by the solid-phase phosphite triester method using an automated oligonucleotide synthesizer (Applied Biosystems, Model 380-B). The oligonucleotides were purified by electrophoresis on 20% polyacrylamide/7 M urea gels and labeled with either [γ-^{32}P]ATP or [α-^{32}P]dNTPs.

Plasmid Construction. The wild-type and mutant −911/+29 apoA-II CAT constructs containing the wild-type (wt) human apoA-II promoter sequences or deletions of the regulatory elements A−M have been described (Chambaz et al., 1991; Cardot et al., 1993). The construct containing the minimal thymidine kinase (tk) promoter under the control of four copies of the AIIJ element (J4tk) was generated by using pBLCAT2 as a vector (Luckow & Schutz, 1987). This plasmid derivative contains the herpes simplex virus tk promoter sequence from −105 to +51 nucleotide fused upstream of the coding region of the CAT gene. A synthetic double-stranded oligonucleotide corresponding to the AIIJ −739 to −716 bp region (Table 1) and carrying an *Xba*I half-site was phosphorylated and cloned into the *Xba*I site of the polylinker region upstream of the tk promoter in the pBLCAT2 plasmid. Plasmid construction was verified by DNA sequencing and contains three copies of the AIIJ sequence in the 5′ to 3′ and one in the 3′ to 5′ orientation. RARα (Giguere et al., 1987), RXRα (Mangelsdorf et al., 1990), and $T_3R\beta$ (Weinberger et al., 1986) full-length cDNA sequences cloned into the *Eco*RI site of the pMT2 eukaryotic expression vector have been described (Ladias et al., 1992). The *Hin*dIII−*Xba*I usp sequence was excised from the pcDNA-I plasmid (Khoury Christianson et al., 1992), was blunt-ended with the Klenow fragment of DNA polymerase I, and was cloned into the *Bam*HI site of the pSG5 expression vector (Stratagene) to generate the pSG5-usp plasmid. The inserted sequence contained the *Pst*I, *Eco*RV, *Bst*XI, *Not*I, *Xho*I, *Sph*I, and *Nsi*I polylinker sites of the parent plasmid on its 3′ end, thus generating a modified pSG5 vector. Two additional eukaryotic expression vectors were generated using PCR amplification and the above modified pSG5 vector. The *Kpn*I−*Pst*I fragment, containing the usp coding sequence, was excised from the pSG5-usp plasmid. Either an ATG or a FLAG cassette was inserted into the *Kpn*I−*Pst*I sites of the remaining vector to create pSG5-ATG or pSG5-FLAG derivatives, respectively. The *Kpn*I−*Pst*I ATG cassette provided a strong translation initiation sequence (Kozak, 1989) that contains the consensus Kozak translation initiation sequence (Table 1, underlined). To generate the ATG cassette, the single-stranded ATG KP oligonucleotide of Table 1 was used as a template for Taq polymerase in one cycle of PCR synthesis using the oligonucleotide F3PR of Table 1 (complementary to the 3′ end of the ATGKP) as primer. The FLAG epitope cassette was created in the same way, using the single-stranded oligonucleotide FLAG of Table 1 as a template in one cycle of PCR synthesis by the Taq polymerase employing the F3PR oligonucleotide of Table 1 as a primer. The FLAG sequences contain the consensus Kozak ATG translation initiation sequence (Table 1, underlined) and the FLAG epitope (DYKDDDDK) encoding sequence, which is recognized by anti-FLAG antibodies. After synthesis of the complementary strand of the ATGKP and FLAG, the double-stranded oligonucleotide was digested with *Kpn*I and *Pst*I restriction enzymes, purified through a low-melting agarose gel (2%), and ligated to *Kpn*I−*Pst*I sites of the modified pSG5 vector. The sequences of the resulting pSG5-ATG and pSG5-FLAG plasmids were verified by DNA sequencing. To generate usp86−508, the usp derivative that lacks amino acids 1−85, pSG5-usp was digested with *Pst*I. The *Pst*I fragment, containing the DNA and the ligand binding domains of usp,was subcloned into the *Pst*I site of the pSG5-ATG vector. Two subcloning steps were required to generate the pFLAG-$T_3R\beta$ derivative. The amino-terminal fragment of $T_3R\beta$ encoding amino acids

Table 1: Oligonucleotides Used as Primers for PCR Amplification and Mutagenesis and in Plasmid Constructions

name	sequence
Ja	ctag(−715)TCTACCAGGGTAAAGGTTGAA-GGCA(−739)
Js	ctag(−739)TGCCTTCAACCTTTACCCTGGT-AGA(−715)
ATGKP	ggggGTACCATGGAGTTAACACGTGCTG-CAgggg
F3PR	ccccTGCAGCACGTGTTAAC
FLAG	ggggGTACCATGGACTACAAGGACGACGATGACA-AGTTAACACGTGCTGCAgggg
TRNH4D	ATCGAGCTAGTCCAAGTGGTC
TRNH4U	GTGAAAATGGCCTTACAGCTTG
mutPD	CCTTGCAGCCCTCACAG-TGTACACGCC
mutPU	GCTGCAAGGGCTTCG*TTTAAACGCACA
KS	TCGAGGTCGACGGTATC
T7	GTAATACGACTCACTATAGGGC

3−61 was amplified by PCR using the pMT-$T_3R\beta$ as a template and the oligonucleotides of Table 1 TRNH4U (which contains a *Pml*I half-site) and TRNH4D (which converts an *Ssp*I site to an *Eco*RV half-site) as sense and antisense primers, respectively. The resulting PCR fragment was treated with T4 DNA polymerase and subcloned into pSG5-FLAG vector digested with *Pml*I and *Eco*RV to generate pFLAG-T03−61 plasmid. The $T_3R\beta$ cDNA sequence encoding amino acids 62−456 of $T_3R\beta$ was excised with *Ssp*I−*Eco*RI digestion, blunted with the Klenow fragment of DNA polymerase I, and inserted into the *Eco*RV site of plasmid pFLAG-T03−61 to generate the pFLAG-$T_3R\beta$ derivative.

The mutant usp derivative containing mutations in the DNA binding domain of usp (uspmutP) was generated as follows. Sequences corresponding to the DNA binding domain of usp were excised from the pSG5-usp plasmid by *Pst*I/*Bam*HI digestion and subcloned into the corresponding sites of the pBluescript KS± plasmid (Stratagene) to generate the pKS-usp-DBD derivative. This derivative was utilized as a template for PCR-based mutagenesis using two sets of mutagenic and two sets of external amplification primers. The sequences of the mutagenic primers designated mutPD and mutPU with 9-nucleotide overlapping sequences and the external 5′ and 3′ primers corresponding to vector sequence KS and T7, respectively, are described in Table 1. The mutPU primer corresponds to the coding sequence of usp and carries a single base deletion (indicated by − in Table 1), leading to a frameshift mutation, whereas the mutPD primer corresponds to the noncoding region of usp with a single base insertion (indicated by * in Table 1), which restores the correct reading frame. The 5′ KS primer and the mutPD mutagenic primer were used to amplify the 5′ region of the DNA binding domain of usp. The 3′ T7 primer and the mutPU mutagenic primer were used to amplify the 3′ region of the DNA binding domain of usp. The two amplified fragments were purified and combined for a second round of amplification using the external amplification primers KS and T7. The overlapping sequence of the mutagenic primers allows annealing of the primary PCR products after mixing, denaturation, and reannealing. The reannealed sequences with recessed 3′ ends were initially filled with Taq polymerase, thus generating the new template for PCR amplification using the external primers. The amplified fragment alters the amino acid sequences SCEGCK-GF of usp to TVRAARAS. This fragment, containing the mutagenized DNA binding domain of usp, was digested with *Pst*I/*Bam*HI, purified, and cloned into the corresponding sites of pBlueScript KS± to generate pKSmutP derivative. The pSG5-usp plasmid was digested with *Pst*I to excise the carboxy-terminal sequence of usp and the remaining vector containing the usp cDNA fragment encoding amino acids 1−86 was religated to generate the pU1−86 usp derivative. This derivative was linearized by *Xho*I digestion and was blunted by treatment with the Klenow fragment of DNA polymerase I to generate the pU1−86X derivative. The mutated *Pst*I/*Bam*HI fragment of usp was excised from pKSmutP derivative and inserted in-frame into the *Pst*I/*Bam*HI sites of pU1−86X plasmid to generate the pUX-mut derivative. Finally, the carboxy-terminal fragment of usp encoding residues 87−508 and containing the 3′ untranslated region was excised with *Bam*HI digestion from pSG5usp and cloned in-frame into the *Bam*HI site of the pUX-mut derivative to generate the puspmutP derivative. Plasmid integrity was verified by DNA sequencing.

