THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 51, Issue of December 23, pp. 32649–32654, 1994
Printed in U.S.A.

ETS Transcription Factors Regulate the Expression of the Gene for the Human Mitochondrial ATP Synthase β-Subunit*

(Received for publication, June 23, 1994, and in revised form, September 30, 1994)

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Elements responsible for the transcriptional activity of the human ATP synthase β-subunit (ATPsynβ) gene promoter have been studied through transient expression in HepG2 hepatoma cells of a *CAT* gene connected with various 5′-deletion mutants of the 5′-flanking region. Promoter activity was mostly dependent upon a single CCAAT motif as well as a nearby Ets domain binding region. This last region contains two sites that bind Ets-related proteins present in liver nuclear extracts as well as recombinant purified Ets-1 protein. The ATPsynβ promoter was trans-activated by Ets-1 and Ets-2 expression vectors, and this effect was lost when the Ets binding region was deleted. The Ets binding region of the ATPsynβ promoter increased basal expression and conferred Ets-1- and Ets-2-dependent trans-activation to the herpes symplex thymidine kinase minimal promoter. A double-point mutation of the main Ets-binding site, which suppresses Ets binding, blocks Ets-dependent trans-activation. It is concluded that the gene for the mitochondrial ATPsynβ is a target of transcriptional activation by members of the Ets family of transcription factors. It is suggested that Ets transcription factors may be involved in the enhanced expression of the ATPsynβ gene in highly proliferating cells and in the coordinate transcription of nuclear genes for mitochondrial proteins.

The mitochondrial respiratory chain and oxidative phosphorylation activities rely on enzyme complexes present in the mitochondrial inner membrane of eukaryotic cells. In mammals each of these enzymatic complexes contains different protein subunits encoded by either the mitochondrial or the nuclear genomes. The mitochondrial respiratory chain/oxidative phosphorylation system is present in most mammalian cells and constitutes the main source of ATP synthesis.

The expression of nuclear genes for proteins of the respiratory chain and oxidative phosphorylation complexes shows several particular characteristics: a certain degree of ubiquitous constitutive synthesis of each component is necessary to maintain regular mitochondrial function, the amount of each component must be modulated according to the cell type and physiological demands for ATP, and functional complexes can be constructed only when the expression of nuclear genes is coordinated both between each other and with the expression of the mitochondrial genome. Changes in the transcription rates of the nuclear genes for subunits of the respiratory chain and oxidative phosphorylation complexes play an important role in the modulation of their expression (for reviews see Refs. 1, 2).

The ATPsynβ[1] is a major component of the catalytic site of the mitochondrial F_0F_1-ATP synthase complex, and it is encoded by the nuclear genome (3). Whereas many nuclear-encoded components of the respiratory chain and oxidative phosphorylation systems show multiple isoforms encoded by several different genes (4–7), the ATPsynβ is encoded by a single copy gene which is ubiquitously expressed in mammalian cells (8). Therefore, the identification of the elements responsible for the transcriptional regulation of the ATPsynβ gene is expected to provide substantial information on the molecular mechanisms responsible for the coordinated expression of components of the respiratory chain and the oxidative phosphorylation systems. The human ATPsynβ gene has been isolated and cloned (8, 9). The structure of the promoter shows particular features: it lacks a canonical TATA box, it contains four CCAAT boxes and a GC box sequence and, depending on the authors, a single (8) or two (9) transcription start sites. Several elements involved in the transcription activity of this gene have been reported. An enhancer in the far upstream region of the gene has been identified (10, 11) as well as a more proximal region (OXBOX-REBOX complex) that includes muscle-specific and non-tissue-specific regulatory elements (12). A GABP/NRF-2-binding site has also been identified in an intron of the ATPsynβ (13).

Here we report that an Ets domain-binding site is a major element for the ATPsynβ promoter activity, and this gene is found to be transactivated by members of the Ets family of transcription factors. The Ets proteins resemble the v-ets oncogene in the "ets domain." This domain interacts specifically with purine-rich sequences of DNA in viral or cell gene promoters. In addition to the c-ets-1 protooncogene product, Ets-1, about 30 Ets proteins have been found in different species, including humans, and they are involved in the regulation of gene expression during a variety of biological processes including growth control, transformation, and developmental programs of many organisms (for review, see Ref. 14). Recent reports have shown that transcription of the nuclear genes for two subunits of the cytochrome oxidase complex also depends on Ets-binding sites in their promoter regions (13, 15). The present finding that ATPsynβ is a target for transcriptional regulation by Ets proteins suggests that these transcription factors are involved in coordinating the expression of components of the respiratory chain and oxidative phosphorylation systems in the mammalian cell.

