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Upstream Stimulatory Factor 2 Activates the Mammalian F_1F_0 ATP Synthase α-Subunit Gene Through an Initiator Element

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The F_1F_0 ATP synthase is the central enzyme complex of the mitochondrial oxidative phosphorylation system synthesizing ATP from ADP and P_i. Our laboratory has been studying the transcriptional regulation of the nuclear gene that encodes the α-subunit of the mammalian mitochondrial ATP synthase complex (*ATPA*). We have previously identified an initiator element in the core promoter that plays an important role in expression of this gene. In this article, we demonstrate that ectopic expression of the transcription factor, upstream stimulatory factor 2 (USF2), transactivates the *ATPA* gene through this initiator element. Importantly, cotransfection of a dominant-negative USF2 mutant significantly reduces both the basal activity and the level of activation of the *ATPA* initiator by coexpressed USF2 demonstrating the role of endogenous USF2 proteins in this activation. We also identify several nucleotides in the *ATPA* initiator element that are important for both basal activity and USF2-dependent transactivation. We have also previously determined that the binding of the multifunctional regulatory protein, YY1, to this initiator element can positively regulate the *ATPA* gene. Here, we show that expression of YY1 together with USF2 results in a decreased level of activation of the *ATPA* initiator relative to expression of USF2 alone, suggesting competition between these two regulatory proteins.

ATP synthase Initiator element *ATPA* gene Upstream stimulatory factor 2

IN mammalian cells, most cellular ATP is synthesized in the mitochondria by the oxidative phosphorylation system. The F_1F_0 ATP synthase, the central enzyme of the oxidative phosphorylation system, synthesizes ATP from ADP and P_i utilizing energy stored by the electron transport chain [for review, see (2)]. Synthesis of ATP varies widely among different tissues and in response to a number of stimuli, including development, differentiation, and cellular proliferation [for reviews, see (13,23)]. ATP synthesis is regulated primarily at two levels: by fast respiratory control and by slow synthesis of enzyme complexes (13,23).

The mammalian mitochondrial ATP synthase is a multisubunit enzyme complex made up of 14 different polypeptides, two of which are encoded by the mitochondrial genome (1). Due to its central role in ATP production, we have begun analyzing the transcriptional regulation of the nuclear gene that encodes the α-subunit of the mammalian ATP synthase complex (*ATPA*). Using transient transfection assays, we have previously identified several positive *cis*-acting regulatory regions in the *ATPA* promoter that are important for expression of this gene. These include a region surrounding several of the sites of transcription initiation and an upstream E-box element (37). We have also begun experiments to identify the *trans*-acting regulatory factors that bind to each of these *cis*-acting elements. We have determined that the multifunctional regulatory protein, YY1, binds to an initiator element in the core promoter of the *ATPA* gene (4). We have also demonstrated that the transcription factor, upstream stimulatory factor 2 (USF2), binds to and stimulates transcription of the *ATPA*

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gene through the upstream E-box element (3). Our previous studies also suggested that USF2 can activate the *ATPA* promoter through a second site separate from the E-box element (3).

In this article, we identify this second site for USF2 activation as an initiator element in the core promoter of the *ATPA* gene. Ectopic expression of USF2 efficiently transactivated the *ATPA* gene through this initiator element. Upstream stimulatory factor 1 has previously been shown to stimulate several viral and cellular genes through interactions with initiator elements, although the physiological significance of these findings has not been demonstrated (6,16,24). The development of dominant-negative mutants of USF that lack DNA binding capacity but can still dimerize with endogenous USF proteins now enables the identification of transcriptional processes that are directly dependent on USF (15,22). Here, through the use of a dominant-negative mutant of USF2, we demonstrate that USF transcription factors are utilized as positive activators of the *ATPA* initiator element in vivo.

MATERIALS AND METHODS

Plasmids and Expression Vectors

The expression plasmids used in this study have been described previously: pSVUSF2a, USF2b, USF2aΔN, and USF2aΔB (19,22); pCMVUSF2a and USF2b (15,38); pCXUSF1 (26); pCMV-YY1 (32); and pCMV-β-gal (37).