Gel electrophoretic Mobility Shift Assay. Whole-cell extracts were prepared from COS-1 cells transfected with the pMT2 vector carrying the full-length cDNA for $T_3R\beta$ or a pSG5 vector carrying usp as described (Kumar & Chambon, 1988). Gel electrophoresis mobility shift assays were performed as described (Fried & Crothers, 1981).

Dimethylsulfate and Potassium Permanganate Interference Assays. The probe for the methylation interference was a double-stranded oligonucleotide AIIJ end-labeled at the noncoding strand. For the permanganate interference assay, the same probe was labeled on the coding strand. The interference assays were performed as described (Tzameli & Zannis, 1996).

Transient Transfection Experiments and CAT Assays. Monolayers of COS-1 cells were maintained as stocks in DME medium supplemented with either 10% fetal calf serum or charcoal-stripped 5% fetal calf serum. Cells (0.3×10^6) were plated on 30 mm dishes and the following day were transfected using the calcium phosphate/DNA coprecipitation method. A total of 8.5 μg of plasmid DNA was used, containing 5 μg of the wild-type or mutant apoA-II-CAT or the J4tk reporter plasmid, 2 μg of phosphoglycerate kinase β-galactosidase plasmid (gift of Dr. Fulvio Mavilio; PGK-β-gal) or 1 μg of SV40 β-galactosidase (PCH110-β-Gal) plasmid to be used as internal control and various concentrations of pMT2 or pSG5 expression plasmids carrying the cDNAs of RARα and $T_3R\beta$ or usp wt and mutants, respectively. pBluescriptII KS± was used as carrier DNA. Sixteen hours posttransfection, COS-1 cells were rinsed and fresh medium was added, supplemented with 5% charcoal-stripped serum, in the absence or presence of 10^{-7} M T_3 or retinoic acid. The cells were harvested 24 h after treatment with hormone in TEN buffer (40 mM Tris, pH 7.4, 1 mM EDTA, and 150 mM NaCl) and lysed in 200 μL of 1× reporter lysis buffer (Promega). An aliquot of the cell extracts was heated at 65 °C for 10 min prior to the CAT assays. The assays were performed in a 7 mL plastic scintillation vial, in a total volume of 250 μL, containing 100 mM Tris-HCl, pH 7.8, 1 mM chloramphenicol, 0.25 μCi of ^{3}H-labeled acetyl coenzyme A, and 10−50 μL of extracts. One blank sample and one sample containing 5 milliunits of purified CAT enzyme were always included. The reaction mixture was overlaid with 4 mL of water-immiscible scintillation fluid and incubated at 37 °C for 2−4 h before it was counted in a liquid scintillation counter. The amount of active CAT enzyme in the cell extracts was determined according to the radioactivity counted in the samples containing the cell extracts and in the sample containing 5 milliunits of purified CAT enzyme. The background values of the blank sample were always subtracted from the counts per minute values of the different samples. Each experiment was repeated 2−5 times in duplicate or triplicate. The β-galactosidase activity of the cell lysates was determined spectrophotometrically by monitoring the hydrolysis of the synthetic substrate *o*-nitrophenyl galactoside, at 410 nm. Control samples containing 0.5, 2, and 10 milliunits of purified β-galactosidase allowed the conversion of OD units in the different samples into β-galactosidase units. The β-galactosidase activity of the cell extracts was utilized to normalize for variability in the efficiency of transfection.