* This research was supported in part by Grant PB-92–0865 from Dirección General de Investigacion Cientifica y Tècnica, Ministerio de Educación y Ciencia Spain. 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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[1] The abbreviations used are: ATPsynβ, ATP synthase β-subunit; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes and poly(dI-dC) were purchased from Boehringer Mannheim or Promega. [α-^{32}P]dCTP and [α-^{35}S]dATP were purchased from Amersham Corp. and D-threo-[1,2-^{14}C]chloramphenicol was from ICN. Tissue culture media and fetal calf serum were obtained from Whittaker. Synthetic oligonucleotides were obtained from Oligos Inc. and Promega.

Polymerase Chain Reaction Amplification of Human ATPsynβ Promoter—A fragment of the human ATPsynβ gene was amplified by polymerase chain reaction using 300 ng of human DNA. The complementary (3′) primer corresponded to bases from +69 to +89, considering the transcription start site commonly reported (8, 9) as +1 for numbering. The complementary (5)′ primer corresponded to bases −786 to −763. The 3′ and the 5′ complementary primers included six-base pair non-complementary extensions capable of generating *Bam*HI and *Xba*I restriction sites, respectively. Reaction was performed in a final volume of 50 μl containing 40 pmol of each primer, 1.5 mM each of the four deoxynucleotide triphosphates in 67 mM Tris-HCl, pH 8.3, 6.7 mM $MgCl_2$, and 4 units of Thermus aquaticus DNA polymerase (Cetus). 30 cycles were performed at 92, 48, and 72 °C for 1 min each. The resulting DNA product of approximately 0.9 kilobase was purified, digested by *Xba*I and *Bam*HI restriction enzymes, and cloned into pUC19 using the *Xba*I/*Bam*HI sites of the polylinker (plasmid −786/+89 β-ATPase pUC). The whole fragment was sequenced by the dideoxy method using flanking and internal synthetic oligonucleotides as primers for sequencing. The sequence of the cloned human ATPsynβ gene fragment from −786 to +89 was identical to that previously published (8).

Plasmid Constructions—The *Xba*I/*Bam*HI fragment of the plasmid −786/+89 β-ATPase pUC was inserted into the *Xba*I/*Bam*HI sites of PBLCAT3 thus resulting in the β786-CAT plasmid which contained the −786/+89 fragment of the human ATPsynβ upstream of the CAT region. Plasmids containing 5′ deletion mutations of this β786-CAT were generated by polymerase chain reaction using different (5′) primers and the same (3′) primer used for initial cloning of the −786/+89 fragment. The 5′ primers corresponded to sequences −381/−361, −339/−319, −246/−226, and −220/−200, and they all included a 5′ non-complementary tail generating a *Xba*I restriction site. Reactions were performed using 10 ng of β786-CAT as template, 30 cycles of 30-s denaturation at 92 °C, 30 s annealing at 42 °C, and 1 min extension at 72 °C. DNA products of the expected sizes were purified, digested with *Xba*I/*Bam*HI, and cloned in these same sites of PBLCAT3. A further 5′ deletion mutant plasmid was constructed by religation of the remaining β-786-CAT after elimination of the *Pst*I fragments spanning from the *Pst*I site of the PBLCAT3 polylinker to the −11 internal *Pst*I site.

A synthetic, double-stranded oligonucleotide, corresponding to the −306/−266 sequence in the human ATPsynβ gene flanked by *Xba*I ends (see Fig. 3*A* for full sequence), was cloned upstream of the herpes symplex virus thymidine kinase promoter driving CAT, using the *Xba*I site of PBLCAT2. The two randomly originated possible orientations of the cloned fragment were identified by sequencing thus resulting in the isolation of plasmids −306/−266-TK-CAT and −266/−306-TK-CAT. A plasmid containing the same sequence except for TT instead of GG at sites −301 and −300 was cloned into PBLCAT2 using the same strategy.

Expression vectors for chicken c-ets-1, human c-ets-2, and an expression vector for a deletion mutant of c-ets-1 lacking DNA binding ability (plasmid 369–388) and used as a negative control (16) were provided by Dr. J. Ghysdael. The expression vector for murine Pu-1 (pMSV-Pu1) (17) was obtained from Dr. R. A. Maki.

Cell Culture, DNA Transfection, and Determination of CAT Activity—HepG2 cells were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum. Transfection was carried out by the calcium phosphate precipitation procedure (18). Each individual transfection contained 10 μg of CAT vector, 3 μg of RSV-β-galactosidase and, where appropriate, 5 μg of the expression vectors for c-ets-1, c-ets-2, or the 369–388 negative control. After transfection cells were incubated for 36 h, CAT activity was analyzed as described (19, 20). The amount of cell extract used was adjusted to maintain a percentage conversion of chloramphenicol between 1 and 20%. The CAT activity was normalized for variation in transfection efficiency using the β-galactosidase activity as internal standard.

