Transcriptional Regulation of the Nuclear Gene Encoding the α-Subunit of the Mammalian Mitochondrial F_1F_0 ATP Synthase Complex: Role for the Orphan Nuclear Receptor, COUP-TFII/ARP-1†

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ABSTRACT: Our laboratory has been studying the transcriptional regulation of the nuclear gene (*ATPA*) that encodes the α-subunit of the mammalian mitochondrial F_1F_0 ATP synthase complex. We have previously determined that the regulatory factor, upstream stimulatory factor 2 (USF2), can stimulate transcription of the *ATPA* gene through the *cis*-acting regulatory element 1 in the upstream promoter of this gene. In this study, we used the yeast one-hybrid screening method to identify another factor, COUP-TFII/ARP-1, which also binds to the *ATPA cis*-acting regulatory element 1. Binding of the orphan nuclear receptor, COUP-TFII/ARP-1, to the *ATPA* regulatory element 1 was confirmed using electrophoretic mobility shift experiments, and COUP-TFII/ARP-1-containing complexes were detected in HeLa cell nuclear extracts. A mutational analysis indicated that the binding site for COUP-TFII/ARP-1 in the *ATPA* regulatory element 1 is an imperfect direct repeat of a nuclear receptor response element (A/GGGTCA) with a spacer of three nucleotides. Functional assays in HeLa cells showed that COUP-TFII/ARP-1 represses the *ATPA* promoter activity in a dose- and sequence-dependent manner. Furthermore, cotransfection assays demonstrated that COUP-TFII/ARP-1 inhibits the USF2-mediated activation of the wild-type *ATPA* gene promoter but not a mutant promoter that is defective in COUP-TFII/ARP-1-binding. Overexpression of USF2 reversed the COUP-TFII/ARP-1-mediated repression of the *ATPA* promoter. Mobility shift assays revealed that COUP-TFII/ARP-1 and USF2 compete for binding to the *ATPA* regulatory element 1. Thus, the *ATPA* gene is regulated by a multifunctional binding site through which the transcription factors, COUP-TFII/ARP-1 and USF2, bind and exert their antagonistic effects.

The activities of the enzymes of the mammalian mitochondrial oxidative phosphorylation system vary in response to a number of physiological conditions, including development, differentiation, hormonal stimulation, oxygen tension, and cellular proliferation (for reviews, see refs *1* and *2*). The levels of these enzymes are regulated, in part, at the level of gene transcription, although posttranscriptional regulation also plays an important role in mitochondrial biogenesis (for reviews, see refs *1* and *2*).

We have been studying the transcriptional regulation of the nuclear gene (*ATPA*)[1] that encodes the α-subunit of the mammalian mitochondrial F_1F_0 ATP synthase complex, the central enzyme complex of the oxidative phosphorylation system. By using DNAse I footprinting assays together with deletion and mutagenesis analyses, we have identified several *cis*-acting regulatory elements that are important for expression of the *ATPA* gene (*3*). We have also identified a protein factor(s), termed ATPF1, which binds to the *cis*-acting regulatory element 1 in the upstream promoter of the *ATPA* gene (*3*). Furthermore, we have determined that the transcription factor, upstream stimulatory factor 2 (USF2), is a component of ATPF1 (*4*). Using transient transfection assays, we have demonstrated that USF2 stimulates transcription of the *ATPA* gene through an E-box element (CANNTG) located in the *cis*-acting regulatory element 1 (*4*).

Upstream stimulatory factor was originally identified as an activator of the adenoviral major late promoter (*5*). Purification of USF revealed two polypeptides, USF1 and USF2, which are encoded by two distinct genes (*6*−*12*). USF1 and USF2 belong to Myc family of basic helix−loop−helix−leucine zipper (bHLH-zip) transcription factors (*7*, *8*, *11*). USF1 and USF2 bind as homo- and heterodimers to E-box elements with a core sequence of CACGTG or CACATG (*7*, *8*, *13*). USF has been found to regulate the expression of a large number of cellular and viral genes (see references in refs *10*−*12*, and *14*). Analyses of the function(s) of USF have revealed that USF1 and USF2 have overlapping and essential functions during embryonic development (*15*).

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[1] Abbreviations: *ATPA*, F_1F_0 ATP synthase α-subunit gene; USF, upstream stimulatory factor; bHLH-zip, basic helix−loop−helix−leucine zipper; COUP-TF, chicken ovalbumin upstream promoter transcription factor; ARP-1, apolipoprotein regulatory protein-1; CAT, chloramphenicol acetyltransferase; bp, basepair; EMSA, electrophoretic mobility shift assay; SDS, sodium dodecyl sulfate; L-PK, L-type pyruvate kinase; YY1, Yin Yang 1.

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Our previous mutagenesis experiments and electrophoretic mobility shift assays (EMSA) indicated that proteins other than USF2 are bound to the *ATPA cis*-acting regulatory element 1 (*3*, *4*). In this study, we used a one-hybrid screen in yeast with the *ATPA* regulatory element 1 as bait to isolate cDNA clones that encode proteins that bind to this element. Using this screen, we identify the orphan nuclear receptor COUP-TFII/ARP-1 as a new regulatory factor that binds to the *ATPA* regulatory element 1. EMSA confirmed the binding of COUP-TFII/ARP-1 to a paired nuclear receptor response element (A/GGGTCA) oriented as a direct repeat with a spacer of three nucleotides in the *ATPA cis*-acting regulatory element 1. Mobility shift studies with HeLa cell nuclear extracts demonstrated the presence of COUP-TFII/ARP-1 in HeLa protein *ATPA* complexes. Using transient transfection assays, we determined that COUP-TFII/ARP-1 inhibits the *ATPA* promoter activity in a dose-dependent and sequence-dependent manner. Furthermore, using cotransfection assays, we found that COUP-TFII/ARP-1 also represses the USF2-mediated activation of the *ATPA* promoter in a dose-dependent and sequence-dependent manner. We also determined that overexpression of USF2, but not a USF2 mutant with a deletion in the DNA-binding domain, could reverse this COUP-TFII/ARP-1-mediated repression. EMSA using COUP-TFII/ARP-1 together with USF2 revealed that COUP-TFII/ARP-1 and USF2 bind in a mutually exclusive manner to the *ATPA* regulatory element 1. Thus, the *ATPA* gene is regulated by a composite genetic element to which USF2 and COUP-TFII/ARP-1 (and possibly other factors) bind and exert their regulatory functions. It is likely that the level of the expression of *ATPA* gene will represent a balance between the positive and negative factors that bind and regulate this gene.