ATPA-*CAT Reporter Plasmids.* The *ATPA* promoter-CAT plasmids have been described previously (3,4,37). The nucleotide sequence of this region of the bovine *ATPA* gene (25) is shown in Fig. 1.

ATPA *Inr Mutants.* Oligonucleotides containing base pair substitutions in the +68 to +86 bp region of the *ATPA* gene (25) were synthesized, annealed together, and then ligated into the reporter vector, pCAT Basic (Promega). The mutations are diagrammed in Fig. 5.

Cell Culture and Transfections

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum. Cells were transfected using the calcium phosphate precipitation method as described previously (3,4,37). Briefly, HeLa cells were transfected with 5–10 μg of the CAT reporter plasmid DNA together with various concentrations of effector DNAs and 2 μg of a β-galactosidase expression plasmid (pCMV-β-gal). Cells were harvested approximately 48 h after transfection. All transfections were repeated at least three times.

Chloramphenicol Acetyltransferase and β-Galactosidase Assays

Chloramphenicol acetyltransferase (CAT) and β-galactosidase activities were determined as described previously (3,4,7). The CAT activities of the extracts were normalized relative to the β-galactosidase activities to correct for transfection efficiencies (relative CAT activity).

RESULTS

USF2 Transactivates the ATPA *Promoter Through an Initiator Element*

We have previously demonstrated that the transcription factor, USF2, can transactivate the *ATPA* promoter through an E-box element (3). However, our previous experiments also suggested that USF2 might interact with an additional site(s) in the *ATPA* promoter since deletion or mutation of this E-box element did not completely abolish transactivation by USF2 (3). To identify this second site(s), we examined the effect of additional 5′ and 3′ deletions of the *ATPA* promoter on activation by USF2 using transient transfection assays. We determined that se-

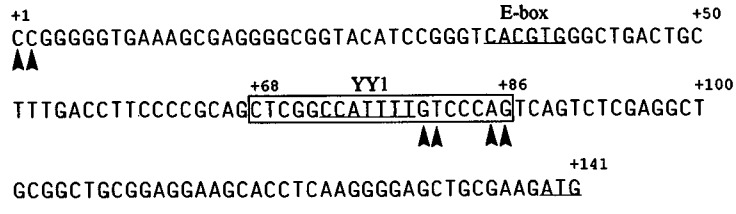

FIG. 1. Sequence of a portion of the bovine *ATPA* gene. The nucleotide sequence of the +1 to + 141 bp region of the bovine *ATPA* gene (25) is shown. The E-box element (3) and the YY1-binding site (4) are indicated. The initiator methionine is underlined. The +68 to +86 bp region of the *ATPA* promoter is boxed. The major sites of transcription initiation of the bovine *ATPA* gene (4,25) are indicated by arrowheads.

quences at both the 5′ and 3′ ends of the *ATPA* promoter could be deleted and still retain activation by USF2 (Fig. 2). Indeed, we identified a 19-bp fragment that was efficiently stimulated by ectopic expression of USF2 (Fig. 2). This fragment contains several of the predominant sites of transcription initiation of the *ATPA* gene (Fig. 1) (4,25) and has previously been shown to function as an initiator element (4). To further examine the activation of the *ATPA* core promoter by USF2 through this initiator element, we tested the effect of expressing increasing concentrations of USF2 in transient transfection assays. The results of these experiments revealed that the *ATPA* initiator element was efficiently transactivated by USF2 in a dose-dependent manner (Fig. 3A).

USF2a Isoform Also Transactivates the ATPA *Initiator Element*

USF2 exists in cells as two alternatively spliced forms, USF2a and USF2b, resulting from the presence or absence of the fourth exon (15,19,22,38). The previous transactivation experiments were carried out using the USF2 isoform that lacks the fourth exon, termed USF2b. We next tested the transactivation of the *ATPA* initiator element by the USF2a isoform. As shown in Fig. 3B, USF2a also efficiently stimulated the *ATPA* core promoter in a dose-dependent manner. The level of activation of the *ATPA* initiator element by the two isoforms of USF2 was comparable (Fig. 3). We also determined that transactivation of the *ATPA* core promoter by USF2a required the amino-terminal transactivation domains because a mutant truncated USF2a protein devoid of the first 198 amino acids (USF2aΔN) (22) did not activate the *ATPA* gene (Fig. 3C). These results suggest that USF2a and USF2b are activators of the *ATPA* initiator in vivo.