Preparation of Nuclear Protein Extracts and Ets-1 Protein—Nuclear proteins were isolated from rat liver as reported (21, 22). Recombinant Ets-1 protein was prepared after expression in *Escherichia coli* of a pET3 vector containing the full-length cDNA for murine c-ets-1 (23), kindly provided by Dr. B. Graves, and purification was performed as reported (23). The resulting Ets-1 protein preparation was estimated to be 80–90% pure based on Coomassie Blue staining of an SDS-polyacrylamide gel electrophoresis.

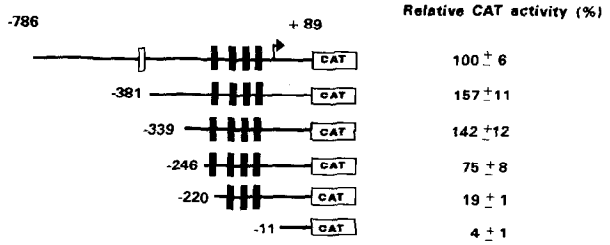

FIG. 1. **Effects of progressive 5′ deletions of the ATPsynβ gene promoter on CAT expression in transiently transfected HepG2 cells.** HepG2 cells were transiently transfected with 10 μg of the different chimeric ATPsynβ gene-CAT constructs depicted on the left and 3 μg of RSV-β-gal. After 36 h the CAT activity was measured in cell extracts as described under "Experimental Procedures." *Boxes* represent previously identified GC (*open box*) and CCAAT (*dark boxes*) sequences (8, 9). Results are expressed as percentages of the activity obtained with the β786-CAT construct (100%) and shown as means ± S.E. of at least four independent transient transfection experiments performed in duplicate for each construct.

DNase I Footprint and Electrophoretic Gel Mobility Shift Assays—The DNA probe for DNase I footprint assays was prepared by end-labeling the *Xba*I-digested β381-CAT plasmid DNA using [α-^{32}P]dCTP and Klenow enzyme. The DNase I footprinting assays were performed as described (18, 24). Binding reactions contained from 1 to 25 μg of protein from rat liver nuclear extracts. Maxam and Gilbert G and G+A reactions were used to identify the position of protected sites. For electrophoretic gel mobility shift assays, double-stranded synthetic oligonucleotides containing 5′ extruding ends were end-labeled using [α-^{32}P]dCTP and the Klenow enzyme. 20,000–30,000 counts/min of labeled probe were incubated in each single assay with with 5 μg of protein from rat liver nuclei or with 0.5 μg of the highly purified recombinant Ets-1 preparation. In the competition experiments different fold molar excess of unlabeled oligonucleotides were included in binding reactions. Reactions were carried out in a final volume of 20 μl containing 20 mM HEPES, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 50 mM NaCl, and 1 μg of poly(dI-dC), and after 15–20 min at 22 °C they were loaded onto 5% polyacrylamide gels in 0.5 × TBE (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA). Electrophoreses were performed at 250 V for 60–80 min at 4 °C. Gels were analyzed by autoradiography.

Statistical Analysis—Where appropriate, statistical analysis was performed by the Student's *t* test and significance is indicated in the text.

RESULTS

Deletional Analysis of the 5′-Flanking Sequence of the ATPsynβ Gene—Various portions of the ATPsynβ promoter were placed upstream of the *CAT* reporter gene (Fig. 1) and transfected in the HepG2 human hepatoma cell-line. The construct containing the fragment from −786 to +89 yielded significant levels of CAT expression with respect to the promoterless CAT vector PBLCAT3. The levels of CAT activity driven by this fragment of the ATPsynβ gene were within the range of those of other mammalian or viral promoters expressed in HepG2 cells (80 and 75% of the CAT activities driven by the −490/+73 rat phosphoenolpyruvate carboxykinase and the −105/+51 HSV thymidine kinase promoters, respectively). All deletion constructs gave significant CAT expression with the exception of the −11/+89 construct, the CAT activity of which was indistinguishable from PBLCAT3 expression background. The fragment spanning nucleotide −786 to −381 appeared to contain weak negative control elements, since its deletion was associated with a small but significant ($p \leq 0.001$) stimulation of CAT activity. Deletion of this fragment suppressed a GC box, putative Sp1-binding site, at −388/−382 (9) which does not appear to have a positive functional role here. Deletion of the region spanning from −381 to −339 did not further affect CAT activity, whereas the suppression of the fragment between −339 and −246 resulted in a significant ($p \leq 0.001$) reduction. Further deletion of the fragment between −246 and −220 caused a