EXPERIMENTAL PROCEDURES

Plasmids. The *ATPA* (+21 to +136 bp)−CAT promoter construct containing the +21 to +136 bp region of the bovine *ATPA* gene promoter cloned into pCAT Basic was described previously (*3*). The *ATPA* (+21 to +46 bp)$_3$−CAT promoter construct contains three copies of the +21 to +46 bp region of the bovine *ATPA* promoter (*18*) cloned into the vector, pCAT Basic (Promega, Madison, WI).

One-Hybrid Screening of a HeLa cDNA Library. The yeast one-hybrid system (CLONTECH, Palo Alto, CA) was used to screen for proteins that bind to the *ATPA cis*-acting regulatory element 1. The *ATPA* promoter sequence from bp +21 to +46 (*18*) was used as the bait to select DNA-binding proteins encoded in a human HeLa cDNA library. The bait was trimerized and inserted into the reporter plasmids, pHISi-1 and pLacZi (CLONTECH). The recombinant plasmids were integrated sequentially into the genome of the yeast strain, YM4271. The target-reporter yeast strain was transformed with a HeLa Matchmaker GAL4 activation domain fusion cDNA library (CLONTECH) using a high-efficiency transformation method. Transformants were selected for growth on histidine-minus and leucine-minus medium supplemented with 45 mM 3-aminotriazole. Transformants were tested for β-galactosidase activity using a filter-binding assay (CLONTECH). Plasmid DNA was isolated from putative positive clones, and cDNA inserts were sequenced by the dideoxy sequencing method (*19*). DNA sequences were used to query the GenBank and EMBL databases.

In Vitro Transcription−Translation Reactions. Coupled in vitro transcription−translation reactions were performed using a TNT kit (Promega, Madison, WI) according to the manufacturer's instructions. For these reactions, one μg of pCR3.1/COUP-TFII (a generous gift of Dr. M. -J. Tsai) or pSVUSF2a (a generous gift of Dr. M. Sawadogo) plasmid DNA was incubated at 30 °C for 60 min with the T7 TNT coupled transcription−translation system in the presence of methionine.

Electrophoretic Mobility Shift Assays. EMSA were performed at room temperature with 0.1 ng of a ^{32}P-labeled double-stranded oligonucleotide as a probe. Binding reactions contained 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 60 mM KCl, 1 μg of poly(dI-dC), 8% glycerol, and 1−10 μL of reticulocyte lysate extract or 1−2 μg of HeLa cell nuclear extract. For supershift assays, 0.5−2 μL of antiserum was included in the binding reactions before the addition of the probe. For competition analyses, a 100-fold molar excess of competitor DNA was added to the binding mixtures. Complexes were resolved on 4% polyacrylamide gels using 1× Tris-borate-EDTA as the running buffer. Polyclonal antiserum against COUP-TFII was a generous gift of Dr. M. Vasseur-Cognet (INSERM, France). Rabbit polyclonal anti-USF2 antiserum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfections. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% calf serum in a humidified atmosphere at 37 °C (*3*, *4*). HeLa cells were transfected with 5 to 10 μg of an *ATPA*-CAT reporter plasmid DNA together with various concentrations of effector DNAs and 2 μg of pCMV-β-galactosidase DNA using the calcium phosphate coprecipitation method, as previously described (*3*, *4*, *20*). In some transfection experiments, human or mouse COUP-TFII/ARP-1 cloned in a eukaryotic expression vector (*16*, *17*; generously provided by Drs. J. Ladias or M.-J. Tsai) and/or mouse USF2a or USF2b cloned in a eukaryotic expression vector (*9*; a generous gift of Dr. M. Sawadogo) was added. In all transfection experiments, the total amount of added DNA was kept constant by adding varying amounts of pcDNA3 control DNA. Cells were harvested approximately 48 h after transfection and cellular lysates were prepared. Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were carried out as described previously (*3*, *4*, *20*). The CAT activities of the cellular lysates are expressed as a ratio relative to the β-galactosidase activities (relative CAT activities) to correct for transfection efficiencies. All transfections were repeated a minimum of three times with at least two or more different plasmid preparations. The promoter activity values represent the average of at least three transfections ± SD. All plasmid DNAs were purified using Qiagen columns according to the manufacturer's instructions (Qiagen, Valencia, CA).

Western Blot Hybridization Analysis. HeLa cell extracts were separated by using electrophoresis in 12% sodium dodecyl sulfate (SDS)−polyacrylamide gels and transferred to a nitrocellulose membrane (*19*). The membrane was blocked overnight in Tris-buffered saline (TBS) containing 5% nonfat dry milk, and then incubated for 3 h at room temperature with anti-USF2 antiserum (1:1000 dilution; Santa

Cruz Biotechnology, Santa Cruz, CA). After three washes were performed at room temperature in TBS, peroxidase-conjugated goat anti-rabbit antiserum was added for 1 h. The blots were washed again and the peroxidase activity was detected with the ECL enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

Oligonucleotides. The following oligonucleotides were used in this study:

COUP-TFII-binding site: 5′ TCGAGGGTAGAGGTCA-GAGGTCACTCG 3′ (DR1; *21*, *22*);

Adenoviral major late promoter E-box element: 5′ TCC-GGTCACGTGACCGA 3′;

Wild-type *ATPA* regulatory element 1: 5′ GTACATC-CGGGT**CACGTG**GGCTGACT 3′ (+21 to +46 bp region of the bovine *ATPA* promoter; *18*). The E-box element is in bold and the COUP-TFII/ARP-1-binding site is underlined.

ATPA mutant 1-2: 5′ GTACATCCaaaTCACGTGGGCT-GACT 3′;

ATPA mutant 1-3: 5′ GTACATCCGGGctgCGTGGGCT-GACT 3′;

ATPA mutant 1-4: 5′ GTACATCCGGGTCAtacGGGCT-GACT 3′;

ATPA mutant 1-5: 5′ GTACATCCGGGTCACGTaaaCT-GACT 3′;

ATPA mutant 1-6: 5′ GTACATCCGGGTCACGTGGG-tcaACT 3′.

