An analysis of the promoter of a bovine and human nuclear-encoded mitochondrial ATP synthase α-subunit gene (ATPA) revealed the presence of a positive control element. DNase I footprinting and electrophoretic mobility shift assays demonstrated that this cis-acting regulatory element contains a binding site for a protein present in human HeLa nuclei, termed ATP factor 1 (ATPF1). The ATPF1 binding site contains the sequence, CANNTG, a sequence identical to the recognition site for a family of transcription factors containing a basic region adjacent to a helix-loop-helix domain. Site-directed point mutations of the basic helix-loop-helix binding site demonstrated the critical role of this element for both binding of ATPF1 and also for transcriptional activation of the ATPA gene.

The mitochondrial ATP synthase complex is a key enzyme in the production of cellular energy in eukaryotic cells. This enzyme complex synthesizes ATP from ADP and F0, utilizing the electrochemical potential gradient generated by the electron transport chain. The biosynthesis of the ATP synthase enzyme is a complex process requiring the coordinate expression of both nuclear and mitochondrial genes. For example, two of the subunits of the ATP synthase are encoded by the mitochondrial genome in animal cells, whereas the remaining subunits of this enzyme complex are encoded by nuclear genes and imported into mitochondria (for review see Ref. 1). The ATP synthase complex offers a model system to study the molecular mechanism(s) of nuclear and mitochondrial gene interactions.

A regulatory system must exist to coordinate the expression of the nuclear and mitochondrial genes that encode proteins of the oxidative phosphorylation system to meet varying energy demands. It has been found in the yeast Saccharomyces cerevisiae that transcriptional control of nuclear genes encoding proteins of the oxidative phosphorylation system is mediated through specific cis- and trans-acting elements in response to heme metabolism, oxygen, and catabolite repression (see Refs. 2 and 3). In contrast, little is known about the regulation of the expression of the nuclear genes that encode proteins of the mitochondrial oxidative phosphorylation system in mammalian cells. To approach this problem, we have begun isolating nuclear genes that encode subunits of the mammalian mitochondrial ATP synthase complex.

Recently, we reported the isolation and characterization of a nuclear gene (ATPA) that encodes a bovine (4) and human (5) mitochondrial ATP synthase α-subunit protein. We have determined that the 5′-flanking region of each of these genes acts as a promoter element (4, 5). In this study, we have analyzed the cis-acting sequences which regulate the expression of these genes. Using a deletion analysis, we have identified a positive cis-acting regulatory element, that is required for basal expression of the ATPA gene. We have determined that a trans-acting factor present in human HeLa nuclei, termed ATP factor 1 (ATPF1), binds to this cis-acting regulatory sequence. The ATPF1 binding site contains the sequence, CANNTG. This sequence is identical to the consensus recognition sequence for a large family of DNA-binding proteins that contain a common structural feature consisting of a basic region adjacent to a helix-loop-helix domain (bHLH) (see Ref. 6). By site-directed mutagenesis, we have found that binding of ATPF1 to specific residues within this bHLH binding site is critical for basal transcription of the ATPA gene.

**MATERIALS AND METHODS**

Oligonucleotides—Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 380B DNA Synthesizer. Synthetic DNA oligomers used in this work were as follows.

ATPA +23/+50: GATCATTTGTTACGTGCTGATCGC
ACGTCATATGCACGGCACTGACGGATC

**Sequence 1**

COXIV +13/+36: GGATCCGGGACCCGCTCTTCTGCGAAGGGCAAGCGGCGCTTTCGA

**Sequence 2**

USF: GATTCCTGGCAGCTGGACGGGA
AGCCAGTNGACCTGCTTCTTAG

**Sequence 3**

DNA Sequence Analysis—DNA sequences of alkaline-denatured double-stranded plasmid DNAs (7, 8) were determined by the dideoxy chain termination method (9) using Sequenase kits (U. S. Biochemical Corp.) and the method recommended by the manufacturer.

