We have identified cis-acting elements and trans-acting factors that regulate constitutive expression of the human antithrombin gene. The activity of the sequences flanking the first exon of the gene was investigated using a luciferase-based reporter assay in transiently transfected HepG2, COS1, BSC40, and HeLa cells. Deletion analysis allowed the mapping of two elements able to promote antithrombin gene transcription in HepG2 and COS1 cells. The first element is located upstream of the first exon (~150/68 nucleotides). The second element is in the first intervening sequence (~300/700 nucleotides) and functions in an orientation opposite to that of the first. Footprint analysis showed three protected areas in the 5' upstream element at -92/-68 (element A), -14/+37 (element B), and -126/-100 nucleotides (element C). These elements acted as enhancers in luciferase reporter assays. Gel retardation analysis demonstrated that two liver-enriched transcription factors, hepatocyte nuclear factor 4 (HNF4) and CCAAT enhancer-binding protein (C/EBPα), bound to element A and B. C/EBPα also interacted with the ubiquitous nuclear hormone receptors chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1), thyroid hormone receptor α (TRα), peroxisome proliferator-activated receptor α (PPARα), and retinoid X receptor α (RXRα). In HepG2 and BSC40 cells, HNF4, C/EBPα, and RXRα activated luciferase expression from a reporter construct containing the 5'-upstream minimal antithrombin gene promoter, while COUP-TF1, TRα, and HNF3 (α or β) repressed such expression. Our results show that constitutive expression of the human antithrombin gene depends in part upon the interplay of these transcription factors and suggest that signaling pathways regulated by these factors can modulate antithrombin gene transcription.
tion factors have been described: human COUP-TF1 (7), a truncated version of human COUP-TF1 (t-COUP-TF1) lacking its first 51 amino acids (7), rat HNF4α (8), rat PPARα (9), and human RXRα (9). Vectors for the expression of mouse C/EBPα and rat HNF3α and β were a generous gift of Drs. P. Hoodless and J. Darnell, The Rockefeller University. Large scale plasmid preparations were made either by two rounds of centrifugation on CsCl or by anion exchange chromatography (Quiagen, Chatsworth, CA).

Cell Culture and Transfection—HepG2, COS1, BSC40, and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg of streptomycin/ml, and 100 μg of penicillin G/ml. Cells at 50–60% confluence were transfected with 25 μg of DNA (20 μg of plasmid plus 5 μg of carrier DNA) by calcium phosphate precipitation (10), followed by a 10% glycerol shock 16 h post-transfection (with the exception of COS1 cells). Lipofection transfections were performed as described (11). In cotransfection experiments, all cells were incubated for 24 h before and during transfection in medium without phenol red and containing 5% charcoal-stripped fetal bovine serum (9). Cells were cotransfected with 5 μg of the AT minimal promoter construct pAT−150/68 nt-luc and 1–8 μg of the expression vectors for various transcription factors. The amount of plasmid transfected was normalized to 20 μg with the parental vectors pSG5 or pRC/CMV (2.5 μg each) and carrier DNA. Luciferase activity was measured 48 h post-transfection and was normalized to the protein content of the lysates and to β-galactosidase activity.

Footprint Analysis—The −150/68 nt DraI/EcoI fragment of the AT gene was made blunt and subcloned in both orientations into the plasmid pGEM-3Zf(+) (Promega). The plasmid was digested with EcoRI, and the 3′-ends were labeled with the Klenow fragment of DNA polymerase I and [α-32P]dATP. The plasmid was then digested with HindIII, and the probe was purified from agarose gel. DNase I cleavage and protection by total cellular extracts of HepG2, COS1, and HeLa cells were performed in the presence of bovine serum albumin, poly(dI-dC)•poly(dI-dC), and carrier DNA, as described (12). The amounts of extract and DNase I used are indicated in the corresponding figure legends.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays (EMSA) were performed with nuclear extracts of HepG2, COS1, BSC40, and HeLa cells (13). The oligonucleotides tested (wild-type; mutant elements A, B, and C; and oligonucleotides for competition) are listed in Table I. Additional oligonucleotides for binding of CTF/NF1, NFκB, TFIID, SP1, AP1, AP2, and AP3 were obtained from Promega. Each oligonucleotide and its complementary strand (with added restriction sites) were annealed and subcloned into pUC18 (Stratagene). For element B, a −28/−47 nt NlaIV/BstXI fragment of the AT promoter subcloned into the plasmid pGEM-7Zf(+) (Promega) was also tested. Restriction fragments of each element were labeled at their 3′-ends with [α-32P]dATP and the Klenow fragment of DNA polymerase I. Extracts containing 10 μg of protein were incubated for 15 min with