Mutations are indicated in lower case letters.

RESULTS

Molecular Cloning of COUP-TFII/ARP-1 Using a One-Hybrid Selection in Yeast. We have previously identified several *cis*-acting regulatory elements that are important for expression of the *ATPA* gene in human HeLa cells (*3*). We have also determined that the transcription factor, USF2, is a component of the proteins in HeLa cell nuclear extracts that bind to the *ATPA cis*-acting regulatory element 1 (*4*). Our EMSA and mutagenesis studies suggested that additional *trans*-acting factors can bind and regulate the expression of the *ATPA* gene through *cis*-acting regulatory element 1 (*3*, *4*).

To identify additional proteins that could recognize the *ATPA* regulatory element 1, we used the yeast one-hybrid screening method which enables the cloning of DNA-binding factors. The *ATPA* regulatory element 1 was trimerized and inserted into the reporter plasmids, pHISi-1 and pLacZi. These recombinant plasmids were introduced sequentially into the chromosome of the yeast strain, YM4271. This target-reporter yeast strain was transformed with a HeLa GAL4 activation domain cDNA fusion library (CLONTECH), and transformants were selected for growth on medium lacking histidine and leucine containing 3-aminotriazole. From three screenings of approximately 2×10^7 transformants, 35 His^+ and $LacZ^+$ dual positive colonies were obtained. Plasmid DNA was recovered from each of these colonies. Sequencing of these plasmid DNAs revealed that 13 clones encoded a USF2 cDNA fused in frame to the Gal4p activation domain. These results indicate that USF2 can bind to the *ATPA cis*-acting regulatory element 1 in our genetic screen. Sequencing of additional cDNA clones revealed that nine encoded the regulatory factor, COUP-TFII (also known as ARP-1) (*16*, *17*).

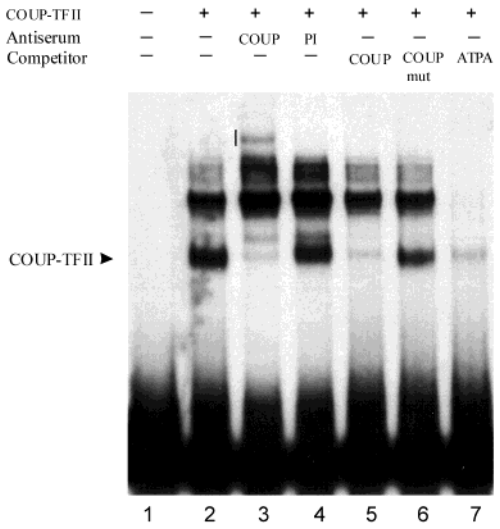

FIGURE 1: COUP-TFII/ARP-1 binds to the *ATPA cis*-acting regulatory element 1. A double-stranded ^{32}P-labeled oligonucleotide containing the regulatory element 1 from the *ATPA* gene promoter (lanes 1−7) was incubated together with in vitro translated COUP-TFII/ARP-1 (lanes 2−7). In some reactions, anti-COUP-TFII antiserum (COUP; lane 3) or preimmune serum (PI; lane 4) was added. The positions of the "supershifted" COUP-TFII-*ATPA* complexes are indicated by a vertical line. In lane 5, an oligonucleotide containing a known COUP-TFII-binding site (COUP; *21*) was added as a competitor and in lane 6, an oligonucleotide containing a mutation in the COUP-TFII-binding site (COUP mut) was added as a competitor. In lane 7, the *ATPA* oligonucleotide (ATPA) was added as a competitor. The positions of the COUP-TFII−*ATPA* complexes are indicated by an arrowhead.

COUP-TFII/ARP-1 Protein Binds to the ATPA Regulatory Element 1. COUP-TFII/ARP-1 is an orphan member of the nuclear receptor superfamily (for reviews, see refs *23*−*25*). COUP-TFs primarily bind as homodimers to paired nuclear receptor binding sites (A/GGGTCA) arranged as direct repeats or palindromes with various spacings (*21*, *22*). Examination of the sequence of the *ATPA* regulatory element 1 promoter revealed a perfect half-site (GGGTCA) and an imperfect half-site (GGGCTG) for COUP-TFs. To verify that COUP-TFII/ARP-1 binds to the *ATPA cis*-acting regulatory element 1, we used EMSA with in vitro translated COUP-TFII/ARP-1. As shown in Figure 1, we found that incubation of in vitro translated COUP-TFII/ARP-1 with a double-stranded oligonucleotide containing the *ATPA cis*-acting regulatory element 1 resulted in the formation of a prominent faster migrating protein−DNA complex and a prominent slower migrating protein−DNA complex, which can be resolved into at least three complexes. We also found that the faster migrating protein−*ATPA* complex could be "supershifted" using a polyclonal antiserum generated against COUP-TFII but not by preimmune serum (Figure 1, lanes 3 and 4). Furthermore, an oligonucleotide containing a known COUP-TFII-binding site (*21*, *22*) effectively competed for the binding of this protein−*ATPA* complex (Figure 1, lane 5). In contrast, an oligonucleotide containing a mutation in the COUP-TFII-binding site did not compete for the binding of any of the protein−*ATPA* complexes (Figure 1, lane 6). We also determined that an oligonucleotide containing the *ATPA cis*-acting regulatory element 1 competed for the binding of all of the slower migrating protein−DNA complexes and most of the faster migrating COUP-TFII−*ATPA* complexes (Figure 1, lane 7). These results demonstrate that