A Dominant-Negative Mutant Demonstrates the Role of USF2 in Activation of the ATPA *Initiator Element In Vivo*

Experiments were next carried out to assess the direct involvement of USF2 in activation of the *ATPA* gene because it is possible that other basic helix–loop–helix leucine zipper transcription factors are responsible for this initiator-dependent promoter activation in vivo. We therefore used an eukaryotic expression plasmid that expressed a dominant-negative USF2 protein in transient cotransfection assays. This mutant protein lacks the basic region required for DNA binding but can still dimerize with endogenous USF1 or USF2 proteins or with itself (15,22). Because the binding of USF to cognate sites requires dimers possessing two functional DNA binding domains (7,8,33), the ectopically expressed mutant USF2 protein sequesters wild-type endogenous USF2 and USF1 in complexes that are unable to bind DNA, thereby allowing the identification of transcription processes that are directly dependent upon USF2. As shown in Fig. 4, expression of the dominant-negative TDUSF2aΔB mutant resulted in a marked reduction in the basal activity of the *ATPA* initiator, presumably

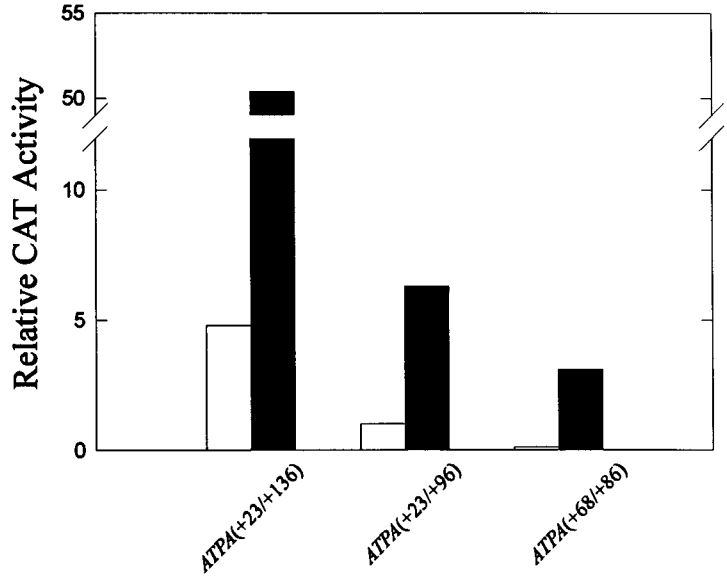

FIG. 2. USF2 transactivates the *ATPA* promoter through an initiator element. HeLa cells were cotransfected with 10 μg of either *pATPA* (+23/+136 bp)/CAT, *pATPA* (+23/+96 bp)/CAT, or *pATPA* (+68/+86 bp)/CAT (37) together with 10 μg of pCMV (□) or pCMV-USF2b (■). Cells were harvested approximately 48 h after transfection and the levels of CAT activity were determined.