![FIG. 1. Deletional analysis of AT gene sequences flanking exon 1.](image-url)

The mean percent luciferase activity (± standard deviation) relative to the −1100/+68 nt construct (F) is shown. ▶, transcriptional start site; ▼, constructs inserted in a reverse orientation; ■, exon 1; =, luciferase cDNA.
Mapping of Regulatory Elements Upstream of Exon 1—Fig. 1 shows the results of deletional analysis of cis-elements flanking exon 1 of the AT gene in the reporter plasmid pSVOA-LΔ5. The data shown were generated following calcium phosphate-mediated transfection of HepG2 and COS1 cells. The largest construct assayed was a 6800-base pair HindIII fragment of the AT gene (Fig. 1A). This fragment was unable to promote luciferase activity. Removal of exon 1 and downstream sequences by cleavage with HindIII at −4800 nt and EneI at +68 nt, 3 nt upstream of the translational start site of the AT mRNA, provided a construct with detectable luciferase activity (Fig. 1D). Further 5′ deletions to −3500 (Fig. 1E) or −1100 nt (Fig. 1F) did not noticeably change luciferase values. Because slightly lower luciferase expression was obtained for a −4800/−68 nt construct (Fig. 1D) than for a −2500/−68 nt construct (Fig. 1E), the effect of sequences between −4800 and −2500 nt was tested directly in the pGL plasmid system. A −4800/−2500 nt construct did not reduce luciferase activity driven by the SV40 minimal promoter, confirming the absence of a negative element in this region of the gene (data not shown). To facilitate further deletional analysis, the activities of all constructs were reported relative to the activity of the −1100/+68 construct, which was given an arbitrary luciferase value of 100% (Fig. 1F).

5′ deletions of the −1100/+68 construct up to −150 nt did not noticeably alter luciferase activity in either HepG2 or COS1 cells (Fig. 2). Further deletions of the −150/+68 nt element were selected to encompass the area protected in DNA footprints. Deletions to −101, −70, and −28 nt progressively abolished luciferase activity in HepG2 and COS1 cells. A −28/+68 nt construct was unable to promote transcription by itself (Fig. 2). Deletions from the 3′-end and upstream of +68 nt did not show markedly altered luciferase activity up to +11 nt. In contrast, decreased luciferase activity was seen at, and upstream of, −28 nt (Fig. 2).

The three elements A, B, and C mapped in footprints were unable to promote luciferase expression when subcloned into pSVOA-LΔ5′ in their natural orientation relative to the luciferase reporter gene (Fig. 3). In contrast, two tandemly repeated copies of element A or three tandemly repeated copies of element B enhanced luciferase expression driven by heterologous minimal promoters when inserted upstream of TK-luc or CPS-luc (Fig. 3).

Mapping of Regulatory Elements in IVS1—Data presented in Fig. 1 support the possibility of a second regulatory element within IVS1 of the AT gene. This element was identified following subcloning of the +300/+2100 nt region of IVS1 in an inverse orientation upstream of the luciferase gene (Fig. 1F). Effectively, insertion of this fragment led to an increase in