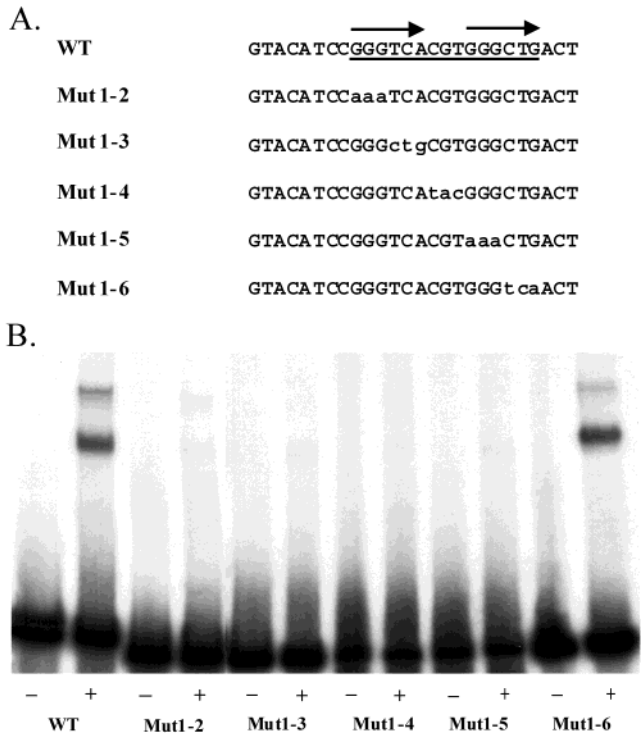

FIGURE 2: Characterization of the COUP-TFII/ARP-1-binding site in the *ATPA* regulatory element. EMSA were carried out as described in Experimental Procedures using various ^{32}P-labeled DNA probes. (A) Sequences of the oligonucleotide probes. WT is the wild-type *ATPA* sequence between +21 bp and +46 bp (*18*). Mut 1-2 to Mut 1-6 contain 3 bp mutations (indicated as lower case letters) within the *ATPA* sequence (*3*). The COUP-TFII/ARP-1-binding site is underlined and the nuclear receptor response element half-sites are indicated by arrows. (B) EMSA were performed either in the presence (+) or the absence (−) of in vitro translated COUP-TFII/ARP-1. The reactions with the Mut 1-6 probe were run on a separate gel.

COUP-TFII/ARP-1 can bind to the *ATPA cis*-acting regulatory element 1.

We next examined the nucleotides required for the binding of COUP-TFII/ARP-1 to the *ATPA cis*-acting regulatory element 1. For these experiments, three bp mutations were introduced into the *ATPA* regulatory element 1, and the effect of these mutations on the binding of in vitro translated COUP-TFII/ARP-1 was examined using EMSA. As shown in Figure 2, we found that mutation of the GGG residues (Mut 1-2) or the TCA residues (Mut 1-3) in the 5′-nuclear receptor response element half-site resulted in a dramatic decrease in the binding of in vitro translated COUP-TFII/ARP-1. Similarly, mutation of the GGG residues (mut 1-5) in the 3′-nuclear receptor half-site also caused a large decrease in the binding of COUP-TFII/ARP-1 (Figure 2). In contrast, mutation of the CTG residues (mut 1-6) in the 3′-half-site did not affect the binding of in vitro translated COUP-TFII/ARP-1 (Figure 2). Mutation of the CTG residues to TCA would result in an oligonucleotide containing a perfect match to the consensus nuclear receptor binding site and should, therefore, not reduce the binding of COUP-TFII/ARP-1 (as was observed; Figure 2). We also found that mutation of the three nucleotides (CGT) between the two-half-sites (mut 1-4) resulted in a dramatic decrease in the binding of in vitro translated COUP-TFII/ARP-1 (Figure 2). In contrast, mutation of the two C residues that flank the 5′-nuclear receptor half-site did not alter the binding of in vitro

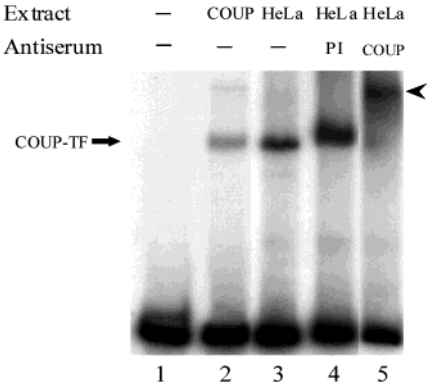

FIGURE 3: COUP-TFII/ARP-1 present in HeLa cell nuclear extracts binds to the *ATPA* regulatory element 1. HeLa cell nuclear extracts were incubated together with a ^{32}P-labeled oligonucleotide containing the regulatory element 1 from the *ATPA* promoter (lanes 3−5). In the reactions in lane 4, preimmune serum (PI) was added and in the reactions in lane 5, anti-COUP-TFII antiserum was added. In lane 2, in vitro translated COUP-TFII/ARP-1 (COUP) was incubated together with the *ATPA* oligonucleotide. The positions of the COUP-TFII−*ATPA* complexes are indicated by an arrow and the positions of the "supershifted" COUP-TFII−*ATPA* complexes are indicated by an arrowhead.

translated COUP-TFII/ATP-1 (data not shown). These results suggest that the binding site for COUP-TFII/ARP-1 in the *ATPA* regulatory element 1 is an imperfect direct repeat of a nuclear receptor response element (A/GGGTCA) with a spacer of three nucleotides.

We next tested if COUP-TFII/ARP-1 is a component of the proteins in nuclear extracts from HeLa cells that bind to the *ATPA cis*-acting regulatory element 1. As shown in Figure 3, incubation of HeLa cell nuclear extracts with the *ATPA* oligonucleotide resulted in the formation of a major protein−DNA complex (lane 3). This HeLa protein−*ATPA* complex comigrated with the predominant in vitro translated COUP-TFII-*ATPA* complex (Figure 3). Upon addition of anti-COUP-TFII antiserum, a "supershift" in this HeLa protein−DNA complex was observed (Figure 3, lane 5), whereas in control experiments using preimmune antiserum, no "supershift" of this complex was seen (Figure 3, lane 4). Thus, endogenous COUP-TFII/ARP-1 from human HeLa cells can bind to the *ATPA cis*-acting regulatory element 1.

We also tested if in vitro translated USF2 can bind to the *ATPA cis*-acting regulatory element 1. As shown in Figure 4, addition of in vitro translated USF2 to the *ATPA* oligonucleotide resulted in the formation of several predominant protein−*ATPA* complexes (lane 2). Furthermore, a polyclonal antiserum raised against USF2 specifically recognized these protein−*ATPA* complexes resulting in the generation of "supershifted" complexes (Figure 4, lane 3). In addition, an oligonucleotide containing an E-box element from the adenoviral major late promoter competed for most of the binding of in vitro translated USF2 to the *ATPA* regulatory element 1 (Figure 4, lane 5).