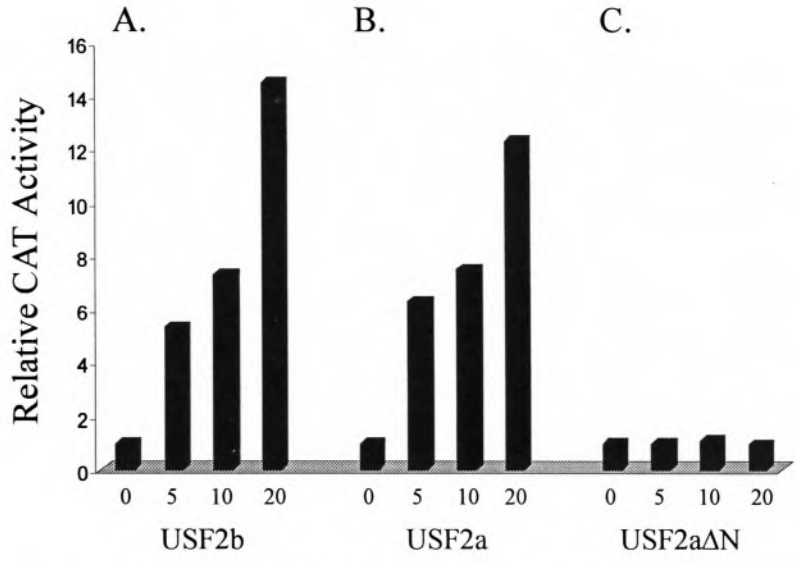

FIG. 3. Transacting properties of USF2b, USF2a, and a mutant USF2a protein. (A) Transactivation of the *ATPA* initiator element by USF2b. Ten micrograms of the *pATPA* (+68/+86 bp)/CAT reporter plasmid was cotransfected with the indicated amounts (in μg) of an expression vector expressing isoform USF2b (22). (B) Transactivation of the *ATPA* initiator element by isoform USF2a. Ten micrograms of the *pATPA* (+68/+86 bp)/CAT plasmid was cotransfected into HeLa cells together with the indicated amounts (in μg) of an expression vector expressing USF2a (22). (C) A mutant form of USF2a lacking the transactivation domains does not activate the *ATPA* core promoter. Ten micrograms of the *pATPA* (+68/+86 bp)/CAT plasmid was cotransfected with the indicated amounts (in μg) of an expression vector expressing a mutant form of USF2a that lacks the amino-terminal transactivation domains, USF2aΔN (22).

by dimerizing with endogenous USF proteins. Furthermore, transfection of the dominant-negative TDUSF2aΔB mutant together with USF2a reduced the level of activation of the *ATPA* initiator caused by the ectopically coexpressed USF2a by greater than 80% (Fig. 4). The results of these experiments demonstrate that the binding of USF2 to the *ATPA* initiator is directly involved in activation of the *ATPA* promoter in vivo.

Mutations in the ATPA *Initiator Reduce Transactivation by USF2*

A comparison of the sequence of the *ATPA* initiator with other core promoters that have been shown to be stimulated by USF reveals that they are homologous (Table 1). To identify nucleotides in the *ATPA* initiator element that are required for USF2-dependent transactivation, we examined the effect of three

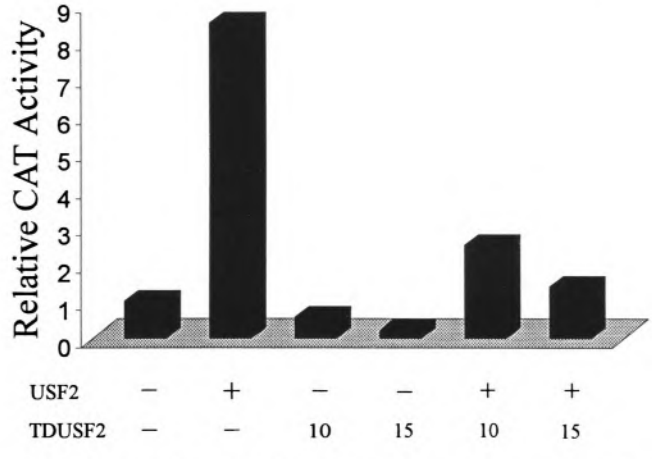

FIG. 4. A dominant-negative mutant of USF2a reduces both the basal activity and the activation of the *ATPA* initiator by USF2a. HeLa cells were cotransfected with 10 μg of the *pATPA* (+68/+86 bp)/CAT reporter plasmid together with 5 μg of the expression plasmid, pSVUSF2a, or the equivalent amount of empty vector, and 10 or 15 μg of pSVTDUSF2aΔB or the equivalent amount of empty vector. TDUSF2aΔB is a dominant-negative mutant of USF2a that is defective in DNA binding but is still capable of dimerization with USF2 and USF1 (22). Cells were harvested after 48 h and assayed for CAT activity.