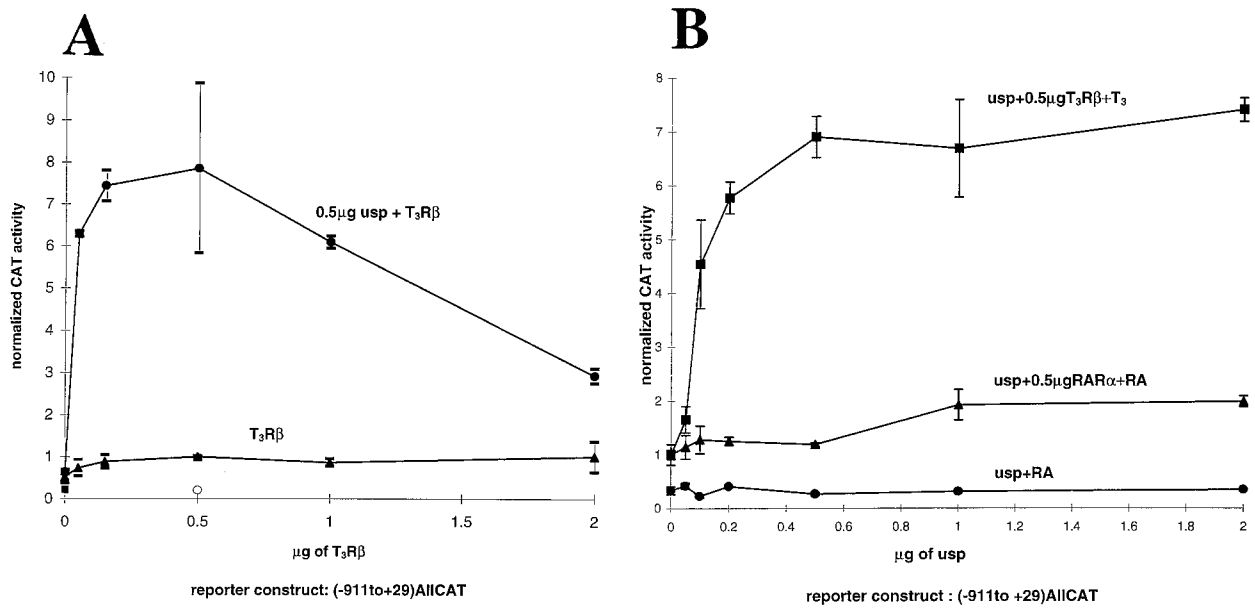

FIGURE 1: A,B. (A) Transactivation of apoA-II promoter by usp/$T_3R\beta$ heterodimers in COS-1 cells. Cotransfection experiments of COS-1 cells with vectors expressing $T_3R\beta$ and usp. ● indicates that cotransfection titration experiments were performed with constant amounts of the usp plasmid (0.5 μg), constant amounts of the reporter plasmid −911 to +29 apoA-II CAT (5 μg), and increasing concentrations of the $T_3R\beta$ plasmid (0.05−2 μg). Titrations were performed in the presence of 0.1 μM thyroid hormone (T_3). ▲ indicates cotransfection experiments with increasing amounts of $T_3R\beta$ in the presence of T_3 and the −911 to +29 apoA-II CAT plasmid. ■ indicates cotransfection experiments with either usp or $T_3R\beta$ alone in the absence of T_3. ○ indicates cotransfection experiments with usp + $T_3R\beta$ in the absence of T_3. CAT activity of apoA-II wt + T_3 corresponds to 0.941 milliunit of CAT/milliunit of βGal. Note that optimal transactivation is achieved in the range 0.25−0.50 μg of $T_3R\beta$ and 0.5 μg of usp in the presence of T_3. The $T_3R\beta$ alone did not transactivate the apoA-II promoter in the presence or absence of T_3. (B) Cotransfection experiments of COS-1 cells with vectors expressing usp and $T_3R\beta$ or usp and RAR. Cotransfection titration experiments were performed with constant amounts of the reporter plasmid −911 to +29 apoA-II CAT (5 μg) and increasing concentrations of the usp plasmid alone (0.05−2 μg) (●) or constant amounts of $T_3R\beta$ (0.5 μg) and increasing amounts of usp (■) or constant amounts of RARα plasmid (0.5 μg) (▲) and increasing amounts of usp. Titrations were performed in the presence of 0.1 μM thyroid hormone (T_3) or 1 μM retinoic acid (RA) as indicated. Note that optimal transactivation is achieved with 0.5 μg of usp for the usp/$T_3R\beta$ heterodimers and 1 μg of usp for the usp/RAR heterodimers. Usp alone did not transactivate the apoA-II promoter. The normalized CAT activity in this figure and Figures 7−9 represent the ratio as milliunits of CAT/milliunit of βGal. CAT enzyme and βGal enzyme of known specific activity were analyzed in parallel for each set of assays. RAR alone caused approximately 3.5-fold transactivation of the apoA-II promoter; a small (1.5-fold) additional increase in transactivation occurred with usp/RAR heterodimers.

In vitro Transcription, Translation, Immunoprecipitation, SDS−Polyacrylamide Gel Electrophoresis, and Immunoblotting. Usp, uspmutP, and $T_3R\beta$ were transcribed and translated *in vitro* by using rabbit reticulocyte lysates in the presence or absence of [^{35}S]methionine as specified by the manufacturer (TNT kit, Promega). Equal volumes of unlabeled and labeled proteins were mixed in buffer containing 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 80 mM KCl, 2.5 mM $MgCl_2$, 5% glycerol, 0.05% Triton X-100, and 0.5% BSA (w/v) and precleared with protein γ−Sepharose beads (Pharmacia) for 1 h at 4 °C. The cleared proteins were incubated with either a rat polyclonal antibody raised against the ligand binding domain of usp (a2T) or a murine IgG monoclonal antibody against FLAG fusion protein (M2 or IBI) for 2 h at 4 °C, followed by incubation with protein γ−Sepharose beads for 1 h at 4 °C. Precipitated material was washed 5 times, eluted from the beads, and analyzed by SDS−polyacrylamide gel electrophoresis followed by autoradiography. For immunoblotting following electrophoresis, the proteins were electrotransferred onto PVDF Immobilon-P membranes in 25 mM Tris-HCl, 0.192 M glycine, and 20% (v/v) methanol. The membrane was washed twice for 30 min each time in TBS/Tween-20 buffer (10 mM Tris-HCl pH 7.4, 0.9% NaCl, and 0.05% Tween-20) containing 5% dry milk. The membranes were then incubated with a 1:500 dilution of the primary anti-rat usp polyclonal antibody for 1 h at room temperature and were subsequently washed three times, 15 min each, with TBS/Tween-20. The membrane was then treated for 1 h at room temperature with anti-mouse IgG secondary antibody conjugated to horseradish peroxidase, and washed three times, 15 min each, with TBS/Tween-20 and twice with H_2O. The protein bands were detected by incubation with 0.12 mg/mL diaminobenzidine tetrahydrochloride and 0.03% H_2O_2 in 10 mL of H_2O for 5 min. The membranes were then rinsed with H_2O and dried.

RESULTS

Drosophila Protein usp Can Heterodimerize with Human $T_3R\beta$ and Transactivate the ApoA-II Promoter in COS-1 Cells. We have previously shown that usp, the *Drosophila* homologue of RXR, can form heterodimers with the human nuclear receptors $T_3R\beta$ and RARα and generate a novel DNA binding activity that recognizes the element AIIJ of the apoA-II promoter (Khoury Christianson et al., 1992). In order to test the activity of these heterodimers *in vivo*, we cotransfected monkey kidney cells (COS-1) with the −911 to +29 apoA-II promoter CAT plasmid and plasmids expressing the *Drosophila* usp and the human $T_3R\beta$. This analysis showed that the combination of *Drosophila* usp and the human $T_3R\beta$ transactivated the human apoA-II promoter 8-fold in the presence of thyroid hormone (T_3) but failed to transactivate in the absence of T_3 (Figure 1A). Control experiments showed that usp alone did not transactivate the apo-AII promoter and $T_3R\beta$ had a small effect on the apoA-II promoter activity in the presence of T_3. In addition,

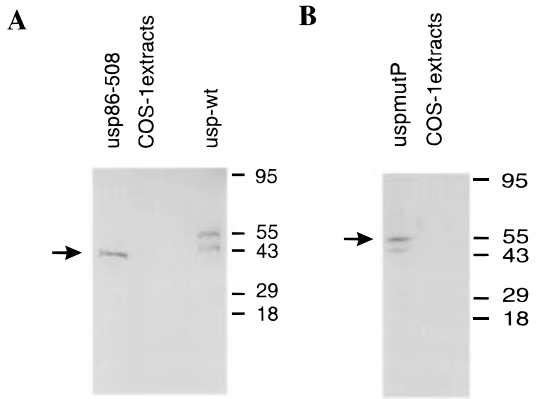

FIGURE 2: A-B. Expression of mutant usp forms. The first mutant lacks the amino-terminal residues 1−85 (usp86−508 mutant). The second mutant carries the following amino acid substitutions (SCEGCKGF to TVRAARAS) in the P box (uspmutP mutant). (A). Western blot analysis of extracts of COS-1 cells transiently transfected with expression plasmids carrying either the wild-type usp or the usp86−508 cDNA sequence. The blot was treated with rat anti-usp polyclonal antibody, followed by incubation with an anti-mouse IgG secondary antibody conjugated to horseradish peroxidase. Note that the wild-type (wt) usp produces two protein bands and the usp86−508 produces one band that migrates slower than the wt usp. (B). Western blot analysis of extracts of COS-1 cells transiently transfected with expression plasmid carrying the mutated form of usp (uspmutP), or extracts of mock-transfected COS-1 cells (COS-1 extracts). The blot was treated as described in panel A. Note that the uspmutP produces two protein bands, as the wt usp shown in panel A. These bands are absent in the mock-transfected COS-1 extracts.

cotransfection with usp/RARα did not efficiently transactivate the apoA-II promoter in the presence of *all-trans*-retinoic acid (RA), despite the fact that usp/RARα heterodimers can bind to the regulatory element AIIJ *in vitro* (Figure 1B) (Khoury Christianson et al., 1992). Usp alone had no effect on the apoA-II promoter activity in the presence of RA, and RARα alone resulted in approximately 3.5-fold transactivation in the presence of RA (Figure 1B).