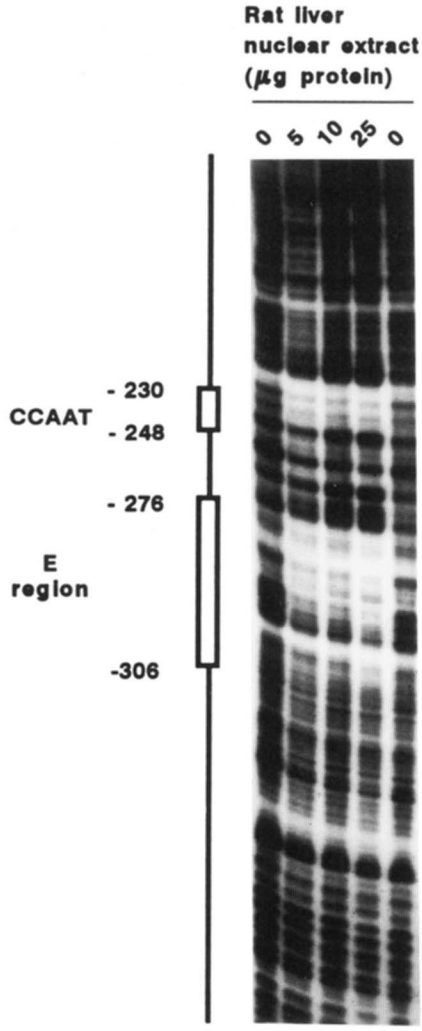

FIG. 2. **DNase I footprint analysis of the ATPsynβ promoter DNA.** The DNA fragment was end-labeled by using Klenow enzyme activity over the 5′ extruding end resulting from the *Xba*I digestion of the p381CAT plasmid (see "Experimental Procedures" for details). It was subjected to DNase I footprinting using 0, 5, 10, or 25 μg of nuclear protein extract from rat liver and electrophoresed using a 6% denaturing acrylamide gel. The *boxes on the left* enclose the regions protected from DNase I digestion.

significant ($p \leq 0.001$) 4-fold decrease in CAT activity. Therefore, positive elements for the promoter expression are present in both regions. Deletion of −246 to −220 suppresses the upstream CCAAT sequence in the promoter, and the low CAT activity levels found in this construct indicates that this CCAAT box may be an essential element for ATPsynβ promoter activity. The region between −220 to +89, which contains three additional CCAAT motifs, drove a CAT activity significantly above the background, but it was 4-fold lower than that found when the −246/−220 region was present.

Identification of Protein-binding Sites in the ATPsynβ Gene Promoter—Since the region between −339 to −220 was responsible for most of the ATPsynβ promoter activity in HepG2 cells, DNase I protection assays were performed to identify binding sites for potential regulatory proteins using nuclear extracts from rat liver. Fig. 2 shows an example of this analysis. Two distinct areas of protection were detected. The first corresponded to −248/−230 and overlapped the upstream CCAAT sequence in the promoter. Nuclear proteins binding at that region showed a high affinity and/or high abundance in the rat liver extracts as amounts as low as 1 μg of nuclear protein extracts yielded a marked footprint signal (not shown). The second area of protection, named E region, was the only one found within the remaining DNA region from −339 to −248 where positive acting elements for promoter activity had been detected. It was placed between −306 and −276, and the analysis of the corresponding DNA sequence indicated the presence of two sites with a close homology with the DNA binding sequence for the ETS family of transcription factors (see below).

The −306/−266 Region of the ATPsynβ Gene Promoter Binds Ets-related Proteins—Two sequences (E1 and E2) homologous to the DNA-binding sites for Ets transcription factors were detected within the E region of the ATPsynβ promoter. A third Ets-related sequence (E3) was also detected in the 3′ immediate vicinity although it did not correspond to a DNase I footprint protected site (Fig. 3*A*). They are compared with the core Ets-binding consensus DNA sequence (25), and they show a full conservation of the A/C GGAA core motif characteristic of the Ets-binding sites.

Identification of the proteins binding the −306/−266 region as members of the Ets family was first achieved by using competition gel shift analysis. When two DNA fragments containing the E1 sequence (−306/−293) or the E2 (+E3) sequences (−292/−266) were incubated with protein extracts from rat liver nuclei, several retarded bands were observed in the gel-shift assay (Fig. 3*B*). Oligonucleotides corresponding to DNA-binding sites for Ets-2 and GABP/NRF2, two members of the Ets family known to be expressed in the liver (26, 27), were used for competition. These were the Ets-1/Ets-2-binding site (−208/−192) in the stromelysin promoter (28) and the GABP/NRF2-binding site (+19/+35) of the rat cytochrome oxidase subunit IV promoter (13, 29) (Fig. 3*B*, *right*). An AT-rich, MEF-2 consensus oligonucleotide was used as a negative control (30). Results indicated that the E1 site originated a main retarded band that was lost when an excess of either GABP/NRF2 or Ets-1/Ets-2 oligonucleotide was added to the incubation mixture (see Fig. 3*B*, *left*). However, lower amounts of the GABP/NRF2 oligonucleotide were able to compete fully for binding when compared with the Ets-1/Ets-2 oligonucleotide action. This indicates that the E1 site of the ATPsynβ promoter binds Ets proteins present in liver nuclei, and they have a somewhat higher affinity for GABP/NRF2 sites than for Ets-1/Ets-2-binding sites. Conversely, only high amounts of the Ets-1/Ets-2 oligonucleotide caused a loss of shifted bands when the fragment containing the E2 and E3 sequences was analyzed (see Fig. 3*B*, *center*) thus indicating that the E2+E3 region has a weaker affinity for the Ets-related proteins present in liver nuclear extracts.