TABLE 1
COMPARISON OF INITIATOR SEQUENCES (Inr) IN DIFFERENT GENES

Gene	Sequence	Reference
	+1	
Ad MLP	TCCTCACTCTCT	Du et al. (6)
HIV-1	TACTGGGTCTCT	Du et al. (6)
Cyclin D1	TGGCCATTCGCG	Philipp et al. (24)
C/EBPα	TGGCCATTCGCG	Li et al. (16)
Albumin	CGATCACCTTTC	Li et al. (16)
ATPA	CGGCCATTTTGT	Pierce et al. (25)
Consensus	YANTYY	Lo and Smale (18)
Inr	A	

mutations in this initiator. Mutant constructs were compared to wild-type by using in vivo transfection assays to examine both basal activity and responsiveness to USF2. Mutant m1 contains a quintuple point mutation at residues +71, +72, +73, +74, and +75, mutant m2 a quadruple point mutation at residues +76, +77, +78, and +79, and mutant m3 a quintuple point mutation at residues +80, +81, +82, +83, and +84 of the *ATPA* initiator. As shown in Fig. 5, all three mutations reduced the basal activity of the *ATPA* core promoter, to a level approximately 30% of that of wild-type in mutant m1, 50% in mutant m2, and 28% in mutant m3. Furthermore, the level of activation of the *ATPA* initiator by coexpressed USF2 was dramatically reduced in mutant m1 but was essentially unaffected in mutants m2 and m3 (Fig. 5). These experiments demonstrate that nucleotides +71 to +75 in the *ATPA* core promoter are important for both basal activity and USF2-dependent transactivation.

Expression of the Multifunctional Regulatory Factor, YY1, Together With USF2, Reduces the Level of Activation of the ATPA *Initiator*

We have previously shown that the binding of the multifunctional regulatory factor, YY1, to this initiator element in the *ATPA* core promoter can activate expression of the *ATPA* gene to a modest extent (4). We therefore tested the effect of expressing YY1, together with USF2, on activation of the *ATPA* gene in transient transfection assays. The results of these experiments revealed that coexpression of YY1 and USF2 resulted in a reduction in the level of activation of the *ATPA* initiator relative to expression of USF2 alone, suggesting competition between these two factors (Fig. 6).

DISCUSSION

In this article, we describe the ability of USF2 proteins to stimulate transcription of the *ATPA* gene via

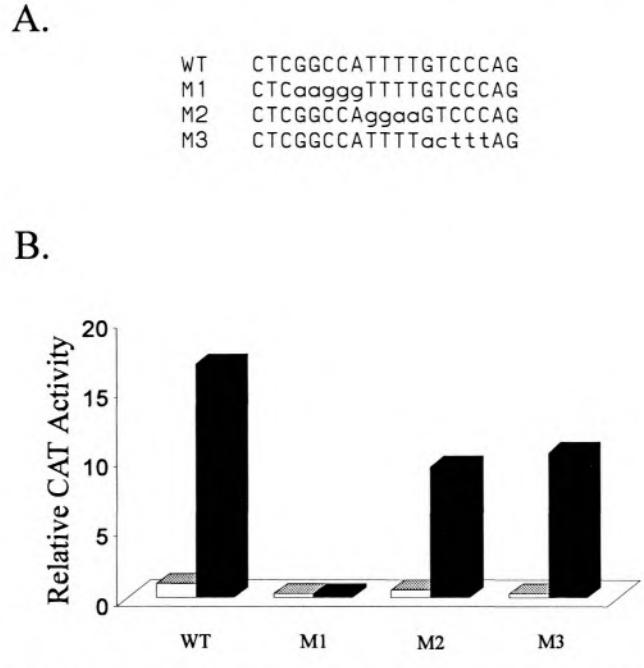

FIG. 5. The effect of mutations in the *ATPA* initiator element. (A) Diagram of the mutations in the *ATPA* initiator element. The sequence of the +68 to +86 bp fragment of the wild-type (WT) *ATPA* gene is shown on the top line. Mutations in this sequence (M1, M2, and M3) are indicated by lower case letters. (B) Diagram of the basal activity and the stimulatory effects of USF2a. HeLa cells were cotransfected with 1 μg of either pSV ((□) or pSVUSF2a (■) together with 1 μg of either the wild-type *pATPA* (+68/+86 bp)/CAT vector (WT) or the mutant *pATPA*/CAT vectors (M1, M2, and M3). Cells were harvested after 48 h and the levels of CAT activity were determined.