Polymerase Chain Reaction Amplification—Polymerase chain reactions (PCR) were carried out in a total volume of 100 μl using 100 ng of each primer, 100 ng of a208 DNA (4), 200 μM of each dNTP, Taq DNA polymerase buffer (Promega, Madison, WI), and Taq DNA polymerase (2.5 units, Promega). Samples were heated at 95 °C for 3 min to denature the template DNA and subjected to 30 cycles of amplification of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Mutations were introduced into the ATPA gene promoter using oligonucleotides containing specific mismatches and PCR reactions.

Plasmid Construction—To facilitate cloning of the 5′-flanking region of the ATPA gene into the reporter plasmid pCAT-Basic (Promega), primers were synthesized containing HindIII and XbaI restriction sites.

* This work was supported by United States Public Health Service Grant GM47738 from the National Institute of Health (to G. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: bHLH, basic helix-loop-helix; PCR, polymerase chain reaction; bp, base pair(s); CAT, chloramphenicol acetyltransferase.
After PCR amplification, the products were digested with HindIII and XbaI (Life Technologies, Inc.) and ligated to pCAT-Basic DNA that had been digested with HindIII and XbaI.

Transfections and Enzyme Assays—Human HeLa cells were used in the transfection experiments. Cells were maintained in Dulbecco’s modified Eagle’s medium (Irvine Scientific, Irvine, CA) supplemented with 10% fetal bovine serum. For DNA transfections, 20 μg of cesium chloride-purified pATPA-CAT DNA and 5 μg of pCMV-β-galactosidase DNA (10) were co-precipitated with calcium phosphate (8). After 2 days, the cells were harvested and the lysates were assayed for β-galactosidase (7) and CAT (11) activities. Prior to assaying for CAT activity, cell extracts were heated at 65 °C for 15 min. CAT activity was assayed using [3H]isocetysil-CoA (Amersham Corp.) as a substrate. Promoter activity values represent the average of at least three separate transfections of three plates each.

DNase I Footprinting Assays— Fragments were prepared from the plasmid pTPA-B/CAT by digestion with either XbaI (to label the coding strand) or HindIII (to label the noncoding strand) and 5'-end-labeled with [α-32P]dNTPs using Klenow enzyme. Binding reactions contained approximately 10 fmol of labeled fragment in a 100-μl reaction volume containing 25 mM Tris, pH 7.9, 6.25 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.1 μg of poly[d(1-dC)], plus approximately 30 μg of HeLa nuclear extract (12). After incubation at room temperature for 15 min, 50 μl of 10 mM MgCl2, 10 mM CaCl2, was added and incubated at room temperature for 1 min. DNase I (Promega) was then added (0.10 unit for reactions without extract and 1.5 units for reactions with extract) and incubated for an additional 1 min at room temperature. Reactions were then terminated, extracted with phenol, and precipitated with ethanol. Samples were then separated by electrophoresis on denaturing polyacrylamide-urea gels.

Electrophoretic Mobility Shift DNA-Protein Binding Assays—A double-stranded oligonucleotide encompassing the +23 to +50-bp region of the bovine ATPA gene was synthesized and used as a probe for electrophoretic mobility shift protein binding assays. The probe was labeled at its 3'-end using the Klenow fragment of DNA polymerase I and the appropriate [α-32P]dNTP. Approximately 0.1 ng of probe (5,000–10,000 cpm) was incubated with 1 μg of HeLa cell nuclear extract as described (8). Complexes were resolved on 4% polyacrylamide gels using 1 x Tris-boric acid-EDTA (7) as the running buffer.

RESULTS

Mapping the Basal Promoter Activity of the ATPA Gene—We have shown previously that a 700-bp fragment from the upstream region of the bovine ATPA gene (from −638 to +161 bp) is sufficient to drive expression of a reporter CAT gene in HeLa cells. Furthermore, we have demonstrated that this fragment acts in an orientation-dependent manner (4). This plasmid was used as the starting point for mapping cis-acting DNA regulatory elements required for controlling expression of the ATPA gene. Potential cis-acting elements were initially identified using a series of 5'-deletion mutants. These deletion mutants were constructed using either naturally occurring restriction endonuclease sites or synthetic oligonucleotides and PCR reactions. Each of these deletion mutants was transfected into human HeLa cells, and the levels of the reporter CAT enzyme were determined. The results of these transfection experiments indicated that deletion of the 5'-flanking sequences of the ATPA gene to within 9 bp of the most 5'-transcription start site had essentially no effect on promoter activity (Fig. 1). Furthermore, deletion of sequences to +25 bp downstream of this start site also had little effect on promoter activity (Fig. 1). However, further deletion to +49 bp dramatically reduced promoter activity and deletion to +71 bp essentially reduced promoter activity to background levels (Fig. 1). These results indicate the importance of the +25 to +70-bp region of the ATPA gene for basal promoter function.