Analysis of the DNA Binding Properties and the Transactivation Potential of Heterodimers of Wild-Type $T_3R\beta$ and Mutant usp Forms. The amino-terminal domain of nuclear hormone receptors has been implicated in ligand-independent transactivation (Nagpal et al., 1992, 1993). In addition, the P box, present in the first zinc finger of the DNA binding domain of nuclear receptors, has been shown to determine the DNA binding specificity of different pairs of hormone nuclear receptors (Umesono et al., 1991). To determine the importance of the amino-terminal domain of usp in the observed transactivation of the human apoA-II promoter as well as the requirement of DNA binding of usp for the observed transactivation, we generated two mutant usp forms. The first mutant, designated usp86−508, lacks the amino-terminal residues 1−85, and the second mutant, designated uspmutP, carries an eight amino acid substitution in the P-box (SCEGCKGF to TVRAARAS). The expression of usp86−508 and uspmutP in COS-1 was tested by immunoblotting using lysates of 0.2 million cells expressing the wild-type (wt) or the two usp mutants. This analysis showed that the levels of expression of the wt and mutant usp forms are similar (Figure 2). The ability of the DNA binding domain mutant (uspmutP) to form heterodimers *in vitro* with $T_3R\beta$ was assessed by immunoprecipitation of the usp/$T_3R\beta$ heterodimers and detection by SDS−PAGE and autoradiography. For this purpose the wt usp or the uspmutP was

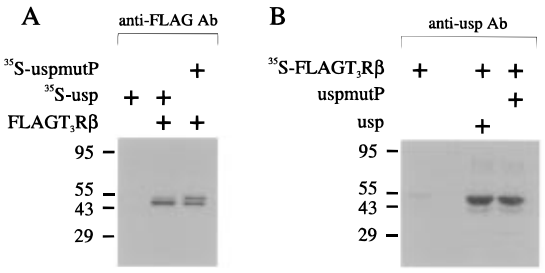

FIGURE 3: A-B. SDS−polyacrylamide gel electrophoresis and autoradiographic detection of ^{35}S-labeled usp/$T_3R\beta$ or uspmutP-$T_3R\beta$ dimer following immunoprecipitation. (A). Wild-type usp or uspmutP was labeled with ^{35}S-methionine *in vitro* and was mixed with unlabeled $T_3R\beta$ containing the FLAG epitope (FLAG$T_3R\beta$), produced by *in vitro* transcription−translation assay. The mixture was immunoprecipitated with anti-FLAG antibody and analyzed with SDS−PAGE and autoradiography. (B). $T_3R\beta$ was labeled with ^{35}S-methionine *in vitro* and was mixed with unlabeled wt usp or uspmutP, produced by *in vitro* transcription−translation. The protein complex was immunoprecipitated with anti-usp polyclonal antibody and analyzed with SDS−PAGE and autoradiography as described in Methods. Note that $T_3R\beta$ can form heterodimers in solution with either wt usp or uspmutP (compare the last two lanes of panels A and B).

labeled with ^{35}S-methionine using *in vitro* transcription−translation. The ^{35}S-labeled usp or uspmutP protein was mixed with unlabeled $T_3R\beta$ containing the FLAG epitope, produced by *in vitro* transcription−translation. The protein complex was immunoprecipitated with an anti-FLAG antibody, resolved by SDS−PAGE, and detected by autoradiography (Figure 3A). Similarly, the $T_3R\beta$ was labeled with ^{35}S-methionine by *in vitro* transcription−translation. The ^{35}S-labeled $T_3R\beta$ protein was mixed with either unlabeled wt usp or uspmutP, produced by *in vitro* transcription−translation. The protein complex was immunoprecipitated with anti-usp polyclonal antibody, resolved by SDS−PAGE, and detected by autoradiography (Figure 3B). This analysis showed that the mutation in the P-box of usp did not affect its ability to heterodimerize with $T_3R\beta$ (Figure 3; compare the last two lanes of panels A and B).

DNA binding gel electrophoresis assays showed that DNA binding of the usp/$T_3R\beta$ heterodimers is not affected by deletion of the 1−85 amino-terminal residues (Figure 4, lane 11), whereas the mutation in the P-box abolished the DNA binding of the usp/$T_3R\beta$ heterodimer *in vitro* (Figure 4, lane 8). Additionally, DNA binding of the usp/$T_3R\beta$ complex does not require T_3 (Figure 4, lanes 5, 6, 11, and 12). Cotransfection experiments using the mutant usp forms showed that the deletion of residues 1−85 of usp (usp86−508 mutant) did not affect its ability to transactivate the apoA-II promoter (Figure 5). In contrast, cotransfection with the DNA binding domain mutant (uspmutP mutant) resulted in significantly lower transactivation of the apoA-II promoter as compared to that achieved with wt usp (Figure 5). As shown in Figure 4, this DNA binding domain mutant has impaired DNA binding properties but can form heterodimers *in vitro* (Figure 3B). Taken together, these findings suggest that the *Drosophila* protein usp can mobilize through heterodimer formation the human $T_3R\beta$ and transactivate the human apoA-II promoter. It appears that the transactivation function is provided by the transcriptional activation domain-(s) of $T_3R\beta$, whereas the DNA recognition sequences *in vitro* are provided by both moieties of the usp/$T_3R\beta$ heterodimer.

usp/$T_3R\beta$ Heterodimers Recognize a DR1 HRE. Previous DNA binding assays and cotransfection experiments identi-

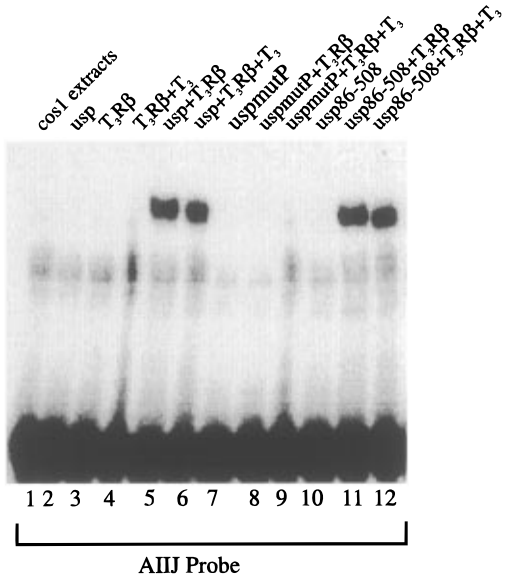

FIGURE 4: DNA binding gel electrophoretic assay using the apoA-II regulatory element AIIJ (−734 to −716) as probe and extracts from COS-1 cells expressing wt and mutant usp forms or $T_3R\beta$. Lanes 1−12 contain the following combinations of COS-1 extracts: (1) 6 μg of mock-transfected COS-1; (2) 4 μg of usp + 2 μg of mock-transfected COS-1; (3) 2 μg of $T_3R\beta$ + 4 μg of mock-transfected COS-1; (4) 2 μg of $T_3R\beta$ + 4 μg of mock-transfected COS-1 + 0.1 μM T_3; (5) 4 μg of usp + 2 μg of $T_3R\beta$; (6) 4 μg of usp + 2 μg of $T_3R\beta$ + 0.1 μM T_3; (7) 4 μg of uspmutP + 2 μg of mock-transfected COS-1; (8) 4 μg of uspmutP + 2 μg of $T_3R\beta$; (9) 4 μg of uspmutP + 2 μg of $T_3R\beta$ + 0.1 μM T_3; (10) 4 μg of usp86−508 + 2 μg of mock-transfected COS-1; (11) 4 μg of usp86−508 + 2 μg of $T_3R\beta$; (12) 4 μg of usp86−508 + 2 μg of $T_3R\beta$ + 0.1 μM T_3. Note that DNA binding requires the presence of both $T_3R\beta$ and usp, is not affected by the deletion of the amino-terminal residues 1−85 of usp, is abolished by a mutation in the DNA binding domain of usp, and does not require T_3.