We also examined the nucleotides important for the binding of USF2 to the *ATPA cis*-acting regulatory element 1. For these experiments, we used the same mutant oligonucleotides that were used previously to test the binding of COUP-TFII/ARP-1, together with in vitro translated USF2 in EMSA. As shown in Figure 5, we found that there was reduced binding of in vitro translated USF2 to mutants 1-3, 1-4, and 1-5. In contrast, the binding of in vitro translated

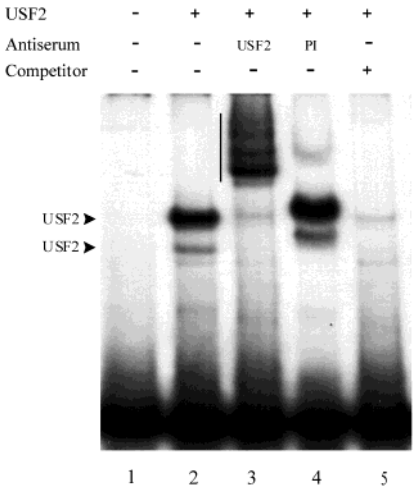

FIGURE 4: USF2 binds to the *ATPA cis*-acting regulatory element 1. In vitro translated USF2a was incubated together with a ^{32}P-labeled oligonucleotide containing the regulatory element 1 from the *ATPA* promoter (lanes 2−5). In some reactions, anti-USF2 antiserum (USF2; lane 3) or preimmune serum (PI; lane 4) was added. In the reactions in lane 5, an oligonucleotide containing the E-box element from the adenoviral major late promoter was added as a competitor. The positions of the "supershifted" USF2−*ATPA* complexes are indicated by a vertical line and the positions of the USF2−*ATPA* complexes are indicated by arrowheads.

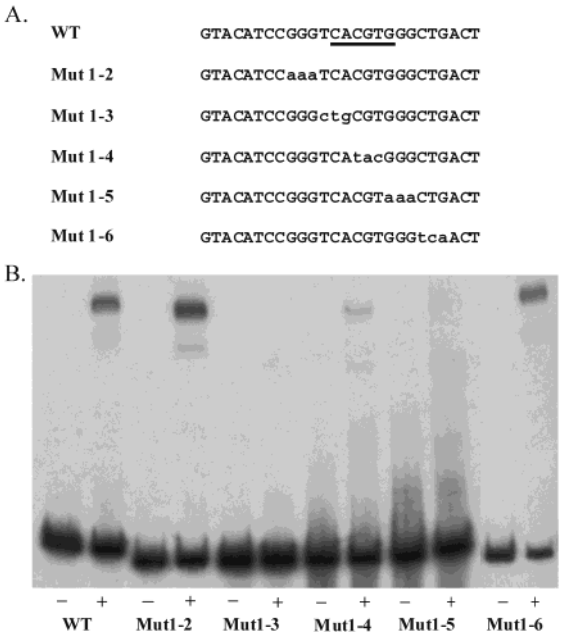

FIGURE 5: Characterization of the USF2-binding site in the *ATPA* regulatory element 1. EMSA were carried out using various ^{32}P-labeled probes as described in Experimental Procedures. (A) Sequences of the oligonucleotide probes. WT is the wild-type *ATPA* sequence between +21 and +46 bp (*18*). Mut 1-2 to Mut 1-6 contain 3 bp mutations (indicated in lower case letters) within this *ATPA* sequence. The E-box element (CACGTG) is underlined. (B) EMSA were performed either in the presence (+) or the absence (−) of in vitro translated USF2a. The reactions with the Mut 1-6 oligonucleotide were run on a separate gel.

USF2 to mutants 1-2 and 1-6 was comparable to that of wild-type (Figure 5). Our combined results indicate that the USF2 binds to an E-box element (CACGTG) in the *ATPA cis*-acting regulatory element 1. We have previously determined that USF2 is a component of the proteins in HeLa nuclear extracts that bind to the *ATPA* regulatory element 1 (*3*). These combined results indicate that both USF2 and COUP-TFII/ARP-1 bind to the *ATPA cis*-acting regulatory element 1 and that the COUP-TFII/ARP-1-binding site overlaps the USF2-binding site.

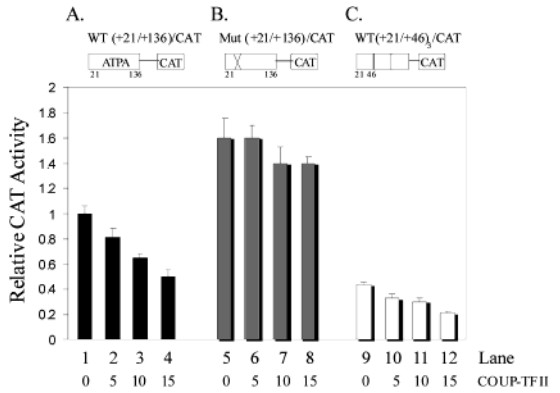

FIGURE 6: COUP-TFII/ARP-1 represses transcription of the wild-type *ATPA* gene promoter but not a mutant *ATPA* promoter. HeLa cells were cotransfected with either 5 μg of the wild-type (WT) *ATPA* (+21 to +136 bp)/CAT plasmid DNA (A; lanes 1−4, black bars), 5 μg of the mutant (Mut) 1-4 *ATPA* (+21 to +136 bp)/CAT plasmid DNA (B; lanes 5−8, gray bars), or 10 μg of the wild-type (WT) *ATPA* (+21 to + 46 bp)$_3$/CAT plasmid DNA (C; lanes 9−12, white bars), together with either an empty expression vector (lanes 1, 5, and 9) or increasing concentrations (μg) of an expression vector encoding COUP-TFII/ARP-1 (lanes 2−4, 6−8, and 10−12), as indicated. The sequence of the mutation in Mut 1-4 is shown in Figures 2 and 5 and in Experimental Procedures.

