Regulation of the Nuclear Gene That Encodes the \( \alpha \)-Subunit of the Mitochondrial \( \text{F}_0\text{F}_1\)-ATP Synthase Complex

ACTIVATION BY UPSTREAM STIMULATORY FACTOR 2

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Gail A. M. Breen‡ and Elzora M. Jordan

From the Department of Molecular and Cell Biology, The University of Texas at Dallas, Richardson, Texas 75083-0688

We have previously identified several positive \( \text{cis} \)-acting regulatory regions in the promoters of the bovine and human nuclear-encoded mitochondrial \( \text{F}_0\text{F}_1\)-ATP synthase \( \alpha \)-subunit genes (ATPA). One of these \( \text{cis} \)-acting regions contains the sequence 5'-CACGTG-3' (an E-box), to which a number of transcription factors containing a basic helix-loop-helix motif can bind. This E-box element is required for maximum activity of the ATPA promoter in HeLa cells. The present study identifies the human transcription factor, upstream stimulatory factor 2 (USF2), as a nuclear factor that binds to the ATPA E-box and demonstrates that USF2 plays a critical role in the activation of the ATPA gene in \( \text{in vivo} \). Evidence includes the following. Antiserum directed against USF2 recognized factors present in HeLa nuclear extracts that interact with the ATPA promoter in mobility shift assays. Wild-type USF2 proteins synthesized from expression vectors \( \text{trans} \)-activated the ATPA promoter through the E-box, whereas truncated USF2 proteins devoid of the amino-terminal activation domains did not. Importantly, expression of a dominant-negative mutant of USF2 lacking the basic DNA binding domain but able to dimerize with endogenous USF proteins significantly reduced the level of activation of the ATPA promoter caused by ectopically coexpressed USF2, demonstrating the importance of endogenous USF2 in activation of the ATPA gene.

Most cellular ATP is synthesized in the mitochondria through the process of oxidative phosphorylation. The mammalian mitochondrial oxidative phosphorylation system requires the functional interaction of gene products encoded by both the nuclear and the mitochondrial genomes (for reviews, see Refs. 1 and 2). The activities of the enzymes of the mammalian oxidative phosphorylation system vary greatly in response to a number of physiological conditions, including cell proliferation, hormonal stimulation, development, and differentiation (for reviews, see Refs. 3 and 4). The levels of these enzymes are controlled, at least in part, at a transcriptional level, although regulation at a post-transcriptional level also plays an important role in mitochondrial biogenesis (1–4).

During the past several years, analysis of mammalian nuclear encoded oxidative phosphorylation genes has resulted in the identification of a number of regulatory factors that contribute to the transcription of these genes. Characterization of these gene regulatory mechanisms should allow the delineation of signals involved in the control of cellular energy production and, possibly, in the coordinate expression of genes encoding proteins of the oxidative phosphorylation system.

Our laboratory has been analyzing the regulation of the nuclear gene that encodes the \( \alpha \)-subunit of the mammalian mitochondrial \( \text{F}_0\text{F}_1\)-ATP synthase complex (ATPA) and has identified several positive \( \text{cis} \)-acting regulatory regions that are important for expression of this gene (5). Furthermore, we have found that protein factor(s) present in HeLa nuclei bind to these \( \text{cis} \)-acting regions. For example, we have determined that the multifunctional regulatory factor, YY1, can activate the ATPA promoter through an initiator-like element (6). In addition, we have identified a protein(s), termed ATPF1, which binds to the \( \text{cis} \)-acting region of the ATPA promoter which contains an E-box element (CACGTG) (5). This E-box element is required for maximum activity of the ATPA promoter in HeLa cells (5). A large family of regulatory proteins with a common structural feature, termed a basic helix-loop-helix motif (bHLH),\(^1\) bind to E-box elements (for reviews, see Refs. 7 and 8). We were interested in isolating and characterizing this regulatory factor(s), ATPF1.

In this paper, we describe the identification of the transcription factor, upstream stimulatory factor 2 (USF2), as a component of ATPF1. We also demonstrate that USF2 can \( \text{trans} \)-activate the ATPA gene through the E-box element. Furthermore, we show using a dominant-negative mutant (9) that USF2 plays an important role in the activation of the ATPA gene \( \text{in vivo} \). USF was initially identified from HeLa nuclei and was shown to stimulate transcription from the adenovirus major late promoter through the core sequence CACGTG (10–12). Purification of USF activity from HeLa cells revealed two polypeptides of 43 and 44 kDa, termed USF1 and USF2, respectively (13, 14). Analyses of cDNA clones indicated that USF proteins are members of the Myc family of regulatory proteins characterized by a COOH-terminal bHLH-leucine zipper (zip) structure responsible for dimerization and DNA binding (13, 14). Other members of this family include the mammalian proteins, Myc (15), Max (16), Mad (17), Mxi1 (18), TFEB (19), and TFE3 (20). Transcriptional activation by USF1 and USF2 can be demonstrated both \( \text{in vitro} \) and \( \text{in vivo} \), and USF proteins appear to play an important role in the transcriptional regulation of a number of different genes (see Ref. 14).