each probe (2.5 ng) at 25 °C in the presence of 1 μg of poly(dI-dC)poly(dI-dC), 2 μg of bovine serum albumin, and 0.5 μg of carrier DNA (unless otherwise indicated). Buffer conditions were as described (14). Electrophoresis was carried at 4 °C on prerun 4% polyacrylamide gels (30:1 acrylamide/N,N′-methylenebisacrylamide weight ratio) with 6.7 mM Tris, 6.7 mM boric acid, 1 mM EDTA as running buffer. In gel electrophoresis was carried at 4°C on prerun 4% polyacrylamide gels (30:1 acrylamide/N,N′-methylenebisacrylamide weight ratio) with 6.7 mM Tris, 6.7 mM boric acid, 1 mM EDTA as running buffer. In competition assays, unlabeled oligonucleotides were used at 100 molar excess of the test oligonucleotide (unless otherwise indicated). In supershift assays, 1 μl of antisera or 0.5 μg of an IgG fraction was used. Antibodies were added immediately after addition of labeled probe, and the reaction was incubated for another 30 min unless otherwise indicated. In vitro transcription-translation assays were carried out with a rabbit reticulocyte lysate-coupled system according to the manufacturer’s instructions (Promega). Reactions were done concomitantly with and without L-[35S]methionine. The efficiency of the translation was determined by SDS-polyacrylamide gel electrophoresis of the labeled products. Unlabeled translation products were used for EMSA, and the total amount of lysate was maintained at 4 μl with unprogrammed lysate. The specificity of the shifts was confirmed by supershift analysis.

RESULTS

Mapping of Regulatory Elements Upstream of Exon 1—Fig. 1 shows the results of deletional analysis of cis-elements flanking exon 1 of the AT gene in the reporter plasmid pSVOA-LΔ5. The data shown were generated following calcium phosphate-mediated transfection of HepG2 and COS1 cells. The largest construct assayed was a 6800-base pair HindIII fragment of the AT gene (Fig. 1A). This fragment was unable to promote luciferase activity. Removal of exon 1 and downstream sequences by cleavage with HindIII at −4800 nt and EneI at +68 nt, 3 nt upstream of the translational start site of the AT mRNA, provided a construct with detectable luciferase activity (Fig. 1D). Further 5′ deletions to −3500 (Fig. 1E) or −1100 nt (Fig. 1F) did not noticeably change luciferase values. Because slightly lower luciferase expression was obtained for a −4800/−68 nt construct than for a −2500/−68 nt construct (Fig. 1E), the effect of sequences between −4800 and −2500 nt was tested directly in the pGL plasmid system. A −4800/−2500 nt construct did not reduce luciferase activity driven by the SV40 minimal promoter, confirming the absence of a negative element in this region of the gene (data not shown). To facilitate further deletional analysis, the activities of all constructs were reported relative to the activity of the −1100/+68 construct, which was given an arbitrary luciferase value of 100% (Fig. 1F).

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Mapping of Regulatory Elements in IVS1—Data presented in Fig. 1 support the possibility of a second regulatory element within IVS1 of the AT gene. This element was identified following subcloning of the +300/+2100 nt region of IVS1 in an inverse orientation upstream of the luciferase gene (Fig. 1F). Effectively, insertion of this fragment led to an increase in
The presence of this second regulatory element was confirmed by further deletional analysis, allowing the narrowing of the active sequences to 1300/1700 nt (Fig. 1J). When the initial 1300/2100 nt construct, or its deletions, were inserted in their natural orientation relative to the luciferase gene, no increase in luciferase expression was observed (Fig. 1, H and K–M). The strength of the element in IVS1 to promote luciferase expression was about 50% and 25% relative to the level of expression of the 59 upstream element in COS1 cells and HepG2 cells, respectively. The lower efficiency in HepG2 cells as compared to COS1 cells was seen with both the original IVS1 construct and with deletions derived from it.

Footprint Analysis of the Upstream Promoter Element—Figs. 4 and 5 define the areas protected by nuclear extracts in the upper and lower strands of the 2150/168 nt probe from the 59 upstream element in COS1 cells and HepG2 cells, respectively. The lower efficiency in HepG2 cells as compared to COS1 cells was seen with both the original IVS1 construct and with deletions derived from it.