Detection of DNA-Protein Interactions Using DNase I Footprinting Assays—To determine whether the regions of the ATPA gene identified by deletion analysis to be critical for promoter activity coincided with the binding of a nuclear factor(s), DNase I footprinting experiments were performed. A fragment of the ATPA gene promoter from −9 to +135 bp was end-labeled and used as a probe in these assays. The results of these experiments revealed that two regions of the ATPA gene promoter were protected from nuclease digestion by the HeLa extracts. One region extended from nucleotide +26 to +49 and coincided with one of the regions identified by the 5'-deletion analysis to be critical for basal promoter activity (Fig. 2). A second (and weaker) protected region of the ATPA gene promoter extended from nucleotide +72 to +81 (Fig. 2).

Detection of DNA-Protein Interactions Using Electrophoretic Mobility Shift Assays—The 5'-deletion analysis and the DNase I footprinting assays identified a region of the ATPA gene within the sequence from +25 to +49 bp that could serve as a potential regulatory site involved in DNA-protein interactions. To further examine the ability of these sequences to interact with trans-acting factors, complementary oligonucleotides that encompass this region were tested in electrophoretic mobility shift assays together with nuclear extracts prepared from HeLa cells. When the products of these binding reactions were separated by electrophoresis in low ionic strength polyacrylamide gels, several complexes were detected (Fig. 3). All of these complexes were competed by excess oligonucleotide (Fig. 3). No competition was detected using nonspecific oligonucleotides (data not shown).

A search for consensus sequences for binding sites of known transcription factors (13) within this region of the ATPA gene revealed two potential binding sites for previously characterized factors. One of these sequences, ACATCCGG, matches the complement of the consensus binding sequence, CCGTTA/TGC, for the ets-1 family of transcription factors (14). The other sequence, CACCCTG, is the consensus recognition sequence for the bHLH-containing family of DNA-binding proteins (6). To determine if either of these sequences contribute to the ATPA-protein complexes observed in the electrophoretic mobility shift assays, oligonucleotides corresponding to the ETS and bHLH binding sites were tested as competitors in mobility shift assays. The results of these experiments revealed that the ETS oligonucleotide competed only weakly for formation of the ATPA-protein complexes (Fig. 3). In contrast, an oligonucleotide containing the bHLH binding site effectively
cis Site Analysis of the Mammalian ATPA Promoter

Fig. 2. Analysis of ATPA-protein complexes using DNase I footprinting assays. A fragment of the ATPA gene (from −9 to +135 bp) was labeled on either the noncoding strand (A) or the coding strand (B), incubated without (−) or with (+) HeLa cell nuclear extracts, and then subjected to digestion with DNase I. DNAs were separated by electrophoresis in 6% polyacrylamide-urea gels. Maxam-Gilbert sequence reactions for purine residues (51) were run alongside. The protected regions of the ATPA gene are indicated by vertical lines.

competed for formation of most of the ATPA-HeLa complexes (Fig. 3).

USF is a member of the bHLH family of DNA-binding proteins (15). USF was originally identified as a cellular factor that binds to an upstream stimulatory element (USS) of the adenovirus major late promoter (16, 17) and has also been found to regulate the transcription of several other genes (18–21). To determine if the protein complexed to the ATPA regulatory sequence was the same as USF, we tested the effect of antiserum to USF (15). The USF antiserum (1:100 dilution) was a polyclonal antiserum raised against the 43-kDa USF (15).