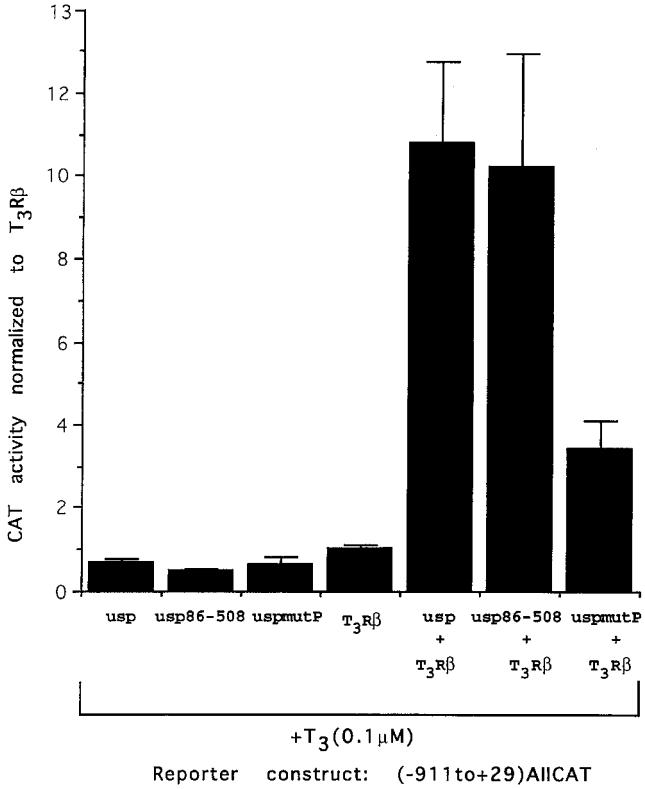

FIGURE 5: Transactivation of the apoA-II promoter by heterodimers of $T_3R\beta$ with either wt usp or the mutated usp forms. COS-1 cells were cotransfected with 5 μg of the −911 to +29 apoA-II CAT construct and 0.25 μg of the plasmids indicated on the *x*-axis [wt usp, usp86−508, uspmutP, $T_3R\beta$, wt usp + $T_3R\beta$ (0.25 μg each), usp86−508 + $T_3R\beta$ (0.25 μg each), and uspmutP + $T_3R\beta$ (0.25 μg each)]. CAT activity for $T_3R\beta$ corresponds to 2.6 milliunits of CAT/milliunit of βGal. Note that the deletion of residues 1−85 of usp does not affect its ability to transactivate the apoA-II promoter. Note also that the mutation in the DNA binding domain reduced significantly the ability of the mutant uspmutP to activate the human apoA-II promoter.

fied the regulatory element AIIJ of the apoA-II promoter as an HRE (Ladias et al., 1992; Khoury Christianson et al., 1992). In order to define the binding site of AIIJ for the usp/$T_3R\beta$ complex, we performed dimethylsulfate and permanganate ($KMnO_4$) interference analysis using AIIJ (Table 2, J_A and J_T) as a probe. Methylation of G residues of the noncoding strand at positions −730, −729, −724, −723, and −722 interfered strongly with DNA binding (Figure 6). Methylation of the G residue at position −733 of the noncoding strand interfered to a lesser extent (Figure 6A). $KMnO_4$ modification of T residues of the coding strand at positions -728, −727, −726, and −721 interfered strongly with DNA binding (Figure 6B). The results of these analyses are summarized in Figure 6C and indicate that the HRE of element AIIJ consists of two direct half-repeats separated by one spacer nucleotide (DR1). Similar to the HRE present in the element AID of apoA-I, this spacer nucleotide participates in DNA−protein interactions with the usp/$T_3R\beta$ heterodimers (Tzameli & Zannis, 1996).

Regulatory Element J of ApoA-II is Necessary but Not Sufficient for the Transactivation of the ApoA-II Promoter by the usp/T3Rβ Heterodimers. To determine whether the regulatory element AIIJ could account for the observed transactivation of the apoA-II promoter by the usp/$T_3R\beta$ heterodimers, we performed cotransfection experiments in COS-1 cells using an apoA-II promoter construct carrying a deletion of element AIIJ (AIIΔJ) (−734 to −716) (Cardot et al., 1993). This analysis showed that the mutant apoA-II promoter which lacks element AIIJ was transactivated only 2-fold by the usp/$T_3R\beta$ heterodimers as compared to the more than 7-fold transactivation observed with the wt promoter (Figure 7).

Table 2: Oligonucleotides Used in DNA Binding and Competition Experiments

name	sequence
Ja	ctag(−715)TCTACCAGGGTAAAGGT-TGAAGGCA(−739)
Js	ctag(−739)TGCCTTCAACCTTTACC-CTGGTAGA(−715)
J_A	aaaa(−742)AGGTGCCTTCAACCTTT-ACCCTGGTAGA(−715)
J_T	tttt(−715)TCTACCAGGGTAAAGGT-TGAAGGCACCT(−742)
alb HNF1	(−70)AGTATGGTTAATGATCTAC-AG(−50)
AIIAB	(−67)AGTCCTGTCACCTGACAG-GGGGTGGGTAAACAGACA(−32)
AIIH	(−572)GTCTCATTACACATTAA-CTC(−553)

To investigate whether the element AIIJ alone can account for the transactivation of the apoA-II promoter by the usp/$T_3R\beta$ heterodimers, a heterologous promoter was constructed where the minimal herpes virus thymidine kinase promoter (tk) was placed under the control of four copies of the element AIIJ (J4tk). The basal activity of this construct was approximately 19-fold higher than that of the intact −911

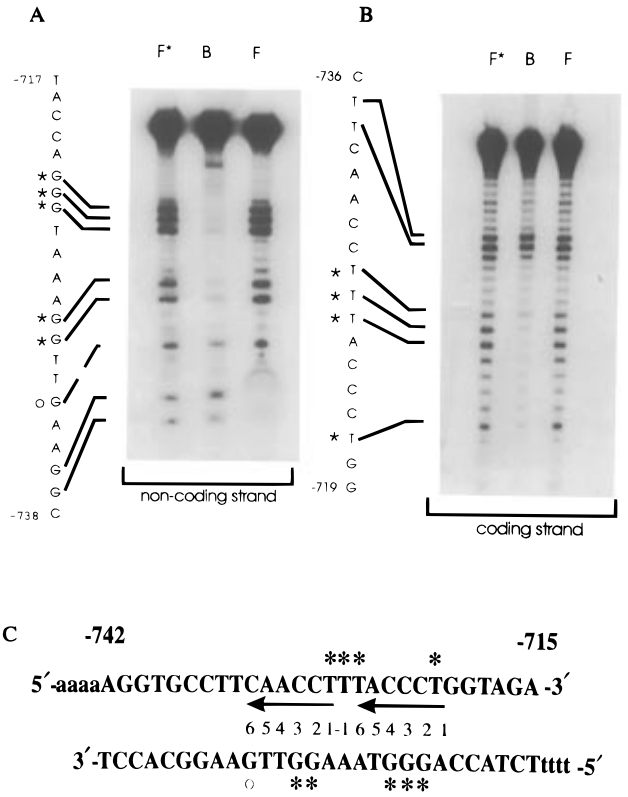

FIGURE 6: A-C. Dimethylsulfate interference and $KMnO_4$ modification patterns of the usp/$T_3R\beta$−DNA complex, obtained with the apoA-II regulatory element J (−734 to −716). (A). Methylation interference patterns of the usp/$T_3R\beta$−DNA complex, obtained with the noncoding strand of a probe spanning element AIIJ. The probe corresponds to nucleotides between −742 and −715 of the apoA-II promoter (Table 2 J_a and J_T). Methylation interference was performed with extracts from COS-1 cells transiently transfected with usp or $T_3R\beta$. F, free DNA before binding; F*, free DNA recovered after binding; B, bound DNA recovered from the DNA−protein complex. The nucleotides involved in strong and weak DNA protein interactions are shown by * or ○, respectively. (B). $KMnO_4$ modification pattern of the usp/$T_3R\beta$-DNA complex obtained with the coding strand of a probe spanning element AIIJ (nucleotides −742 to −715). F, free DNA before binding; F*, free DNA recovered after binding; B, bound DNA recovered from the DNA−protein complex. The asterisks indicate the position of thymine residues that participate in strong DNA−protein interactions with the usp/$T_3R\beta$ heterodimer. The numbers indicate the position of nucleotides in the apoA-II promoter. *Panel C* is a summary of the interference pattern deduced from the findings of panels A and B. The dimethylsulfate and $KMnO_4$ modification patterns show that the HRE of AIIJ has DR1 spacing.