FIG. 3. Activity of elements –92/−68 nt (A) and +1/+37 nt (B) on heterologous promoters. Values (RLU) reported are the mean percent luciferase activity in arbitrary light units of pSV0A-LΔ5'. Black box, element A; gray box, element B; the arrow indicates the presence of the thymidine kinase (TK) or the carbamoyl-phosphate synthetase (CPS) minimal promoter.
HNF4 showed a supershift with HepG2 extract, but not with CO, and the general transcription factors listed in Table I (nucleotides corresponding to the binding sites for elements B, No competition for binding to element A was seen with oligo-
interact with a number of nuclear hormone receptors, including C/EBPα and C/EBPβ (Fig. 6A, lane 1) and members of the CCAAT-binding proteins, such as C/EBPa and C/EBPβ (lane 2 or 10 and lane 4, respectively). The Epstein-Barr Virus nuclear antigen 1-nuclear factor 1 (EBNA1-NF1), another element known to bind members of this protein family, also competed strongly with element B for binding (Fig. 6C, lane 6). Antibodies to isofoms α, β, and γ of HNF3β did not generate supershifts with element B (Fig. 6D for HNF3γ). In contrast, supershift analysis confirmed the interaction of C/EBPa with element B in HepG2 extracts after overnight incubation at 4 °C of the antibody with the nuclear extract mixture before addition of the probe (Fig. 6D). Mutant B3 (+3/+27 nt; Fig. 6C, lane 12), or half-site mutant B1 (+3/+13 nt; Fig. 6C, lane 9) could not compete for binding to element B, while mutant B2 (+10/+27 nt; Fig. 6C, lane 11) competed only slightly.

Mobility shifts were generated with element C and nuclear extracts from all three cell lines (Fig. 6E, data for HepG2 and HeLa nuclear extracts). Binding to element C could be competed with excess unlabeled element C and also partially with element A (Fig. 6E, lanes 3 and 1, respectively). Additional competition assays suggested interactions of element C with HNF4, HNF3, and C/EBPa (Fig. 6E, lanes 6–10). Supershift analysis confirmed a partial interaction of element C with HNF4 (Fig. 6F).

Interaction of Individual Transcription Factors with Elements A, B, and C—Interaction of the AT 5′ upstream promoter was observed with two liver-enriched factors, HNF4 and C/EBPa. HNF4 bound to elements A and C (Fig. 7A and D, respectively), whereas C/EBPa bound to element B (Fig. 7B). 5′ truncation of element A (removal of a nuclear hormone receptor consensus half-site at −92/−88 nt) did not suppress HNF4 binding (Fig. 7A). The additional element A mutations tested were targeted to the nuclear hormone receptor half-site TGACC at −75/−79 nt and to the 5′ nt immediately upstream. None of the mutants tested could bind HNF4.

Interactions between ubiquitous nuclear hormone receptors and element A were also detected by EMSA. Addition of both PPARα and RXRα with element A resulted in a shift, suggest-
ing heterodimerization of these receptors (Fig. 7A). No binding was seen with either receptor alone. No mutant of element A bound the combination of both receptors. In contrast, binding of TRα was seen with the wild-type and all mutant A elements, although TRα binding to mutant A4 was greatly reduced (Fig. 7C). Fig. 7 also presents the binding profile of COUP-TF1 with the wild-type and mutant A elements. COUP-TF1 interacted strongly with element A, with additional species above the primary shifted species (Fig. 7A). COUP-TF1 dimerization on element A was assessed by incubation with full-length COUP-TF1 and with a truncated form of the same receptor (Tr-COUP). This truncated form has been shown previously to retain intact DNA binding properties (7). After EMSA, a new shifted species migrating between the COUP-TF1 and Tr-COUP species was seen, indicating dimerization of the COUP-TF1 receptor (Fig. 7C, arrow).}

![Fig. 6. EMSA of elements A, B, and C.](image)