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To identify specific residues within this cis-acting regulatory sequence that are involved in the DNA-protein interactions, several complementary oligonucleotides were synthesized containing point mutations within this sequence (Fig. 4). These oligonucleotides were tested as competitors in electrophoretic mobility shift assays. The results of these experiments revealed that there was essentially no difference between the mobility of the ATPA-HeLa complexes that were reacted with anti-USF antiserum compared with those that were treated with a preimmune serum, suggesting that the nuclear factor bound to the ATPA regulatory element is distinct from USF (Fig. 3).

To identify specific residues within this cis-acting regulatory sequence that are involved in the DNA-protein interactions, several complementary oligonucleotides were synthesized containing point mutations within this sequence (Fig. 4). These oligonucleotides were tested as competitors in electrophoretic mobility shift assays. The results of these experiments revealed that oligonucleotide mut 1, which contains a 2-bp substitution in the ETS binding site, was not able to compete for binding of HeLa nuclear extracts to the wild-type ATPA probe (Fig. 4). Oligonucleotide mut 2, which has a 3-bp substitution in the sequence adjacent to the core bHLH site, competed partially for formation of the ATPA-HeLa complexes. In contrast, oligonucleotide mut 3, which contains a mutation altering the CA residues of the core bHLH site (CANNTG) together with the 5′-flanking T residue, competed only weakly for formation of the ATPA-protein complexes (Fig. 4). However, oligonucleotides mut 4 and mut 5, which contain base pair substitutions changing the NNTG residues of the bHLH binding site, effectively competed with the wild-type ATPA regulatory element for formation of the DNA-protein complexes (Fig. 4). These data demonstrate the importance of the CA residues of the bHLH binding site for formation of the ATPA-HeLa complexes.

The nucleotide sequence of this cis-acting regulatory element identified in the bovine ATPA gene is highly homologous to the corresponding region of the human ATPA gene (Fig. 5; Ref. 5). It is therefore likely that the corresponding region of the human gene also acts as a regulatory element controlling expression of this gene. In support of this idea is the finding that oligonucleotides containing this region of the human ATPA gene effectively competed for binding of HeLa nuclear extracts to the bovine regulatory element (data not shown).

Transfection of HeLa Cells with Mutated ATPA-CAT Constructs—The electrophoretic mobility shift and DNase I protection assays indicated that a factor(s) in HeLa nuclear extracts could bind to the ATPA promoter in the region from +25 and +49 bp. Furthermore, site-directed mutations in this region identified several nucleotides which appear to be critical for this binding. These in vitro assays do not, however, address the functional consequences of these DNA-protein interactions. In order to assay the effects of mutations in the ATPA cis-acting regulatory element on basal transcription, each of the mutant constructs, pATPA mut 1, mut 2, mut 3, mut 4, and mut 5-CAT, was transfected into HeLa cells in parallel with the corresponding unmutated pATPA-CAT construct. The results of these experiments revealed that the pATPA mut 1-CAT construct had slightly reduced transcriptional activities when compared with the wild-type construct (Table I). The pATPA mut 2-CAT plas-
mid showed a reduction in transcriptional activity to levels approximately 36% of wild-type (Table I). The pATPA mut 3-CAT construct showed an even more dramatic reduction in transcriptional activity to levels approximately 11% of wild-type, whereas the pATPA mut 4-CAT and pATPA mut 5-CAT constructs had activities that were comparable or even greater than those of the wild-type pATPA-CAT plasmid (Table I). These results indicate that residues within this cis-acting regulatory element of the ATPA gene promoter are essential for forming a DNA-protein complex required for expression of this gene.