\(^1\) The abbreviations used are: bHLH, basic helix-loop-helix; zip, zipper; USF, upstream stimulatory factor; bp, base pair; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus.
EXPERIMENTAL PROCEDURES

Materials—The following oligonucleotides were used in this work. ATPA wild-type was 5’-ACATCCGGTTCGACGTGCGTACT-3’ containing the +23 to +46 bp region of the bovine ATPA promoter (21). The wild-type E-box element is underlined. ATPA mutE was 5’-ACATCCGGGTTCGACGTGCGTACT-3’ containing a mutated E-box element of the ATPA promoter (5).

Polyclonal antiserum to USF1 and USF2 were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Antibodies against E12/E2-2 were from Pharmingen, La Jolla, CA.

Oligonucleotide Screening of a Phage Library—cDNA clones encoding proteins with ATPA E-box binding activity were isolated by screening a human HeLa cDNA expression library in phage ggt11 (Clontech, Palo Alto, CA). Approximately 10<sup>6</sup> phage plaques from the cDNA library were screened using a concatamerized 32P-labeled double-stranded oligonucleotide probe containing the ATPA E-box element by an in situ filter detection method as described previously (22, 23). To ensure the binding specificity of positive clones, filters were rescreened with a concatamizered double-stranded oligonucleotide containing a mutated E-site to detect clones that bound nonspecifically. Only those positive plaques that did not bind to the mutated site were analyzed further. The sequences of the oligonucleotides containing the wild-type and mutated ATPA E-sites are shown above.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from cultured HeLa cells, as described (5, 23). Self-complementary oligonucleotides containing the E-box of the ATPA gene were used as probes in mobility shift assays after end filling with Klenow and ATP. ATP. The DNA binding reaction mixtures were performed at room temperature in binding buffer (10 mM Tris, pH 7.5, 60 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 8% glycerol) together with approximately 1–2 µg of HeLa cell nuclear extract, 2 µg of poly(dI-dC), and 0.1–0.5 ng of end-labeled oligonucleotide (23). For supershift assays, 1–2 µl of anti-serum was included in the binding reactions before the addition of probe. For competition assays, 10 ng of cold oligonucleotide with the same sequence was used. Samples were analyzed by electrophoresis on 4% polyacrylamide gels using 1 X Tris borate-EDTA as the running buffer.

Plasmids and Expression Vectors—The ATPA-CAT (chlamyphemonal acetyltransferase) reporter constructs used in these study have been described previously (5). Briefly, ATPA (+23/+136 bp)CAT contains the wild-type E-box element. ATPA mutE (+23/+136 bp)CAT contains a mutated E-box and ATPA ∆E (+49/+136 bp)CAT has a deletion in the E-box element of the bovine ATPA promoter. All expression vectors were driven by either the cytomegalovirus (CMV) immediate early promoter/enhancer or the SV40 promoter/enhancer. The human USF1 expression vector was a kind gift from Dr. R. Roeder (24). The human USF2a and USF2b expression cDNAs (25) and the mouse wild-type USF2a, mutant USF2bAN, and mutant TDSUSF2AB expression cDNAs (9) were generous gifts from Dr. Raymondjean and Dr. Sawadogo, respectively. The E12 expression vector was generously provided by Dr. Kadesch (26).

Transfections and CAT Assays—HeLa cells were cultured as described previously (5). HeLa cells were typically transfected with a 5–10 µg of the CAT reporter plasmid DNA together with various concentrations of effectors DNAs and 2 µg of a β-galactosidase expression plasmid (pCMV-βgal) by the calcium phosphate coprecipitation method (5, 23). Cells were harvested approximately 48 h after transfection. CAT and β-galactosidase assays were carried out as described previously (5, 6), and the CAT activities of the extracts were normalized relative to the β-galactosidase activities. Promoter activity values represent the average of at least three separate transfections of three plates each.