**TABLE I**

| Consensus Oligonucleotide | Oligonucleotide |
|---------------------------|----------------|

| Element A | Consensus Oligonucleotide |
|-----------|---------------------------|
| Wild type | GGTCATCAGCTTGAAGAGGCTGCC |
| 5′ truncated | GGTCATCAGCTTGAAGAGGCTGCC |
| Mutant 1 | TGGTCATCAGCTTGAAGAGGCTGCC |
| Mutant 2 | TGGTCATCAGCTTGAAGAGGCTGCC |
| Mutant 3 | TGGTCATCAGCTTGAAGAGGCTGCC |
| Mutant 4 | TGGTCATCAGCTTGAAGAGGCTGCC |
| PYBP | CTTGAAGGAC |

| Element B | Consensus Oligonucleotide |
|-----------|---------------------------|
| Wild type | TCCAGCTGGCCATTGCTACGTTTAC |
| Mutant 1 | TCCAGCTGGCCATTGCTACGTTTAC |
| Mutant 2 | TCCAGCTGGCCATTGCTACGTTTAC |
| Mutant 3 | TCCAGCTGGCCATTGCTACGTTTAC |

| Element C | Consensus Oligonucleotide |
|-----------|---------------------------|
| Wild type | CAACACTGGGCTCTACACTTTGCCA |
| Competition HNF4 | TGGAGCCAGGTATTTTGTTGAG |
| COUP-TF1 | TCCAGCTGGCCATTGCTACGTTTAC |
| HNF3 | TGGAGCCAGGTATTTTGTTGAG |
| C/EBP | TGGAGCCAGGTATTTTGTTGAG |
| HD-PPRE | TGGAGCCAGGTATTTTGTTGAG |

We attempted to detect receptor heterodimerization on element A. There was no evidence of COUP-TF1 heterodimerization with any receptor (Fig. 7C). Likewise, HNF4 did not heterodimerize with TRα or PPARα, although preliminary evidence from supershift analysis suggested an RXRα-HNF4 interaction. As seen in Fig. 8, addition of antibodies resulted in
the disappearance of the shifted species formed with element A. Supershifted species were formed also with anti-HNF4 antibodies (lanes 3, 10, and 13; single arrowhead) and with anti-RXRα antibodies (lanes 7 and 11; double arrowhead). Anti-PPARα antibodies did not form a readily discernible super-shifted species; however, these antibodies did result in the disappearance in the shifted species formed with element A (Fig. 8, lane 8). Addition of antibodies against RXRα to a reaction containing element A and both HNF4 and RXRα formed a supershifted species (Fig. 8, lane 11). As RXRα alone was unable to bind element A, the results suggest that a complex of HNF4-RXRα can form on element A.

Effects of in Vivo Expression of Nuclear Hormone Receptors and C/EBPα on the Transcriptional Efficiency of the AT 5' Upstream Promoter—Expression of HNF4 and C/EBPα in transient transfections of HepG2 and BSC40 cells activated transcription from the AT 5'-upstream promoter (Fig. 9). RXRα activated transcription in BSC40 cells, but almost not at all in HepG2 cells. COUP-TF1, TRα, HNF3, and, to a lesser extent PPARα, repressed transcription in both cell types. Activation by HNF4 was reduced by coexpression of COUP-TF1, TRα, and PPARα/RXRα. In HepG2 cells, activation by HNF4 was reduced by coexpression of PPARα or RXRα alone. In contrast in BSC40 cells, when HNF4 and RXRα were cotransfected, the increase in luciferase expression was up to 7-fold higher than the increase expected by the sum of activities of both factors, suggesting RXRα-HNF4 synergy. The addition of all the receptors tested resulted in a marked decrease of AT gene transcriptional efficiency.

DISCUSSION

The 5'-upstream element, located at −150/+68 nt, was shown to promote basal transcription. Deletion of the three areas protected in DNA footprint analysis provided evidence for the modular nature of this element. Deletion of element A at −92/−65 nt, element B at +1/+37 nt, and, to a lesser extent, element C at −124/−101 nt dramatically decreased luciferase reporter activity. Elements A, B, and C were not able to promote luciferase expression individually when inserted upstream of the luciferase gene in pSV0A-LA5'. A role for these sequences in transcription initiation is therefore unlikely. In contrast, tandem copies of the two elements A and B enhanced the transcriptional efficiency of two heterologous minimal promoters. This is in agreement with observations of a number of liver promoters, which contain a cluster of modular elements able to initiate and to modulate (principally enhance) transcription in close proximity to the start site(s) of transcription. Examples of promoters homologous to that of the human AT gene include the promoters of genes encoding several coagula-
tion factors, their inhibitors (serpins), apolipoproteins, and transferrin (4, 16–19).