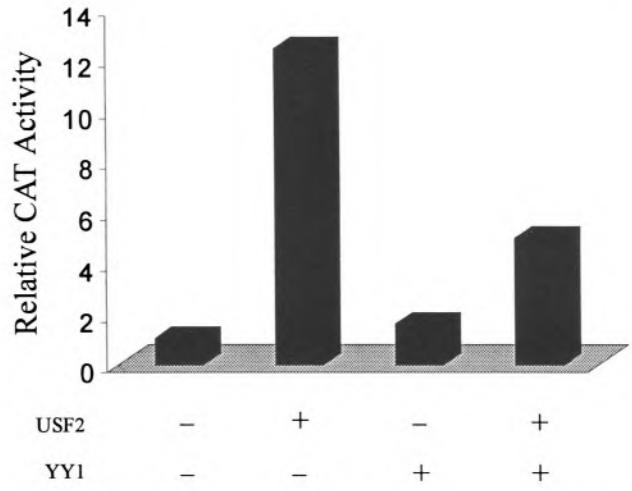

FIG. 6. Expression of YY1 together with USF2 reduces the activation of the *ATPA* initiator element. HeLa cells were cotransfected with 10 μg of the *pATPA* (+68/+86 bp)/CAT reporter plasmid together with either pCMV, pCMV-USF2b (15 μg), pCMV-YY1 (5 μg), or pCMV-YY1 (5 μg) plus pCMV-USF2b (15 μg). The total amount of DNA in each transfection was adjusted to 32 μg using pCMV DNA.

an initiator element in the core promoter. Importantly, through the use of a dominant-negative mutant we show that USF2 proteins are required for activation of the *ATPA* gene through this initiator sequence in vivo. It had been shown previously that USF1 can transactivate several cellular and viral genes, including the adenoviral major late promoter (6), human immunodeficiency virus type I (HIV-1) (6), cyclin D1 (24), C/EBPα (16), and serum albumin (16), via interactions with the pyrimidine-rich initiator elements of these promoters. However, the physiological relevance of these findings was not clear. The recent development of dominant-negative USF mutants now provides a means to assess the direct involvement of USF in the regulation of gene expression through initiator sequences. These dominant-negative mutants are capable of dimerizing with endogenous USF1 and USF2 proteins but cannot bind DNA (15,22). Consequently, in cells transfected with an expression vector for the transdominant USF2 mutant, functional USF oligomers are expected to be progessively replaced by defective oligomers that are unable to bind DNA, thus enabling the identification of transcription processes that are dependent upon USF. In several cases now, including transcriptional activation by the varicella-zoster virus immediate-early protein IE62 (22), the regulation of the L-type pyruvate kinase gene by glucose (15), the expression of the *ATPA* gene (3), the activation of the TGF-β2 gene (31), and the insulin regulation of the fatty acid synthase promoter (39), the use of dominant-negative mutants has demonstrated the participation of USF in the activation of genes via E-box elements. We used a similar approach here to demonstrate the importance of endogeneous USF in the transcriptional activation of the *ATPA* gene via an initiator element.