RESULTS

Isolation of cDNAs for ATPF1—We have determined previously that an E-box element (CACGTG) is required for maximal transcription of the ATPA gene when tested in transient expression assays in HeLa cells (5). We have also determined that a protein factor(s) present in HeLa nuclei, termed ATPF1, binds to this E-box (5). To identify cDNAs that encode ATPF1, we screened a human HeLa cDNA expression library using a multimerized oligonucleotide containing the ATPA E-box as a probe. From this screening, we isolated two phage clones that hybridized to this probe but did not hybridize to a multimerized oligonucleotide containing a mutation in the ATPA E-element. These clones were plaque purified, and the nucleotide sequence of a portion of each cDNA was determined (23). Analyses of these sequences revealed that one cDNA encoded the regulatory factor, E12 (26) and the other cDNA encoded the transcription factor, USF2 (14, 25). Both E12 and USF2 have been shown to bind to E-box elements and to regulate the expression of a number of genes.

USF2 Is Present in ATPA-HeLa Complexes—To determine if E12 or USF2 is a component of the ATPA-protein complexes in HeLa cells, we examined the effect of specific antibodies raised against these proteins using electrophoretic mobility shift assays. As shown in Fig. 1, we found that addition of antibodies against USF2 resulted in a supershift in the mobility of the ATPA-HeLa complexes. In contrast, antibodies against E12/E2-2 had no effect on the mobility of the ATPA-HeLa complex. Similarly, nonimmune serum also did not affect the mobility of the ATPA-HeLa complexes. However, antibodies specific for USF1 could also supershift the mobility of the ATPA-HeLa complexes. To verify that the effects of the antibodies to USF2 and USF1 were specific, we added an unlabeled oligonucleotide containing the ATPA E-box as a competitor. These experiments demonstrated that the supershifted USF-HeLa-ATPA complexes were effectively inhibited by an excess of competitor oligonucleotide (Fig. 1). These results indicate that USF2 and USF1 are components of the endogenous ATPA E-box binding activity in HeLa nuclear extracts.

USF2 Trans-activates the ATPA Promoter—The ability of USF2 and E12 proteins to function as transcriptional modulators of the ATPA promoter was next analyzed using transient transfection assays. These experiments demonstrated that cotransfection of the ATPA/CAT reporter construct together with an expression vector encoding USF2 resulted in an increased expression of the ATPA promoter in a dose-dependent fashion (Fig. 2). In contrast, there was little or no effect of cotransfect-
Fig. 2. USF2b, but not E12, trans-activates the ATPA promoter. HeLa cells were cotransfected with 5 μg of the ATPA(23/+136 bp)/CAT plasmid DNA (5), together with various concentrations (μg) of the expression vectors, pCMVUSF2b (25) or pSVE12 (26). Cells were harvested after approximately 48 h and assayed for CAT activity.

RESULTS

USF2a Isoform Also Activates the ATPA Promoter—Alternatively spliced forms of USF2 resulting from the presence or absence of the fourth exon have been described (9, 25). The previous trans-activation experiments were carried out using the USF2 isoform that lacks the fourth exon, termed USF2b. Experiments were also performed to examine the trans-activation potential of the USF2a isoform on the ATPA promoter. The results of these experiments indicated that USF2a can also activate the ATPA promoter, although not quite as effectively as USF2b (Fig. 4). Furthermore, the concentration of USF2a required to achieve maximum activation of the ATPA promoter was lower than that of USF2b (and higher concentrations were inhibitory). We also determined that trans-activation of the ATPA promoter by USF2a required the amino-terminal trans-activation domains since a construct containing only the amino-terminal 150 amino acids of USF2a was still trans-activating (Fig. 4).

A Dominant-negative Mutant Demonstrates the Role of USF2 in Activation of the ATPA Gene in Vivo—The next series of experiments were carried out to assess the direct involvement of USF2 in activation of the ATPA promoter in vivo since the possibility remains that other bHLH-zip proteins more directly stimulate this promoter. The strategy used relied on the use of a dominant-negative mutant of USF2, TDUSF2aΔN. This mutant lacks the basic region required for DNA binding but can still dimerize with endogenous USF1 or USF2 proteins or with itself (9, 25). Since the binding of USF to cognate sites requires dimers possessing two functional DNA binding domains (13, 28), the sequestration of endogenous USF by ectopically expressed TDUSF2aΔN allows the identification of transcriptional processes that are directly dependent upon USF2. The results of these experiments demonstrated that expression of TDUSF2aΔN significantly reduced the level of activation of the ATPA promoter caused by ectopically coexpressed USF2a (Fig. 5). These cumulative data argue forcefully that the binding of USF2 to the E-box element in the ATPA promoter is involved directly in the activation of the ATPA gene in vivo.