When the AT gene region upstream of exon 1 was searched for classical eukaryotic control sequences, TATA box consensus sequences were found, but only upstream of the −150/+68 nt element (first consensus sequence at −155 nt). In contrast, perfect matching for an initiator element (CCACCC) was found at −43 nt (20). As seen in Fig. 2, −28/+67 nt-luc was unable to promote transcription, while −70/+67 nt-luc retained 20–30% of the activity of the entire −150/+67 nt region. Residual activity (15–35%) was also seen for a 3′-deletion ending at −28 nt. These data suggest the presence of an initiator at −43 nt. Ongoing cotransfection experiments with the transcriptional activators identified here and with deletion mutants of −150/+68 nt-luc should confirm the presence of this initiator. Furthermore, initiator elements mostly encompass mRNA start site(s) (20). Prochownik previously located a single AT mRNA start site 43 nt downstream of the putative initiator CCACCC (21). We wanted to confirm these findings and to reassess the 5′-end of the AT mRNA in tissues and cells of hepatic origin, using different experimental approaches. Mapping of the 5′-end of AT mRNA by S1 nuclease protection, primer extension, or 5′-rapid amplification of cDNA ends (RACE) generated a single major product, which placed the start site of transcription in agreement within 1–2 bases with the previously published start site (data not shown). The sequence encompassing this site, ACCAGTTT (−1/+7 nt), is also homologous to the mammalian initiator of transcription consensus sequence Py-PyCAN(T/A)PyPy (20). We also observed protection in this region, participation of element B (+1/+37 nt) in enhancing promoter strength, and binding by transcription factors. Element B alone was nevertheless unable to initiate luciferase expression. This region, especially in the area immediately upstream of the start site at −14/+1 nt, also shows homology with recently described GAGA boxes, control sequences for growth hormone-induced transcription (22). It has been shown that GAGA boxes also determine, in part, basal promoter activity in non-hormonal responses and bind specific zinc finger proteins such as PUR-1 and MAZ-1 (22, 23).

The three elements A, B, and C are able to bind nuclear proteins from several cell lines. These results are summarized in Fig. 10. None of the elements was protected solely by HepG2 nuclear extracts, contrary to previous results (4). Only differences in protection strength, upstream boundary, or the sequence around the transcription start site in the upper strand could be detected. Our results are somewhat similar to the regulatory features of a transgene AT −680/+24 nt upstream sequences linked to the apolipoprotein A-II gene (24). In the AT region, four protected areas (I-IV, corresponding to −138/−123, −112/−104, −89/−68, and −48/−22 nt, respectively, in the upper strand) were identified by footprint analysis using mouse liver extracts. Two of these areas (II and III) are similar to
elements A and C, respectively, of the AT gene, except that element C extends further upstream to −124 nt. Protection of element Bin our case and of element I of Tremp et al. (24) were not observed, because the 5′ and 3′ boundaries of the probes used for footprint analysis were located directly in these two areas. We did not observe protection of the putative initiator location (element IV at −48/−22 nt in Ref. 24) with extract from any cell line.

EMSA with nuclear extracts or in vitro translated factors allowed identification of interactions of the promoter with two liver-enriched factors: HNF4, a member of the nuclear hormone receptor superfamily, and C/EBPα, a leucine zipper CCAAT-binding protein. These two factors have been implicated with the liver-enriched expression of many other genes (8, 16, 19). HNF4 bound to elements A and C in extracts of HepG2 cells, but not COS1 or HeLa extracts. Interactions of HNF4 with area II of the −680/−24 nt AT sequences (equivalent to element A) have also been reported in the transgenic