COUP-TFII/ARP-1 Represses Transcription of the Wild-Type ATPA Gene Promoter but not a Mutant ATPA Promoter. COUP-TFII/ARP-1 has been shown to regulate the transcription of a number of genes and generally acts as a repressor (for reviews, see refs *23*−*25*). Transient transfection experiments were carried out to examine the effect of COUP-TFII/ARP-1 on the expression of the *ATPA* gene promoter. As shown in Figure 6A, we found that overexpression of COUP-TFII/ARP-1 in HeLa cells repressed the activity of the wild-type *ATPA* promoter in a dose-dependent manner. In contrast, there was much less repression by COUP-TFII/ARP-1 of an *ATPA* promoter construct containing a mutation in the COUP-TFII/ARP-1-binding site in the *ATPA* regulatory element 1 (Figure 6B). The experiments described above were carried out using the +21 to +136 bp region of the *ATPA* promoter. It is possible that this region of the *ATPA* promoter contains an additional COUP-TFII/ARP-1-binding site(s). We therefore repeated these experiments using a construct containing three copies of the wild-type *ATPA* regulatory element 1 driving expression of the CAT reporter gene. As shown in Figure 6C, COUP-TFII/ARP-1 also inhibited the expression of the trimerized *ATPA* (+21 to +46 bp)/CAT construct in a dose-dependent manner. These results indicate that COUP-TFII/ARP-1 negatively regulates the *ATPA* gene through a binding site in the *ATPA cis*-acting regulatory element 1.

COUP-TFII/ARP-1 Represses the USF2-Mediated Activation of the Wild-Type ATPA Gene Promoter but not a Mutant ATPA Promoter. We have previously determined that USF2 can stimulate transcription of the *ATPA* gene through the E-box element in the *ATPA cis*-acting regulatory element 1 (*4*). We next examined the effect of cotransfecting COUP-TFII/ARP-1 together with USF2 on the expression of the *ATPA* gene promoter. As shown in Figure 7A, transfection of USF2 alone increased the CAT activity of the wild-type

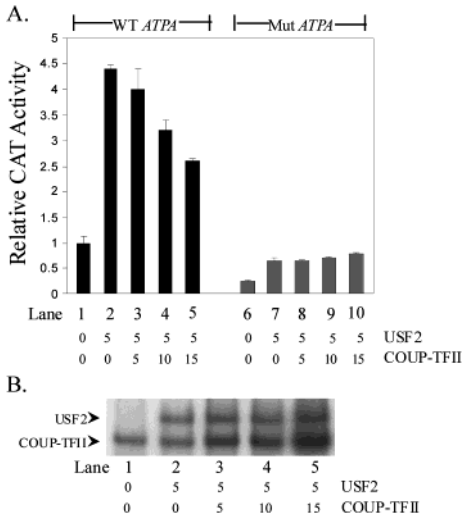

FIGURE 7: COUP-TFII/ARP-1 represses the USF2-mediated stimulation of the wild-type *ATPA* gene promoter but not a mutant *ATPA* promoter. (A) Promoter activities of transfected cells. HeLa cells were cotransfected with either 5 μg of the wild-type *ATPA* (+21 to +136 bp)/CAT reporter plasmid DNA (WT; black bars, lanes 1−5) or the *ATPA* mutant 1−2 (+21 to +136 bp)/CAT reporter plasmid DNA (Mut; gray bars, lanes 6−10) together with either 5 μg of SVUSF2a alone (lanes 2 and 7) or the equivalent amount of the empty expression vector (lanes 1 and 6), or 5 μg of SVUSF2a together with increasing amounts (μg) of a COUP-TFII/ARP-1 expression vector (lanes 3−5 and 8−10), as indicated. Cells were harvested approximately 48 h after transfection, and the CAT activities of the transfected cells were determined as described in Experimental Procedures. The sequence of the mutation in Mut 1-2 is shown in Figures 2 and 5 and in Experimental Procedures. (B) Electrophoretic mobility shift analysis of the binding activities of transfected cells. Nuclear extracts were prepared from either HeLa cells transfected with pcDNA3 control DNA (lane 1), HeLa cells transfected with 5 μg of SVUSF2a alone (lane 2), or HeLa cells transfected with 5 μg of SVUSF2a together with increasing concentrations of a COUP-TFII/ARP-1 expression vector (lanes 3−5). Nuclear extracts were incubated together with a ^{32}P-labeled oligonucleotide probe containing the regulatory element 1 from the *ATPA* promoter and protein−DNA complexes were separated on polyacrylamide gels as described in Experimental Procedures. The positions of the USF2−*ATPA* and COUP-TFII−*ATPA* complexes are indicated by arrowheads.

ATPA (+21 to +136 bp)/CAT reporter construct, in agreement with our previously reported results (*4*). However, expression of COUP-TFII/ARP-1 together with USF2 repressed the USF2-mediated transactivation of the wild-type *ATPA* (+21 to +136 bp)/CAT reporter in a dose-dependent manner (Figure 7A). EMSA and Western blot hybridization experiments were carried out to examine the binding activities and the level of expression of USF2 and COUP-TFII/ARP-1 in these transfected cells. As shown in Figure 7B, we found that there was an increase in the USF2-binding activity in cells transfected with the USF2a expression vector when compared with cells transfected with an empty expression vector (lanes 1 and 2). Furthermore, we determined that the USF2-binding activity was similar in cells transfected with a constant amount of USF2 and increasing concentrations of the COUP-TFII/ARP-1 expression vector (Figure 7B, lanes 2−5). Western blot hybridization analysis also revealed that the levels of USF2 were similar in cells cotransfected with USF2 and COUP-TFII/ARP-1 expression vectors (data not shown). These results demonstrate that the inhibitory effect of COUP-TFII/ARP-1 was not due to an inhibition

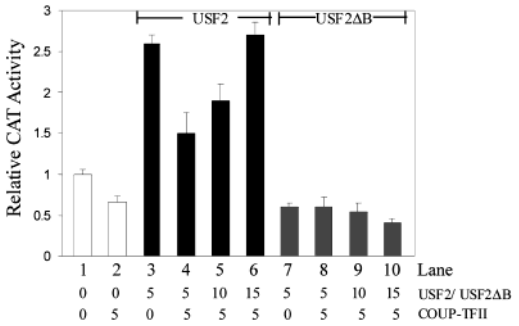

FIGURE 8: Overexpression of USF2, but not USF2ΔB, reverses the COUP-TFII/ARP-1-mediated repression of the *ATPA* gene promoter. HeLa cells were cotransfected with 5 μg of the *ATPA* (+21 bp +136 bp)/CAT reporter plasmid DNA together with either an empty expression vector (lane 1), an expression vector (5 μg) for COUP-TFII/ARP-1 alone (lane 2), an expression vector (5 μg) encoding USF2b alone (lane 3), an expression vector encoding COUP-TFII/ ARP-1 (5 μg) together with increasing amounts (μg) of an expression vector for USF2b (lanes 4−6), an expression vector (5 μg) encoding USF2ΔB alone (lane 7), or an expression vector encoding COUP-TFII/ARP-1 (5 μg) together with increasing amounts (μg) of an expression vector for USF2ΔB (lanes 8−10), as indicated. USF2ΔB is a USF2 protein that lacks the basic region (ΔB) that is required for DNA binding (*26*).

in the expression or the binding of USF2. Interestingly, the binding levels of COUP-TFII/ARP-1 were only slightly increased in cells transfected with increasing concentrations of the COUP-TFII/ARP-1 expression plasmid (Figure 7B). These results probably reflect the high levels of endogenous COUP-TFII/ARP-1 protein present in HeLa cells (Figure 7B) and the relatively low transfection efficiencies obtained. These combined results demonstrate that COUP-TFII/ARP-1 can repress the USF2-dependent transactivation of a reporter gene directed by the wild-type *ATPA* promoter.