**DISCUSSION**

In this paper we define a *cis*-acting regulatory sequence that is required for basal expression of a bovine and human nuclear-encoded mitochondrial ATP synthase α-subunit gene (*ATPA*). Using electrophoretic mobility shift and DNase I footprinting assays, we have determined that a *trans*-acting nuclear factor(s) present in human HeLa nuclei, termed ATPF1, binds to this regulatory sequence. The core of this *cis*-acting regulatory site contains the sequence, CACGTG, which matches the consensus binding site (CANNTG) for a large family of DNA-binding proteins that have a common structural feature termed the bHLH domain. This domain consists of a stretch of basic amino acids followed by a conserved sequence of amino acids presumed to form two α-helices interrupted by a loop. Members of this family include the transcription factor, USF, that binds to an upstream sequence of the adenovirus major late promoter (15, 16); transcription factors, TFE3 (22), TFEF (23), and E12, E47 (24), which bind to specific DNA sequences found in immunoglobulin enhancers; proteins such as MyoD (25), myf-5 (26), myogenin (27), achaete-scute (28), and daughterless (29), which play important roles in cell determination; and the Myc family of oncoproteins (see Ref. 30), together with their binding partners, such as Max/Myn (31). It is likely that the *trans*-acting protein factor, ATPF1, required for basal expression of the ATPA gene is a member of this bHLH family of DNA-binding proteins. Indeed, using a mutational analysis we have determined that binding of ATPF1 to specific residues within the bHLH sequence is critical for transcriptional activation of the ATPA gene.

One member of the bHLH family, transcription factor USF, has been found to play an important role in the regulation of several cellular genes, including genes for mouse metallothionein I (18), rat fibrinogen (19), rat human growth hormone (20), Xenopus transcription factor TFIIIA (32), and duck histone H5 (21). To determine if ATPF1 was the same as USF, we tested the effect of anti-USF antisera (15) on the mobility of the ATPA-HeLa protein complexes. The results of these experiments suggested that ATPF1 is distinct from USF (Fig. 3).

The *cis*-acting region of the ATPA gene found to be important for basal promoter activity also contains a potential binding site for transcription factors with an ETS domain (see Ref. 14). The results of both our *in vitro* (Fig. 3 and 4) and *in vivo* (Table I) experiments suggest that binding of an ETS domain factor to the ATPA regulatory element is not essential for basal transcription of the ATPA gene. This contrasts to the rat and mouse nuclear genes that encode cytochrome *c* oxidase subunit IV, whose basal promoters consist in a large part of two tandemly linked ETS domain binding sites (33–35) (see also below). It is possible that an ETS domain protein acts in concert with ATPF1 to modulate expression of the ATPA gene. ETS proteins have been found to cooperate with other transcription factors in

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**FIG. 4.** The effect of mutations in the ATPA *cis*-acting regulatory element on DNA-protein binding. An oligonucleotide encompassing the +23 to +50-bp region of the ATPA gene (wild-type) was labeled with 32P and used as a probe in electrophoretic mobility shift assays. Binding reactions were performed in the presence of approximately 1 μg of HeLa nuclear extract. Complexes were formed in the absence (none) or the presence of competitor oligonucleotides as indicated above the lanes. A 100-fold molar excess of unlabeled competitor oligonucleotide was used. The sequences of the mutant oligonucleotides are shown below. The bases which were mutated are indicated by an underline.