The presence of shifted bands that are not affected by Ets-related competitors indicated that non-Ets proteins also interact with the −306/−266 region of the ATPsynβ. Databank analysis of the DNA sequence within this region did not yield any consistent homology with previously identified DNA binding motifs, with the exception of Ets. The GC-rich stretch between the E1 and E2 sites showed a single mismatch for full homology with a GC box putative Sp1-binding site. It was tested in competition gel-shift assays using a consensus Sp1-binding site oligonucleotide (31), and results were negative (not shown).

The −306/−266 Region of the ATPsynβ Promoter Contains Binding Sites for Ets-1—For further evaluation of the ability of sequences from −306 to −266 in the ATPsynβ gene to bind Ets-related proteins, the two fragments −306/−293 and −292/−266 were subjected to gel-shift assays using recombinant purified Ets-1 protein. As shown in Fig. 4, incubation of either fragment with Ets-1 protein resulted in a single retarded band. Binding was specific, as an excess of cold Ets-1/Ets-2 oligonucleotide suppressed the appearance of the retarded band, whereas the MEF-2 oligonucleotide did not. When equimolar

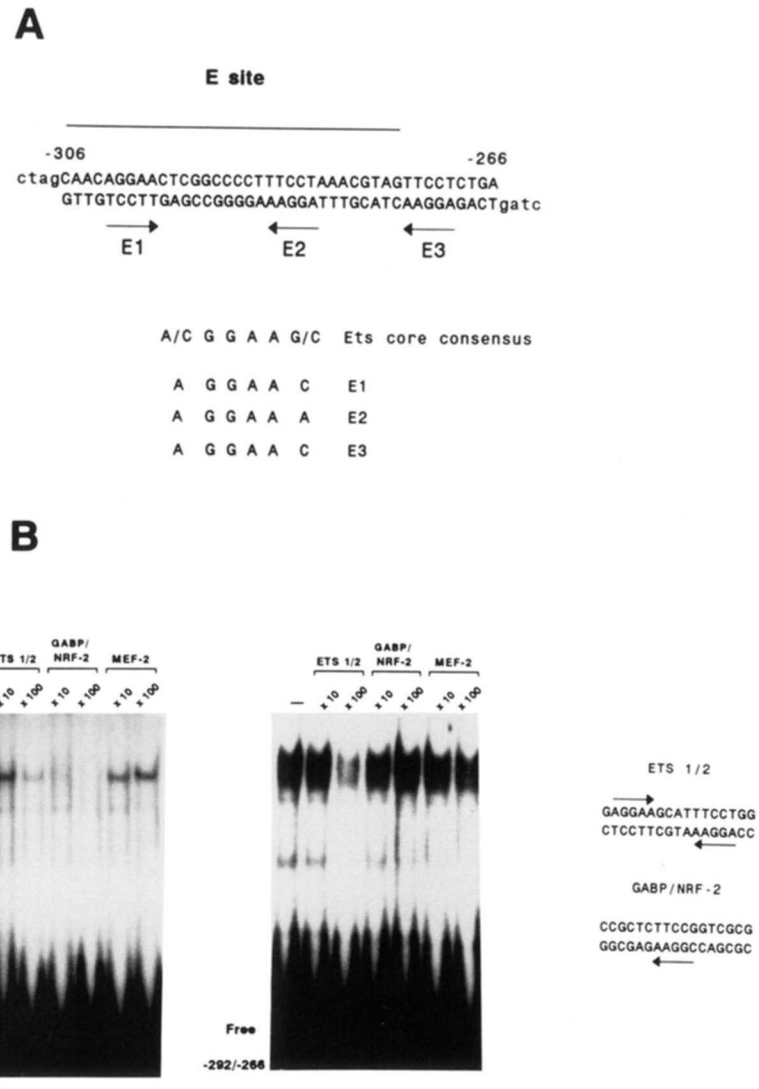

FIG. 3. **Competition gel mobility shift analysis of the −306/−293 and the −292/−266 regions of the ATPsynβ gene promoter.** *A*, synthetic oligonucleotide containing the −306/−266 region of the ATPsynβ gene promoter. The *upper line* shows the E region protected in the DNase I footprint assay (*top*). Comparison of the E1, E2, and E3 sequences in the ATPsynβ gene with the consensus core sequence for Ets binding (25) (*bottom*). *B*, the two end-labeled fragments resulting from the *Hae*III digestion of the −306/−266 double-stranded oligonucleotide depicted in *A* were incubated with 5 μg of protein from rat liver nuclear extracts and electrophoresed on 5% acrylamide gel. The competitor oligonucleotides Ets-1/Ets-2 (region −208/−192 in the stromelysin promoter) (28) and GABP/NRF2 (region +19/+35) in the rat cytochrome *c* oxidase subunit IV promoter) (29) are depicted to the *right*. MEF-2 is an AT-rich oligonucleotide used as a negative control (see "Experimental Procedures"). Competitors were added to binding reactions at the indicated molar excess. The absence of competitor is depicted as −.