Our previous binding studies demonstrated that *ATPA* mutant 1-2 retains normal binding for USF2 but has greatly reduced binding for COUP-TFII/ARP-1 (Figures 2 and 5). We therefore examined the effect of cotransfecting USF2 together with COUP-TII/ARP-1 on the activity of the *ATPA* mutant 1-2/CAT reporter construct. As shown in Figure 7A, we found that expression of USF2 alone increased the CAT activity of the *ATPA* mutant 1-2/CAT reporter construct, in agreement with our binding studies (Figure 5) However, coexpression of COUP-TFII/ARP-1 together with USF2 did not repress the USF2-mediated activation of the *ATPA* mutant 1-2/CAT construct (Figure 7A). Furthermore, expression of COUP-TFII/ARP-1 alone did not inhibit the expression of the *ATPA* mutant 1-2/CAT construct (data not shown). Interestingly, the basal level of expression of the *ATPA* mutant 1-2 promoter was less than that of the wild-type promoter, indicating the release of a positive regulatory factor from this binding site (Figure 7A).

To further examine this repression by COUP-TFII/ARP-1 of the USF2-mediated activation of the *ATPA* gene promoter, we tested whether increasing the amount of added USF2 would reverse the inhibitory effect of COUP-TFII/ARP-1. As shown in Figure 8, when additional USF2-expressing plasmid was added, a dose-dependent reactivation of the *ATPA* (+21 to +136 bp) promoter/CAT construct was observed (lanes 4−6). In contrast, no reactivation of the *ATPA* (+21 to +136 bp)/CAT construct was observed when a mutant form of USF2 with a deletion in the DNA-binding

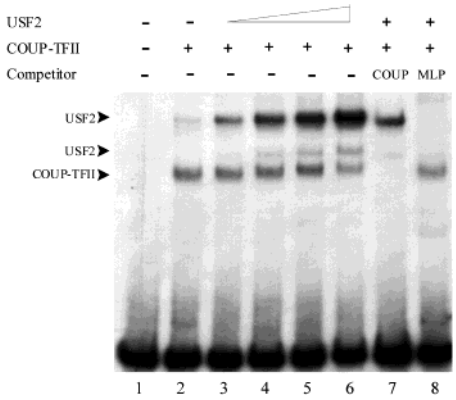

FIGURE 9: COUP-TFII/ARP-1 competes with USF2 for an overlapping binding site in the *ATPA* regulatory element 1. A ^{32}P-labeled oligonucleotide containing the *cis*-acting regulatory element 1 from the *ATPA* gene promoter (lanes 1−8) was incubated together with a constant amount of in vitro translated COUP-TFII (lanes 2−8) and increasing amounts of in vitro translated USF2a (lanes 3−6). The amount of in vitro translated USF2 added in lanes 7 and 8 was the same as in lane 4. In some reactions, an oligonucleotide containing a known COUP-TFII-binding site (COUP; lane 7) or an oligonucleotide containing the E-box element from the adenoviral major late promoter (MLP; lane 8) was added as a competitor. The positions of the USF2−*ATPA* and COUP-TFII−*ATPA* complexes are indicated by arrowheads.

domain (USF2ΔB; *26*) was added, demonstrating the requirement for DNA-binding by USF2 to reverse the repression by COUP-TFII/ARP-1 (Figure 8, lanes 8−10). A similar dose-dependent reactivation by USF2 was observed using an artificial promoter with oligomerized *ATPA* regulatory element 1 binding sites (data not shown).

COUP-TFII/ARP-1 and USF2 Compete for Binding to the ATPA Regulatory Element 1. To further investigate the interaction between the COUP-TFII/ARP-1 and USF2 transcription factors and the *ATPA cis*-acting regulatory element 1, EMSA were carried out using COUP-TFII/ARP-1 together with USF2. As shown in Figure 9, we found that when increasing amounts of USF2 protein were added together with a constant amount of COUP-TFII/ARP-1, there was an increase in the levels of the USF2−*ATPA* complexes and a corresponding decrease in the COUP-TFII−*ATPA* complexes. The USF2−*ATPA* complexes were competed with an oligonucleotide containing an E-box element from the adenoviral major late promoter (Figure 9, lane 8) and the COUP-TFII−*ATPA* complexes were competed with an oligonucleotide containing a known COUP-TFII-binding site (Figure 9, lane 7). No complexes with mobilities other than those corresponding to either protein alone were observed when the two proteins were mixed together that might suggest the formation of a COUP-TFII−USF2−*ATPA* complex. These results indicate that USF2 and COUP-TFII/ARP-1 compete for binding to the *ATPA cis*-acting regulatory element 1.