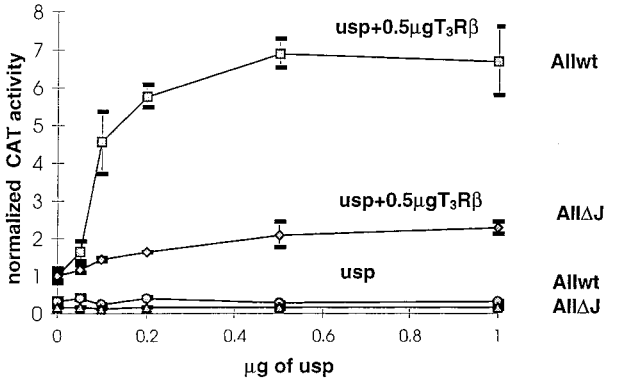

FIGURE 7: Transactivation of the wt apoA-II (−911 to +29) and a mutated apoA-II promoter lacking element AIIJ (apoA-II −911 to +29 ΔJ) by usp/$T_3R\beta$ heterodimers. COS-1 cells were transfected with 5 μg of either wt apoA-II or apoA-II −911 to +29 ΔJ CAT construct, 0.5 μg of $T_3R\beta$ plasmid, and 0.05−1 μg of usp plasmid. □ and ◇ indicate cotransfection of wt and mutant (AIIΔJ promoter), respectively, with usp + $T_3R\beta$ in the presence of T_3. △ and ○ indicate cotransfection of mutant or wt promoter, respectively, with usp alone in the presence of T_3. CAT activity for AIIΔJ is 2.9 milliunits of CAT/milliunit of βGal. Note that deletion of the regulatory element J reduces significantly the transactivation of apoA-II promoter by the usp/$T_3R\beta$ heterodimer.

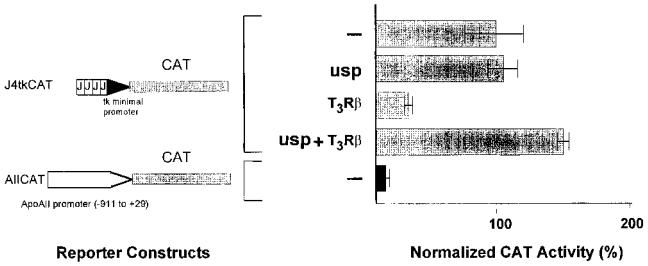

FIGURE 8: Effect of the usp/$T_3R\beta$ heterodimer on the transactivation of a CAT construct containing the thymidine kinase promoter (−105 to +51) under the control of four copies (J4tk) of the regulatory element AIIJ (−734 to −716) of apoA-II. Cotransfection experiments in COS-1 cells were performed with a constant amount of J4tk CAT construct (5 μg), a constant amount of wt usp expression vector (1.5 μg), and a constant amount of $T_3R\beta$ plasmid (0.5 μg), in the presence of T_3 (0.1 μM). The CAT activity of each sample is expressed as percentage of the CAT activity of the J4tkCAT construct. Note that $T_3R\beta$ represses the transcription of the J4tk CAT construct. usp/$T_3R\beta$ heterodimers relieve the $T_3R\beta$-mediated repression. The CAT activity of the J4tk CAT reporter construct in COS-1 cells is 92 milliunits of CAT/milliunit of βGal and is approximately 19-fold higher than the CAT activity of the −911 to +29 AII CAT reporter construct.

to +29 apoA-II promoter in COS-1 cells in the presence of T_3 (Figure 8). Cotransfection with usp alone did not affect the J4tk promoter activity. Cotransfection with $T_3R\beta$ alone repressed the activity of the heterologous J4tk promoter in the presence of T_3. Similar repression is observed in the apoA-I promoter by RXRα/$T_3R\beta$ heterodimers in the presence of T_3 (Tzameli & Zannis, 1996). Cotransfection with usp relieved repression of the heterologous J4tk promoter caused by $T_3R\beta$ and resulted in approximately 1.3-fold transactivation (Figure 8). Since $T_3R\beta$ does not bind to element AIIJ *in vitro* (Figure 4), a plausible interpretation of our findings is that repression of the J4tk construct is due to squelching of endogenous COS-1 activities, probably by the sequestering of the factor(s) into inactive heterodimers with $T_3R\beta$. In the presence of usp, $T_3R\beta$ is driven into heterodimers with the *Drosophila* protein, and the repression is relieved. Thus element AIIJ cannot confer to a heterologous promoter the optimal transactivation that was observed with the wt apoA-II promoter. These findings suggest that interactions of usp/$T_3R\beta$ heterodimers with factors bound to additional apoA-II promoter elements may be required for optimal activity.

Several Regulatory cis-Elements Are Involved in the Transactivation of the Human ApoA-II Promoter by usp/$T_3R\beta$ Heterodimers. To define additional *cis*-elements that may be involved in the transactivation of the apoA-II promoter by the usp/$T_3R\beta$ heterodimers, we performed cotransfection experiments in COS-1 cells using CAT constructs harboring mutants of the −911 to +29 apoA-II promoter lacking previously characterized regulatory elements (Cardot et al., 1993) and plasmids expressing usp and $T_3R\beta$. This analysis showed that deletion of the element AIIJ reduced the promoter strength to approximately 35% of control levels and the magnitude of transactivation to

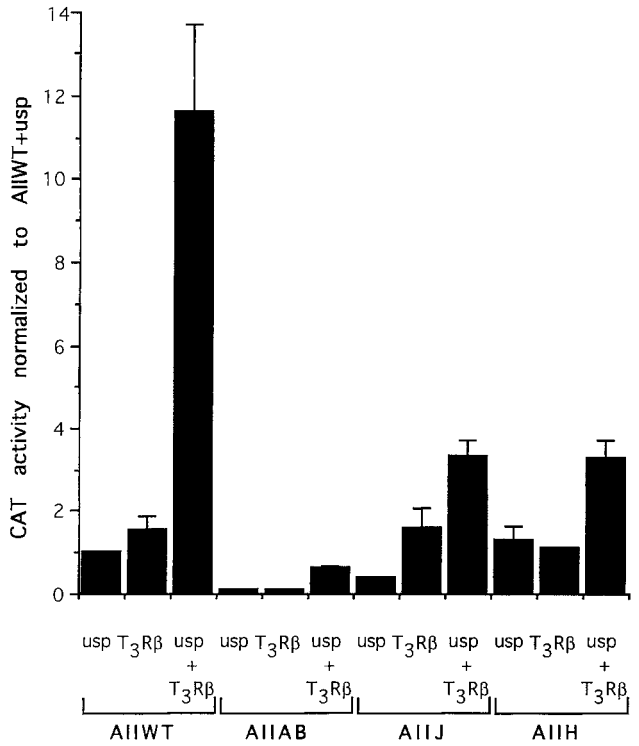

FIGURE 9: Transactivation of the wt (−911 to +29) and mutated apoA-II promoter CAT constructs by usp/$T_3R\beta$ heterodimers. Cotransfection experiments in COS-1 cells were performed with 5 μg of either wt or mutant apoA-II promoter CAT constructs, carrying deletions at regulatory element AB (−65 to −33), J (−734 to −716), and H (−573 to −554), 0.5 μg of usp, and 0.5 μg of $T_3R\beta$ in the presence of T_3 (0.1 μM). CAT activity of AII wt usp is 1.15 milliunits of CAT/milliunit of βGal. Note that deletion of element H or J reduces significantly the 8-fold transactivation of the apoA-II promoter by the usp/$T_3R\beta$ heterodimers.