A comparison of the sequences of the initiator elements of the promoters that have been shown to be transactivated by USF reveals that they are homologous (Table 1). These initiator elements also conform to a functionally derived consensus sequence for initiator activity of Py Py A+1 N T/A Py Py, where Py is a pyrimidine residue (12,18). Previous studies of the initiator element of the adenoviral major late promoter (Ad MLP) indicated that residues −2, −1 and +2, +3, +4, +5 are important for USF1-dependent transactivation (6,16). Previous experiments have also shown that residues −1, −2, and −3 of the Ad MLP initiator are necessary for USF1 binding in vitro (28). In this study, we demonstrate that residues +71, +72, +73, +74, and +75 of the *ATPA* initiator are important for both basal activity and transactivation by USF2. These residues correspond to the −4, −3, −2, −1, and +1 positions of the Ad MLP. In contrast, residues +76, +77, +78, +79 and +80, +81, +82, +83, +84 of the *ATPA* core promoter (which correspond to positions +2, +3, +4, +5 and +6, +7, +8, +9 of the Ad MLP), although important for basal activity, are not required for USF2-dependent transactivation. Previous studies of consensus initiator sequences have shown that an A residue at +1, a T or A at +3, and a pyrimidine at −1 are the most critical residues for determining the strength of an initiator (12,18).

The bovine *ATPA* gene has multiple sites of transcription initiation. The +1 and +2 sites are the predominant initiation sites used in bovine heart (25), whereas the +80, +81, +85, and +86 sites are used predominantly in bovine liver [(25); D. J. Pierce and G. A. M. Breen, unpublished observations]. We have previously determined that +68 to +86 bp region of the *ATPA* gene, which contains several of the major sites of transcription initiation, can function as an ini-

tiator element and direct accurate transcription initiation in the absence of other control elements (4).

Upstream stimulatory factor (USF) is a ubiquitously expressed transcription factor that has been found to play an important role in the expression of a large number of cellular and viral genes [see refs. in (10,17)]. USF was initially characterized as a binding activity able to interact and transactivate the adenovirus major late promoter (29,30). Purification of this binding activity revealed the presence of two polypeptides of 43 (USF1) and 44 (USF2) kDa (8,30) that are encoded by two distinct genes (9,10,17). USF belongs to the Myc family of transcription factors that are characterized by a highly conserved C-terminal basic helix–loop–helix leucine zipper (bHLH-zip) domain important for dimerization and DNA binding (7,8,33). The amino-terminal activation domains of USF1 and USF2 are highly divergent, suggesting that USF1 and USF2 may target different genes (14,19,38). Indeed, studies using USF2 knock-out mice have revealed that USF1 homodimers are not as effective as USF2/USF1 heterodimers in the glucose regulation of L-PK and S14 gene expression (36). Furthermore, it has been shown that whereas USF2 inhibits both *myc*- and E1A-dependent transformation, USF1 only blocks *myc*-dependent transformation (20).

Core promoters of eukaryotic genes consist of a TATA box or an initiator element or both. The *ATPA* promoter is an example of a TATA-less initiator-containing promoter. One of the central issues regarding initiator-mediated transcription is the identity of the functional initiator binding proteins. Several proteins have now been identified that bind to initiator elements, including USF1 (6,16,24,28), YY1 [for review, see (35)], TFII-I (27), c-*myc* (16,24), E2F (21), TAFs [for review, see (34)], and RNA polymerase [for review, see (34)]. It is not known if different initiator binding factors function through different promoters or under different physiological conditions. Furthermore, some initiators may be composed of a consensus initiator element and an overlapping binding site for an additional protein that enhances promoter strength or imparts transcriptional regulation through an initiator. To date, we have determined that the *ATPA* initiator can be stimulated by ectopic expression of either the multifunctional regulatory protein, YY1 (4), or the transcription factor, USF2 (this article). Interestingly, when YY1 was coexpressed together with USF2 we found that the level of activation of the *ATPA* initiator was reduced relative to expression of USF2 alone, suggesting competition between these factors. YY1 and USF2 might compete for binding to the *ATPA* initiator sequence or for a common coactivator, such as p300 [for review, see (11)] or $TAF_{II}55$ (5). Other factors might also regulate the activity of the *ATPA* initiator. For example, it has been shown that USF1 can interact cooperatively with the initiator binding factor, TFII-I (28). Furthermore, it has been found that c-*myc* can antagonize the effect of USF1 through some initiator elements (16,24). It is possible that a number of transcription factors might play an important role in regulating the expression of the *ATPA* gene through this initiator. Such interactions might be important in coordinating cellular responses to diverse physiological stimuli.

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