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**FIG. 9. Effect of expression of transcription factors on the activity of the AT 5′-upstream minimal promoter in BSC40 and HepG2 cells.** The values reported are the mean ± standard deviation of luciferase activity relative to the activity of the AT 5′-upstream minimal promoter in the absence of expressed transcription factors. The numbers 2 and 4 (in parentheses) refer to the amounts of expressing plasmid, in µg.
study of Tremp et al. in extracts from mouse kidney (24). However, the absence of HNF4 in CV1-derived cells (COS1 and COS7) has been well documented (25). In element A, the HNF4 consensus binding site proposed by Sladek (8) matches in 10 of 13 nt of the element. The presence of HNF4, however, has been indicated only in the case of HepG2 cells (data not shown). Mutation 2, which destroys the HNF4-binding region, and data from footprinting and gel retardation assays. The interaction of HNF4 with element A and C is mediated by the pyrimidine strand immediately upstream of the transcription start site. The presumed initiator element is also indicated (start Site). The first ATG is boxed. The interacting transcription factors identified in this study are indicated above each element.

Additional cotransfection experiments showed that HNF4, C/EBPα, and RXRα were able to activate the AT 5′-promoter, while COUP-TF1, TRα, and HNF3 had a repressive effect. Interaction with the transcription initiation machinery, either directly or through bridging cofactors, could explain the activating and repressive effects of these unliganded nuclear hormone receptors (29, 30). HNF4 transactivation potential was also strongly reduced by COUP-TF1, TRα, and PPARα + RXRα. Direct binding competition between HNF4 and these four receptors has been demonstrated, at least in the induced transcription setting (15, 26). Our study suggests that interactions of elements A and C with nuclear hormone receptors could directly modulate initiation of AT gene expression. It is likely that additional members of the nuclear hormone receptor superfamily will interact with elements A and C in a constitutive or an induced setting. For example, heterodimerization of RXRα with several other receptors has been shown to participate in the transcriptional responses induced by retinoic acid, retinoids, peroxisome proliferators, vitamin D, and triiodothyronine (7, 9, 10, 15, 26, 27). Furthermore, interactions with subtypes or isoforms of the factors identified in this study or with newly described receptors, some of which might interact with half-site motifs (26, 27), multiply the number of potential modulatory responses through elements A and C. Moreover, elements A and C contain several putative arrangements of TGACCC half-sites in addition to direct repeats.

DNA-binding proteins other than nuclear hormone receptors, e.g., the single-stranded binding proteins PYBP or pTB (31, 32) originally identified in studies of the promoters of the transferrin gene and other liver-enriched genes, also interacted with element A (data not shown). Mutation 2, which destroyed HNF4 or RXRα-PPARα binding to element A, also destroyed the pyrimidine strand immediately upstream of the −75/−79 nt TGACCC half-site. A similar mutation in the rat aminotransferase gene has been reported to also destroy pTB binding (32). Whether or not PYBP or pTB could modulate nuclear hormone receptor interactions and/or binding to DNA is unknown. Moreover, ubiquitous factors interacting with elements B and C have not been characterized in this study. Preliminary data provided by competition assays (Fig. 6) suggest possible interactions of element B with ubiquitous CCAAT-binding proteins of the NF family. This result will have to be confirmed with supershifts and direct binding assays. For element B (−14/+37 nt), putative crosstalk with C/EBP isoforms and subtypes involved in the acute phase response, and with signal transduction and hormonal pathways (e.g., growth hormone-responsive elements) should be investigated in the induced setting (22–25).

The detection of a promoter-like activity in IVS1 was surprising, but a number of regulatory sequences have been found in IVSs, including IVSs of genes encoding serpins, liver-expressed coagulation factors, and a number of apolipoproteins (33, 34). The orientation of this element in the coding DNA strand of the AT gene suggested an open reading frame was present in the lower strand of IVS1. Computer analysis did not reveal any apparent coding regions. The search for long terminal repeat sequences in this region of the gene was also unsuccessful. The absence of reported activity of a construct including both the 5′-upstream and IVS1 elements could be due to the fact that both elements function in opposite directions and that promoter activity is quenched, at least in reporter assays. It could also suggest that the IVS1 element could silence expres-
sion of the 5′-upstream element. Our findings to date are preliminary. Effectively, exon 1 and the first 300 nt downstream of it were shown to also decrease (Fig. 1G), but not abolish, the activity of the 5′-upstream promoter. Therefore, the actual role of the IVS1 element on AT gene expression remains to be determined.

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