amounts of labeled −306/−293 (E1) or −292/−266 (E2+E3) fragments were incubated with equal amounts of Ets-1, the percentage of radioactivity in the retarded band was always lower in the E2+E3 fragment than in the E1. These results confirm the presence of Ets-binding sites in both fragments, but also the much lower affinity of the E2+E3 fragment than the E1 in binding Ets proteins. On the other hand, the mobility of the retarded bands caused by recombinant purified Ets-1 was always lower than the Ets-related bands obtained using liver nuclear extracts (not shown).

Ets-1 and Ets-2 Expression Trans-activates the ATPsyn β-Subunit Gene Promoter Due to Elements Present at Position −339/−247—The β786CAT plasmid was co-transfected with expression plasmids containing the cDNAs of either c-Ets-1, h-Ets-2, or the distantly related member of the Ets family Pu-1. A deletion mutant form of the c-Ets-1 expression vector unable for DNA binding was used as negative control (16). As shown in Fig. 5, both Ets-1 and Ets-2 trans-activated the ATPsynβ gene promoter whereas the Pu-1 expression vector had no effect. Deletion mutants β381CAT and β339CAT were also trans-activated severalfold over the negative control by c-Ets-1 and h-Ets-2 expression vectors. The β246 CAT and β11 CAT constructs were unresponsive to Ets (Fig. 5). These results indicate that the ATPsynβ promoter is a target for trans-activation by Ets-related transcription factors, and that cis-acting elements responsible for trans-activation occur in the −339/−247 region, where Ets-binding sites had been detected.

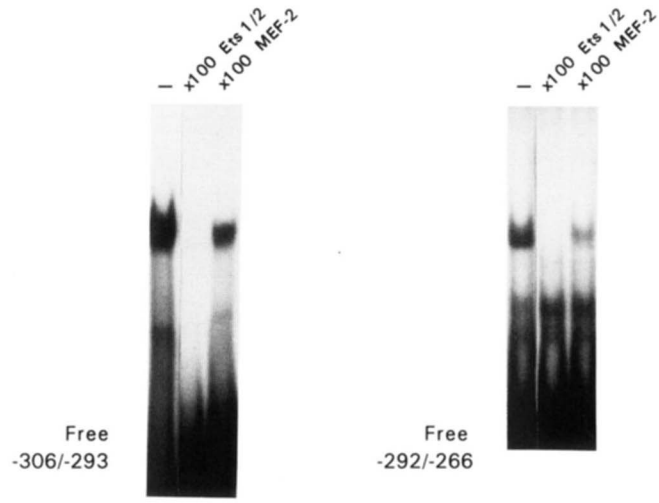

FIG. 4. **Gel mobility shift analysis of the Ets-1 protein binding to the −306/−293 and the −292/−266 regions of the ATPsynβ gene promoter.** The synthetic oligonucleotide corresponding to the −306/−266 region of the ATPsynβ gene and depicted in Fig. 3*A* was subjected to double end labeling followed by digestion with the *Hae*III endonuclease. The two resulting labeled fragments −306/−293 and −292/−266 were incubated with 0.5 μg of highly purified bacterially expressed murine Ets-1 protein and electrophoresed on 5% acrylamide gel. The Ets-1/Ets-2 oligonucleotide competitor and the MEF-2 negative control (see Fig. 3) were added at the indicated molar excess. The absence of competitor is depicted as −.

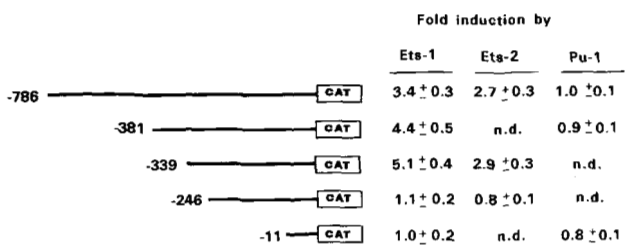

FIG. 5. **Effects of Ets-1, Ets-2, and Pu-1 expression on ATPsynβ promoter activity.** HepG2 cells were transiently transfected with 10 μg of the different ATPsynβ CAT constructs, 3 μg of RSV-β-gal, and 5 μg of either c-Ets-1, h-Ets-2, or Pu-1 expression vectors. Controls were transfected with the expression vector for the 369–388 form of Ets-1, which lacks DNA binding ability. After 36 h, the CAT activity was determined and results are expressed as the fold stimulation elicited by each expression vector on the CAT activity driven by the different extensions of the ATPsynβ gene promoter. Data are means ± S.E. of at least four independent transfection experiments performed in duplicates. *N.D.*, not determined.