**Wild-type:** GTACATCCGGGTCACGTGGGCTGACTGC

**SEQUENCE 4**

**Mut 1:** GTACATTAAGGTTCACGTGGGCTGACTGC

**SEQUENCE 5**

**Mut 2:** GTACATTTAATAACGTGGGCTGACTGC

**SEQUENCE 6**

**Mut 3:** GTACATCCGGGTCACGTGGGCTGACTGC

**SEQUENCE 7**

**Mut 4:** GTACATCCGGGTCACGTGGGCTGACTGC

**SEQUENCE 8**

**Mut 5:** GTACATCCGGGTCACGTGGGCTGACTGC

**SEQUENCE 9**

**DISCUSSION**

In this paper we define a *cis*-acting regulatory sequence that is required for basal expression of a bovine and human nuclear-encoded mitochondrial ATP synthase α-subunit gene (*ATPA*). Using electrophoretic mobility shift and DNase I footprinting assays, we have determined that a *trans*-acting nuclear factor(s) present in human HeLa nuclei, termed ATPF1, binds to this regulatory sequence. The core of this *cis*-acting regulatory site contains the sequence, CACGTG, which matches the consensus binding site (CANNTG) for a large family of DNA-binding proteins that have a common structural feature termed the bHLH domain. This domain consists of a stretch of basic amino acids followed by a conserved sequence of amino acids presumed to form two α-helices interrupted by a loop. Members of this family include the transcription factor, USF, that binds to an upstream sequence of the adenovirus major late promoter (15, 16); transcription factors, TFE3 (22), TFEF (23), and E12, E47 (24), which bind to specific DNA sequences found in immunoglobulin enhancers; proteins such as MyoD (25), myf-5 (26), myogenin (27), achaete-scute (28), and daughterless (29), which play important roles in cell determination; and the Myc family of oncoproteins (see Ref. 30), together with their binding partners, such as Max/Myn (31). It is likely that the *trans*-acting protein factor, ATPF1, required for basal expression of the ATPA gene is a member of this bHLH family of DNA-binding proteins. Indeed, using a mutational analysis we have determined that binding of ATPF1 to specific residues within the bHLH sequence is critical for transcriptional activation of the ATPA gene.

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the transcriptional activation of a number of genes (see Refs. 36-38).

It is becoming evident that not all genes encoding subunits of the oxidative phosphorylation system in mammalian cells are regulated by the same cis- and trans-acting factors. Several distinct cis-acting elements have now been identified which are important for the transcriptional initiation of some mammalian nuclear-encoded oxidative phosphorylation genes. For example, the 5'-flanking region of the rat and human somatic cytochrome c genes contain a binding site for a trans-acting factor, termed NRF-1 (39, 40). A NRF-1 site is also found in the genes that encode rat cytochrome c oxidase subunit VIc-2, human ubiquinone-binding protein, mouse and human MRPl RNA, and bovine ATP synthase γ-subunit (40). Similarly, transcription of the rat and mouse cytochrome c oxidase IV genes requires binding of a trans-acting factor, NRF-2, to a cis-acting element (33). The core of this element has the sequence, GGAAG, which is the consensus binding site for proteins with an ETS domain (14). NRF-2 has been shown recently to be similar or identical to the ETS domain protein, GABPα (34, 35). Other studies have shown that transcription of the mouse cytochrome c oxidase Vb gene involves a cis-acting regulatory sequence to which a factor that is similar or identical to NF-E1 (also called δ, YY1, FACT1) binds (41). This NF-E1 sequence is adjacent to two ETS binding sites which may also play a role in the transcriptional initiation of the COXVb gene (33, 41). In addition, several cis-acting sequences, termed Mt1, Mt3, and Mt4, have been found in the 5'-flanking region of several human oxidative phosphorylation genes, including ATP synthase β-subunit, cytochrome c1, and the ubiquinone-binding protein (42). Similarly, two cis-acting elements, termed the OXBOX site and the REBOX site, have been identified which are important for transcriptional activation of the human ATP synthase β-subunit gene and the adenine nucleotide translocator 1 gene (43, 44). The OXBOX sequence binds a muscle-specific protein that acts as an "enhancer" element is present in the upstream region of the rat and mouse cytochrome c1 oxidase genes. Similarly, two cis-acting elements, termed the REBOX site and the OXBOX site for the binding of a trans-acting factor, NRF-1 (39,40). A NRF-1 site is also found in the genes that encode subunit VII of the ubiquinol-cytochrome c oxidase complex (49). CFIP1 is a member of the bHLH family of transcription factors (50). ABF1 and CFIP1 also bind to a number of genes that encode proteins involved in processes important for cell growth and to elements important for cell division and it has been postulated that ABF1 and CFIP1 are involved in coupling the rate of mitochondrial biogenesis to cellular growth (46). It is possible that some transcription factors which control the expression of nuclear genes encoding mitochondrial proteins are similar between both lower and higher eukaryotic organisms.

Acknowledgments—We thank Dr. Richard Scarpulla, Northwestern University; Dr. Michele Sawadogo, M.D. Anderson Cancer Center; and Dr. Robert Roeder, Rockefeller University for the generous gifts of plasmids, oligonucleotides, and/or antisera. We thank J. Wadas for assistance with the preparation of this manuscript.

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cis Site Analysis of the Mammalian ATPA Promoter

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