DISCUSSION

Our laboratory has been analyzing the regulation of the nuclear gene that encodes the α-subunit of the mammalian mitochondrial F1F0-ATP synthase complex (ATPA). Using a deletion analysis, we have identified several positive cis-acting regulatory regions in the promoter of this gene (5). By site-directed mutagenesis, we have determined that an E-box element (CACGTG) located within one of these cis-acting regions is required for maximum expression of the ATPA gene (5). E-box elements have been found to be critical for the expression of a number of different genes. A large family of transcription factors with a common structural feature, termed the bHLH motif, binds to E-box elements (for reviews, see Refs. 7 and 8).
A subfamily of bHLH proteins also contains a leucine zipper for additional dimerization potential (7). In this paper, we demonstrate that the transcription factor, USF2, a bHLH-zip-containing protein, binds to the E-box sequence in the ATPA promoter. Furthermore, we show that the binding of USF2 to the ATPA E-box activates this promoter. In addition, we demonstrate that endogenous USF2 plays an essential role in the activation of the ATPA gene. Evidence includes the following. Specific antibodies against USF2 (or USF1) revealed that USF proteins are components of the protein(s) in HeLa nuclear extracts bound to the ATPA cis-acting region. Expression of wild-type USF2 proteins had a stimulatory effect on the activity of the ATPA promoter through the E-box sequence. These results demonstrate that USF2 proteins functionally interact in living cells with the E-box of the ATPA promoter. However, it is still possible that ectopically expressed USF2 proteins supplant other E-box-binding proteins that normally participate in the activation process. The use of a dominant-negative mutant, TDUSF2aB, demonstrated that functional USF2 oligomers are important for activation of the ATPA gene in vivo. This mutant protein is able to dimerize through its HLH-zip motif but cannot bind DNA because of a deletion in the basic region (9, 25). Consequently, in cells transfected with the TDUSF2aB mutant, functional USF2 oligomers are expected to be progressively replaced by defective oligomers unable to interact with the ATPA E-box. This titration of functional USF2 oligomers by TDUSF2aB resulted in a decrease in the level of activation of the ATPA promoter caused by ectopically expressed USF2a. The use of such dominant-negative mutants has revealed previously that USF2 is a regulator in vivo of the liver-type pyruvate kinase gene in response to glucose (25) and of transcriptional activation by the varicella zoster virus immediate-early protein IE62 (9).

USF activity in HeLa cells was initially described as a complex consisting of two polypeptides of 43 and 44 kDa, termed USF1 and USF2, respectively (13, 14, 31). Later, an additional level of complexity of USF2 was described since differential splicing generates mRNAs encoding polypeptides of 44 kDa (USF2a) and 38 kDa (USF2b) (9, 25). USF2b lacks an internal 67-amino acid domain present in USF2a because of splicing out of the fourth exon in the primary transcript. It has been reported that the trans-activation potential of the 38-kDa USF2b alternative spliced form depends on the promoter context (29, 30). For example, USF2b was found to be approximately three to four times less active than USF2a or USF1 on a minimal promoter depending on oligomerized USF binding sites (29). However, in the context of the liver-specific pyruvinate kinase promoter, USF2b exhibited a trans-activation potential similar to that of USF2a or USF1 (29). In this paper, we demonstrate that USF2b is as effective (or slightly more effective) an activator of the ATPA promoter as USF2a.

The expression of both nuclear and mitochondrial genes that encode proteins of the mammalian mitochondrial oxidative phosphorylation system changes in response to a number of conditions including cellular proliferation, oxygen tension, neoplastic transformation, hormonal stimulation, development, and differentiation (for reviews, see Refs. 3 and 4). The mechanism(s) by which a cell regulates and coordinates the expression of these genes to meet cellular energy demands is not understood. A number of transcription factors have now been identified which are important for the expression of one or more nuclear genes that encode proteins of the mammalian mitochondrial oxidative phosphorylation system. Examples include: USF2 (this work), NRF-1 (also termed a-Pal; 32–34), GABP (34–37), OXBOX and REBOX-binding factor(s) (38), Sp1 (39), and YY1 (6, 39). The question still remains as to whether any of these regulatory proteins can respond to the energy needs of a cell and, if so, by what mechanism(s). One possibility is that the activity of these factors is affected by the redox potential of the cell. Interestingly, it has been reported that the DNA binding and activation potential of USF1 is strongly affected by redox changes (40). Furthermore, the DNA binding and trans-activation potential of GABP (41) and the binding affinity of the REBOX-binding protein(s) (38) have also been shown to be sensitive to reducing conditions. It is therefore possible that proteins such as USF, GABP, and the REBOX-binding factor(s) may function as important links between the redox state of the cell and the regulation of nuclear and mitochondrial gene expression.

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