The −306/−266 Region of the ATPsynβ Gene Promoter Confers Ets-dependent Responsiveness to a Heterologous Promoter—The synthetic oligonucleotide spanning the −306/−266 region of the ATPsynβ gene was cloned upstream of the HSV thymidine kinase promoter driving the CAT reporter, both in the orientation present in the ATPsynβ promoter and in the opposite one. Basal CAT activities driven by these constructs as well as the effect of c-Ets-1 expression vectors were determined through co-transfection into HepG2 cells. Fig. 6 indicates that the presence of the −306/−266 sequences increased the basal TK-driven CAT activity 5-fold in the correctly orientated construct and approximately 4-fold in the opposite one. Ets-1 expression vector increased the CAT activity above negative controls in constructs containing the −306/−266 fragment irrespective of their orientation toward the TK promoter. This demonstrates that the −306/−266 region of the ATPsynβ gene constitutes an Ets-responsive element. Similar findings were observed in parallel experiments using the Ets-2 expression vector (not shown).

Double Point Mutation from the AGGAA Motif to AttAA in the E1 Element (−302/−297) Suppresses Ets-dependent Transactivation—When the construct transfected to HepG2 cells contained a version of the −306/−266 region in which the AGGAA motif at −302/−297 (E1 site) had been mutated to A*tt*AA, the capacity to be trans-activated by Ets-1 expression was lost (see Fig. 6). This is consistent with the previously reported requirement for an intact GG pair in Ets-responsive elements (23). However, the presence of the double point mutation did not block the enhanced basal expression of the construct with respect to TK promoter alone, previously observed for the wild-type fragment. Band shift analysis of a labeled probe corresponding to the mutated −306/−293 region using recombinant Ets-1 protein indicated that the double point mutation suppressed the ability of the E1 site to bind Ets-1 (not shown). These results indicate that the main Ets-binding site in the ATPsynβ promoter, site E1, is necessary for Ets-dependent trans-activation of the promoter.

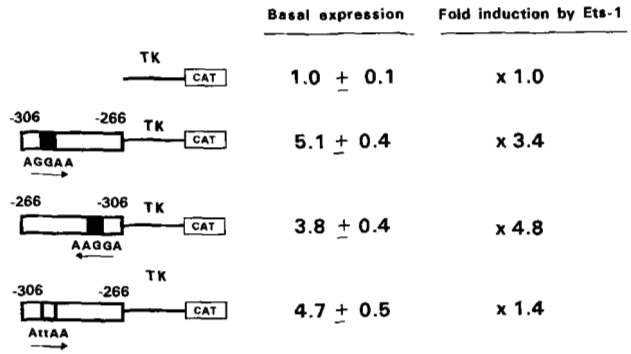

FIG. 6. **Basal expression and Ets-1 responsiveness of chimeric constructs containing the −306/−266 region of the ATPsynβ gene cloned upstream the TK promoter.** Effect of a double-point mutation in the E1 site. HepG2 cells were transiently transfected with 10 μg of plasmid constructs in which the double stranded synthetic oligonucleotide corresponding to wild type −306/−266 region of the ATPsynβ or a mutant version (the E1 site has been changed from AGGAA to AttAA) were placed upstream a TK-driven CAT vector (PBLCAT2). For determination of the effects of Ets-1 expression, 5 μg of the expression vector for c-Ets-1 or the 366–388 negative control were co-transfected. All transfections included 3 μg of the RSV-βgal plasmid. CAT activity was determined after 36 h. Results show the basal activity of the constructs expressed as increase above the activity obtained with TK-CAT (PBLCAT2). The effects of Ets-1 expression are shown as the increase over the control of the CAT expression due to the co-transfection of the c-Ets-1 expression vector. Results are means ± S.E.

DISCUSSION

In this study it is demonstrated that the main basal promoter activity of the ATPsynβ gene in hepatic cells depends on two cis-acting regions: a CCAAT box site, analogous to that present in many eukaryotic gene promoters, and a nearby upstream region containing Ets domain-binding sites. The ATPsynβ gene promoter is identified as a target for Ets-1 and Ets-2 trans-activation due to the presence of an Ets domain binding region.