2-fold, compared to more than 7-fold achieved with the wt apoA-II promoter (Figure 9). Deletion of element A/B reduced the promoter strength to 9.5%; however, it did not affect significantly the magnitude of transactivation by the usp/$T_3R\beta$ heterodimer. Deletion of element H increased the promoter strength slightly but reduced the usp/$T_3R\beta$-mediated transactivation to 3-fold. The findings suggest that the transactivation of the apoA-II promoter by usp/$T_3R\beta$ heterodimers involves complex interactions between these heterodimers and factors bound to other regulatory elements. Previous studies have identified the hepatic activities that recognize these regulatory elements of apoA-II (Cardot et al., 1993). DNA binding gel electrophoresis assays using oligonucleotide probes corresponding to the regulatory elements AIIAB and AIIH have shown binding of the usp/$T_3R\beta$ heterodimer to the element AIIAB but not to the element AIIH (Figure 10A). Previous studies have also shown that element AIIJ binds the usp/$T_3R\beta$ heterodimers (Khoury Christianson et al., 1992). Element AIIH has homology with the consensus sequence that is recognized by a homeodomain protein, hepatocyte nuclear factor 1 (HNF-1) (Frain et al., 1989; Cereghini et al., 1988), and it was previously shown that HNF-1 binds to this element (Chambaz et al., 1991). DNA binding gel electrophoresis showed that element AIIH binds an activity present in COS-1 cells that migrates more slowly than the RXR/$T_3R\beta$ complex (Figure 10B). An antibody that recognizes both HNF-1 and vHNF1 completely supershifted this activity in COS-1 extracts (Figure 10B, lane 4). Another antibody, specific for HNF-1, partially supershifted this activity (Figure 10B, lane 5). Furthermore, this complex was completely competed out by an oligonucleotide containing an HNF-1 binding site in the albumin promoter (Maire et al., 1989) (Figure 10B, lane 2), whereas it was not competed out by an oligonucleotide corresponding to the regulatory element AIIJ (Figure 10B, lane 3). Taken together, these data suggest that the regulatory element AIIH is involved in the transactivation of the apoA-II promoter by usp/$T_3R\beta$ heterodimers through the binding of HNF-1-related factor(s) to this site.

DISCUSSION

Usp/$T_3R\beta$ Heterodimers Recognize a Direct Repeat with One Spacer Oligonucleotide (DR1). The regulatory element AIIJ of apoA-II contains on the noncoding strand two direct repeated sequences homologous to a consensus half-site motif AGG/TTCA, which is the binding site of hormone nuclear receptors (Umesono et al., 1991; Ladias et al., 1992; Giguere, 1993; Mangelsdorf et al., 1994; Glass, 1994). $KMnO_4$ and dimethylsulfate interference experiments showed that five G residues of the noncoding strand participate in strong and one in weak DNA−protein interactions with the usp/$T_3R\beta$ heterodimers. Similarly, four T residues of the coding strand participate in strong DNA protein interactions with the usp/$T_3R\beta$ heterodimers. This analysis classifies the direct repeat of element AIIJ as a DR1 and shows that the spacer nucleotide −727 participates in DNA−protein interactions. Participation of all the residues of the two half-repeats as well as the spacer nucleotide in DNA−protein interactions with homo- and heterodimers of RXR has also been described for the HREs present in the proximal apoA-I promoter (Tzameli & Zannis, 1996). It should be noted that usp/$T_3R\beta$ heterodimers do not recognize exclusively DR1 HREs. In control experiments we have found that the usp/$T_3R\beta$ heterodimers also bind *in vitro* with high affinity to an oligonucleotide spanning a thyroid hormone response element (TRE) found in the α-myosin heavy chain promoter, which contains a DR4 spacing (data not shown). The DNA binding of the usp/$T_3R\beta$ heterodimers to this element is not affected by the presence of T_3. Thus the DNA recognition motifs of the usp/$T_3R\beta$ heterodimers appear to be similar to those of RXR/$T_3R\beta$ heterodimers (Zhang et al., 1992b; Schrader & Carlberg, 1994).

usp/$T_3R\beta$ Heterodimers Can Transactivate the Human ApoA-II Promoter: The Transactivation Function Is Provided by $T_3R\beta$, Is Dependent on T_3, and Requires Binding of the Heterodimer to Its Cognate Site. Cotransfection experiments established clearly that the usp/$T_3R\beta$ heterodimer is functional *in vivo* since it can transactivate the human apoA-II promoter 7−8-fold in COS-1 cells in the presence of T_3. Consistent with other members of the nuclear receptor family (Nagpal et al., 1992, 1993), transactivation does not require the amino-terminal domain of usp between residues 1 and 85. A mutant usp with impaired DNA binding domain that can still form heterodimers with $T_3R\beta$ *in vitro* causes significantly lower transactivation of the human apoA-II promoter. The small transactivation observed with this mutant could be accounted for by weak binding of the uspmutP/$T_3R\beta$ heterodimers *in vivo* to half-repeat sites of the HREs of the apoA-II promoter. Such binding may be stabilized by protein−protein interactions with other transcription factors bound to the apoA-II promoter. In this regard, recent studies have shown weak binding of RXRα/