DISCUSSION

In this study, we used the yeast one-hybrid system to isolate human cDNAs that encode proteins, which bind to the *cis*-acting regulatory element 1 in the *ATPA* gene promoter. Nucleotide sequencing of the cDNAs isolated in this screen revealed that the majority of the clones encoded either the bHLH-zip transcription factor, USF2, or the orphan nuclear receptor, COUP-TFII/ARP-1. We confirmed the binding of both USF2 and COUP-TFII/ARP-1 to the *ATPA* regulatory element 1 using EMSA and "supershift" assays together with specific antibodies. Using functional assays, we found that COUP-TFII/ARP-1 represses the *ATPA* gene promoter, whereas USF2 activates it. We also determined that COUP-TFII/ARP-1 inhibits the USF2-dependent transactivation of the *ATPA* promoter through competition for an overlapping binding site in the *ATPA cis*-acting regulatory element 1. Evidence includes the following. Mutation of the COUP-TFII/ARP-1-binding site in the *ATPA* regulatory element 1 resulted in a loss of repression of the *ATPA* promoter by COUP-TFII/ARP-1. Expression of COUP-TFII/ARP-1 together with USF2 inhibited the USF2-dependent transcription of the wild-type *ATPA* gene promoter but not a mutant *ATPA* promoter with a mutation in the COUP-TFII/ARP-1-binding site. Overexpression of USF2, but not a USF2 mutant that was unable to bind DNA, reversed the COUP-TFII/ARP-1-mediated repression of the *ATPA* promoter. EMSA revealed that in the presence of a constant amount of COUP-TFII/ARP-1 and increasing concentrations of USF2 there was an increase in the amount of the USF2−*ATPA* complexes and a corresponding decrease in the COUP-TFII/ARP-1−*ATPA* complexes, indicating that these two regulatory factors compete for binding to the *ATPA cis*-acting regulatory element 1. These data indicate that both positive (such as USF2) and negative (such as COUP-TFII/ARP-1) regulators compete for an overlapping regulatory element in the *ATPA* promoter thus providing antagonist pathways that might govern the expression of the *ATPA* gene.

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are orphan members of the nuclear receptor superfamily (for reviews, see refs *23*−*25*). In most higher vertebrates, the COUP-TF family contains two highly conserved members, COUP-TFI and COUP-TFII (*23*−*25*). COUP-TFII was also cloned as the apolipoprotein regulatory protein-1, ARP-1 (*17*). Members of the COUP-TF family primarily form homodimers and have been shown to bind to paired nuclear response elements (A/GGGTCA) oriented as direct repeats, inverted repeats, or everted repeats with spacings of 0−12 nucleotides (*21*, *22*). COUP-TFs are widely expressed transcription factors and have been shown to regulate the transcription of a large number of genes, and generally act as transcriptional repressors (*23*−*25*). Studies in several organisms have indicated that COUP-TFs play important roles in many biological processes, including cell differentiation, cell fate determination, cell cycle regulation, and metabolic homeostasis (for reviews, see refs *23*−*25*). For example, homozygous loss of COUP-TFII function in mice results in defects in heart formation and angiogenesis that result in embryonic lethality (*28*). The identification of downstream target genes is critical for understanding how COUP-TFs exert their diverse biological functions.

Several mechanisms have been identified whereby COUP-TFs inhibit gene transcription. These include active repression, competition with other nuclear receptors for the occupancy of DNA-binding sites, and transrepression (for reviews, see refs *23*−*25*). Our data indicate that COUP-TFII/ARP-1 abrogates the activation of the *ATPA* gene by USF2 through competition for an overlapping DNA-binding site. Interestingly, our findings on the regulation of the *ATPA* gene are very similar to results obtained by Vasseur-Cognet and colleagues on the transcriptional regulation of the L-type pyruvate kinase gene (*L-PK*). For example, they have

demonstrated that COUP-TFII/ARP-1 represses the USF2-dependent transactivation of the *L-PK* gene through competition for an overlapping binding site in the upstream promoter of the *L-PK* gene (*27*). A composite regulatory element containing overlapping USF and COUP-TF binding sites has also been identified in the promoter of the follicle stimulating hormone receptor (*29*).

Upstream stimulatory factor is a ubiquitously expressed transcription factor that has been shown to regulate the expression of a large number of cellular and viral genes (see references in refs *9*, *10*−*12*, and *14*). USF activity in HeLa cells was originally purified as two polypeptides that are encoded by two distinct genes, *USF1* and *USF2* (*6*−*12*). Two forms of USF2 have been described, USF2a and USF2b. USF2b lacks an internal 67-amino acid domain present in USF2a due to splicing out of exon 4 (*14*, *30*). USF1 and USF2 bind as homo- and heterodimers to sites on the DNA that contain a CACGTG or CACATG sequence (E-box element) (*7*, *8*, *13*). Studies have indicated that USF family members play important roles in many cellular processes, especially the regulation of cellular growth and proliferation (*31*−*34*).

To date, we have identified several functional *cis*-acting regulatory elements and binding transcription factors that regulate the expression of the mammalian *ATPA* gene (*3*, *4*, *20*, *35*). These *trans*-acting regulatory factors include USF2 (*4*, *20*), YY1 (*35*), and COUP-TFII/ARP-1 (this study). It appears that the transcriptional regulation of the *ATPA* gene is complex and is dependent on a number of different transcription factors. USF2, COUP-TFII/ARP-1, and YY1 are all expressed in a wide variety of tissues and probably often in the same cell type (*9*, *25*, *36*). Indeed, USF2, YY1, and COUP-TFII/ARP-1 have been shown to be coexpressed in a number of cells, including human HeLa cells (*4*, *9*, *17*, *35*, *36*). Although the mammalian *ATPA* gene is ubiquitously expressed, its level of expression varies widely among different tissues (*18*). For example, the bovine *ATPA* gene is expressed at levels approximately 5−10-fold greater in cardiac and skeletal muscle than in liver (*18*). It is likely that the net expression of the *ATPA* gene (at least in some cells) will result from the relative concentration and affinity of these transcription factors (and probably other factors) expressed in a given physiological state. These transcription factors might play important regulatory roles linking changes in expression of the *ATPA* gene with changes in cellular energy needs during development, differentiation, hormonal stimulation, and/or cell growth, etc.

Data from our laboratory using EMSA and mutagenesis experiments indicate that regulatory factors, in addition to USF2 and COUP-TFII/ARP-1, are important for the transcriptional regulation of the *ATPA* gene through *cis*-acting regulatory element 1 (*3*, *4*). For example, mutation of the 5′-nuclear receptor half-site in the *ATPA* regulatory element 1 results in a 60−90% decrease in the *ATPA* promoter activity, indicating the release of a positive factor from this element, whereas mutation of the 3′-half-site results in an increase in the *ATPA* promoter activity, indicating release of a negative factor (*3*; Figures 6 and 7). Indeed, other transcription factors, including other nuclear receptors, were recovered in our genetic screen. Future experiments will be directed toward examining the role of each of these factors in the transcriptional regulation of the *ATPA* gene.

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