The Ets-responsive region of the ATPsynβ gene promoter presents a complex structure as it contains at least two Ets-binding sites (E1 and E2) in opposing orientation, each one showing different affinities for Ets proteins. Ets-responsive elements composed of different arrangements of more than a single Ets-binding site have been previously identified (23, 28, 29, 32, 33). The opposite alignment of the E1 and E2 sites is similar to the palindromic alignments of individual Ets-binding sites in the Ets-1/Ets-2-responsive elements of the stromelysin (28) or the *GATA-1* genes (34). The present observation that either Ets-1 or Ets-2 transactivate the ATPsynβ promoter is also in agreement with previous reports showing that these two closely similar members of the Ets family activate mammalian or viral promoters through common Ets-1/Ets-2-responsive elements (28, 35). The lack of effect of Pu-1, the most divergent member of the Ets family (17), is also consistent with its distinct binding specificity and lack of cross-transactivation with other Ets-responsive elements (36). In this context, the finding that the Ets proteins in liver nuclei which bind the Ets-responsive elements in the ATPsynβ gene have high affinities for both Ets-1/Ets-2 and GABP/NRF2 sites is not surprising. Cross-binding is a characteristic feature of closely related members of the Ets family. Ets-2, which is substantially expressed in the liver, binds the same DNA sequences that can bind Ets-1 (37, 38). In addition, the Ets-1/Ets-2-binding sites of the MSV and the polyoma PEA3 promoters are able to bind GABP/NRF2, another Ets protein expressed in the liver (15, 29). Therefore, the combination of the functional and binding studies shown here does not necessarily point to a single Ets-related protein as the unique mediator of Ets-dependent ATPsynβ promoter activity in liver cells. The higher affinity for GABP/NRF2 suggests that this member of the Ets family might be the main Ets protein in the liver interacting with the E1 region of the ATPsynβ gene. However, the relevance of the binding with other members of the Ets family such as Ets-2 and the possibility of a complex cross-talk of binding and trans-activation activities from different members of the Ets family cannot be ruled out.

The presence, within the Ets-responsive region of the ATPsynβ promoter, of DNA elements which bind proteins other than Ets, indicated by the presence of shifted bands unaffected by Ets competitors, is remarkable. Their functional relevance

might be related to the basal enhancement that the Ets-responsive region confers on the heterologous TK promoter, even when the E1 site is mutated and Ets responsiveness is lost. Many Ets family members require cooperation with other non-Ets DNA-binding proteins for basal enhancer function or Ets-dependent trans-activation (14). Further research is necessary to ascertain the identity of these non-Ets-binding proteins and their functional role within the Ets-responsive region of the ATPsynβ gene promoter.

Recent reports have shown that the promoter activity of nuclear genes for rat and murine cytochrome oxidase subunits IV and Va depends on Ets motifs that bind GABP/NRF2 (13, 15, 29). It has been proposed that GABP/NRF2 is involved in coordinating the expression of nuclear genes encoding for mitochondrial respiratory chain components. The present findings suggest a more extensive role of members of the Ets family in the mitochondrial biogenesis. The identification of the ATPsynβ gene promoter as a target for Ets transcription factors indicates that Ets proteins are likely candidates to provide molecular mechanisms for the coordination of the expression of components of the respiratory chain with the ATP synthase complex. Moreover, the present findings provide the first evidence for a trans-activating action of Ets-1 and Ets-2 upon a nuclear-encoded gene for a mitochondrial protein and indicate that, together with GABP/NRF2, a potentially extensive array of members of the Ets family are involved in the regulation of gene expression of components of the respiratory chain/oxidative phosphorylation systems. Therefore, Ets proteins must join to the previously identified NRF-1 (39), the OXBOX and REBOX proteins (12), and the Mt binding factors (40) in this regulatory role.

Several features of the regulated expression of the ATPsynβ gene in hepatic cells are consistent with the established role of Ets proteins as transcriptional activators. Ets transcription factors are involved in the regulation of gene expression during a variety of biological processes, including cell growth control, cell transformation, and development. Several mammalian and viral genes are activated by mitogenic signals through ets-like motifs present in their promoter region (33, 41, 42). Proliferative signals and transformation increase ATPsynβ gene expression in different mammalian cell types, including hepatic cells in culture (43–45). *In vivo*, increased ATPsynβ mRNA expression occurs during fetal liver development (46) when liver cells proliferate and high levels of expression of Ets-2 occur (26, 47). The expression of Ets proteins, including Ets-2, is enhanced in regenerating liver (26, 48), a model for cell proliferation *in vivo* associated with increased mitochondrial ATP synthase activity (49). It is therefore suggested that the action of Ets proteins upon the ATPsynβ gene could be related to its enhanced expression in highly proliferating cells. Moreover, the high expression of the ATPsynβ gene associated with cell proliferation is part of an overall increase in the expression of many other nuclear and mitochondrial genes for mitochondrial proteins (43, 45, 50, 51). Therefore, Ets proteins are likely candidates to provide the molecular mechanisms eliciting the coordinate enhanced expression of genes for the respiratory chain/oxidative phosphorylation systems in response to proliferative signals.

Acknowledgments—We thank Dr. B. Graves and Dr. J. Ghysdael for generously providing the expression vectors for Ets-1 and Ets-2 and Dr. R. Maki for the expression vector for Pu-1.

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