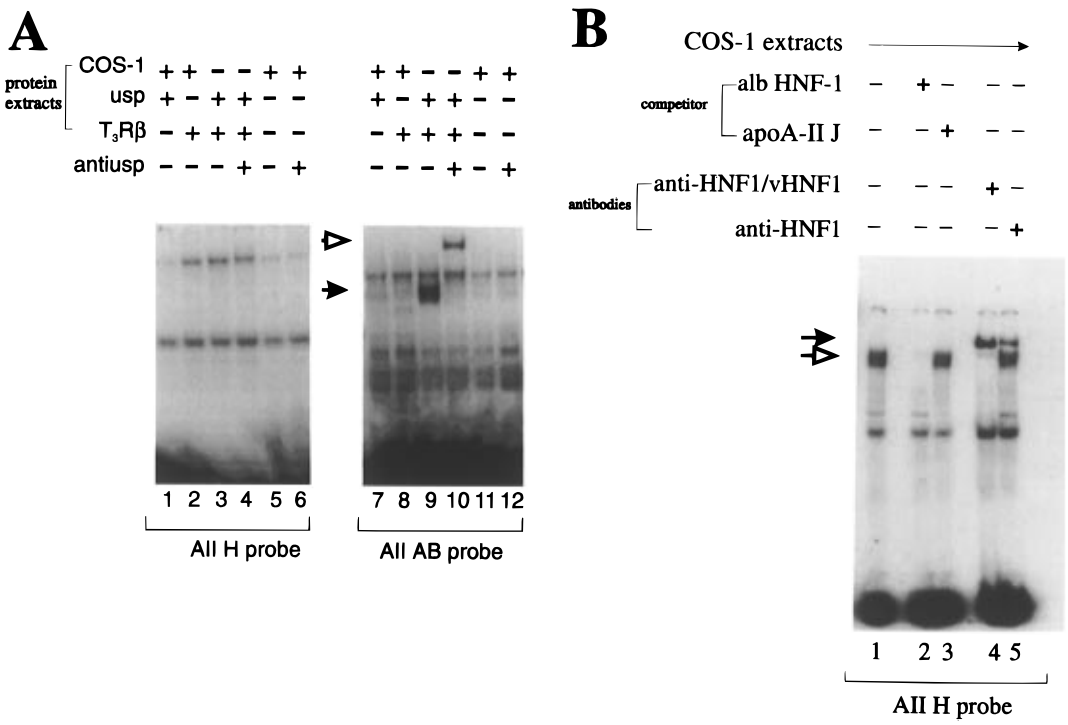

FIGURE 10: A,B. (A). DNA binding gel electrophoretic assay using the apoA-II regulatory elements AB (−65 to −33) or H (−573 to −554) as probe and extracts from COS-1 cells untransfected or following transfection with usp or $T_3R\beta$ plasmids. (*Left panel)* Lanes 1−6 show binding of AIIH probe (Table 2) to the following combination of COS-1 extracts: (1) 4 μg of usp + 2 μg of mock COS-1 extracts; (2) 2 μg of $T_3R\beta$ and 4 μg of mock COS-1 extracts; (3) 4 μg of usp + 2 μg of $T_3R\beta$; (4) 4 μg of usp + 2 μg of $T_3R\beta$+ 1 μL of the anti-usp monoclonal antibody (AB11); (5) 6 μg of mock COS-1 extracts; and (6) 6 μg of mock COS-1 extracts + 1 μL of the anti-usp antibody AB11. (*Right panel)* Lanes 7−11 show binding of AB probe to the following combination of COS-1 extracts: (7) 4 μg of usp + 2 μg of mock COS-1 extracts; (8) 2 μg of $T_3R\beta$ and 4 μg of mock COS-1 extracts; (9) 4 μg of usp + 2 μg of $T_3R\beta$; (10) 4 μg of usp + 2 μg of $T_3R\beta$ + 1 μL of anti-usp monoclonal antibody (ab11); (11) 6 μg of mock COS-1 extracts; (12) 6 μg of mock COS-1 extracts + 1 μL of anti-usp antibody AB11. Note that the usp/$T_3R\beta$ heterodimers bind to the regulatory element AB but do not bind to the regulatory element H. (B). DNA binding gel electrophoresis, competition, and supershift assays using the regulatory element H (−573 to −554) of the apoA-II promoter as a probe and extracts obtained from untransfected COS-1 cells. Competition assays were performed by using oligonucleotides HNF-1 and AIIJ of Table 2 as competitor. Lanes 1−5 contain the following: (1) 6 μg of untransfected COS-1 extracts; (2) 6 μg of untransfected COS-1 extracts + 100-fold molar excess of HNF-1 oligonucleotide; (3) 6 μg of untransfected COS-1 extracts and 100-fold excess of AIIJ oligonucleotide; (4) 6 μg of untransfected COS-1 extracts + 1 μL of anti-HNF-1/vHNF-1 antibody; (5) 6 μg of untransfected COS-1 extracts + 1 μL of anti-HNF-1 antibody. Note that a factor, which is present in COS-1 extracts, is competed out by the HNF-1 oligonucleotide and it is supershifted with anti-HNF-1 antibodies.

$T_3R\beta$ heterodimers *in vitro* to the half-repeat motif of the HRE of the regulatory element AID of the apoA-I promoter (Tzameli & Zannis, 1996). Furthermore, interactions of the transcription factor SP1 bound to DNA with mutant SP1 molecules lacking the DNA binding domain have been shown to cause superactivation of a target promoter (Pascal & Tjian, 1991).

It was shown recently that RXRα which lacks its DNA binding domain can heterodimerize with $T_3R\beta$ or RARα and can be transcriptionally active in the presence of T_3 or *all-trans*-retinoic acid but not in the presence of 9-*cis*-RA. It has been proposed that allosteric interactions of the heterodimers favor binding and activation of one of the partners of the RXR/$T_3R\beta$ or RXR/RAR heterodimers (Forman et al., 1995). Similarly, the present study suggests that in the usp/$T_3R\beta$ heterodimer allosteric interactions favor binding and activation of $T_3R\beta$. In this regard usp may function by recruiting $T_3R\beta$, utilizing domains that have been conserved throughout evolution. The formation of usp/$T_3R\beta$ heterodimers can occur in solution and may not require binding to the HRE *in vivo*. This observation is consistent with previous studies that demonstrated heterodimerization of the mammalian homologue of usp, RXRα, with $T_3R\beta$ *in vitro* (Kliewer et al., 1992c) and *in vivo* (Qi et al., 1995). The transactivation function is specific for the heterodimers of usp with T3Rβ since, as shown in the present study, the usp/RARα heterodimers that also bind to the regulatory element AIIJ (Khoury Christianson et al., 1992) cannot efficiently transactivate the human apoA-II promoter. Recent studies have also shown that the *Drosophila* ecdysone receptor (EcR) is dependent on heterodimer formation with usp for high-affinity DNA binding or transactivation of target promoters (Yao et al., 1993). RXRα could not substitute for usp in transactivating a heterologous promoter driven by ecdysone response elements in CV1 cells in the presence of ecdysone receptor (EcR). However, heterodimers of RXR with EcR could transactivate the target promoter in the presence of another EcR ligand, muristerone (Yao et al., 1993), suggesting distinct ligand binding and activation features between usp and RXRα. It is also important to note that according to the phylogenetic tree constructed by Laudet et al. (1992), ecdysone receptor is closely related to vitamin D receptor. It is probable that additional insect proteins more closely related to $T_3R\beta$ may exist which form functional complexes with usp, in the presence of the appropriate ligand, and function as transcriptional activators or repressors. In this regard it is interesting that a *Drosophila* homologue of the mammalian NGF1-B nuclear receptor, designated DHR38, heterodimerizes with usp and represses transcription by

preventing the formation of the usp/ecdysone receptor heterodimers (Sutherland et al., 1995).

Transactivation of the ApoA-II Promoter by usp/$T_3R\beta$ Heterodimers Requires Complex Interactions of This Heterodimer Bound to Two Promoter Sites with Factors Bound to Other Proximal and Distal ApoA-II Regulatory Sites. Deletion analysis of the apoA-II promoter indicated that element AIIJ which contains a DR1 HRE is necessary for transactivation of the apoA-II promoter by the usp/$T_3R\beta$ heterodimers. A minimal heterologous promoter (tk) under the control of four copies of the element AIIJ could be repressed by $T_3R\beta$ alone to approximately 20% of the control. Usp alone had no effect and usp/$T_3R\beta$ heterodimers relieved the repression of the heterologous promoter. Since $T_3R\beta$ alone does not bind to the element AIIJ whereas usp/$T_3R\beta$ heterodimers bind (Figure 4), the observed activation of the heterologous promoter implies that the basal promoter activity results from the binding of endogenous hormone nuclear receptors to element AIIJ. The repression by $T_3R\beta$ implies squelching of the positive activators by $T_3R\beta$. Finally, the relief of repression by usp/$T_3R\beta$ heterodimers implies activation of the heterologous promoter by these heterodimers bound to the element AIIJ. Different activation properties have also been reported for the HRE of element AID of apoA-I as a component of heterologous promoters (Leng et al., 1994; Zhang et al., 1994) or in the correct promoter context (Tzameli & Zannis, 1996).

Cotransfection experiments with mutant apoA-II promoters lacking different regulatory elements showed that factors bound to the regulatory elements AIIAB and AIIH may also contribute to the transactivation of the apoA-II promoter by the usp/$T_3R\beta$ heterodimers. Element AIIAB binds usp/$T_3R\beta$ and element AIIH binds HNF-1 (Frain et al., 1989; Cereghini et al., 1988) and vHNF-1 (DeSimone et al., 1991) and binds to HNF-1 present in hepatic extracts (Chambaz et al., 1991). The potential involvement of a homeodomain protein (HNF-1-related) in transactivation by nuclear hormone receptors is reminiscent of previous studies that have shown transcriptional synergism between $T_3R\beta$ or RAR and another homeodomain protein, pit1 (Schaufele et al., 1992; Rhodes et al., 1993). The cotransfection experiments using mutant apoA-II promoter constructs suggest that other factors besides HNF-1 bound to proximal and distal apoA-II regulatory sites may also contribute to the transactivation of the apoA-II promoter by usp/$T_3R\beta$ heterodimers.

Our findings suggest that the *Drosophila* usp functions through a highly conserved domain in order to recruit the human $T_3R\beta$ and form heterodimers in solution. Optimal function of this heterodimer in transcription requires an intact DNA binding domain of usp and utilizes the ligand-dependent transactivation functions of $T_3R\beta$. usp may mimic the mammalian RXR, which can form functional heterodimers with a variety of other hormone receptor partners. Usp may form functional dimers with known as well as still unidentified partners in the presence of the appropriate hormonal ligand and thus may participate in the regulation of target *Drosophila* genes. Finally, these data and recent reports by us and others (Hatzivassiliou et al., 1995; Ngoc et al., 1996) suggest that retinoid/thyroid hormone receptors are involved in transactivation of the apoA-II promoter. Optimal transactivation appears to require complex interactions between the hormone nuclear receptors and HNF-1 and possibly other factors that recognize the human apoA-II promoter.

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