Tissue-specific expression of the human α_1 -antitrypsin gene is controlled by multiple cis-regulatory elements

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ABSTRACT

Human α_1 -antitrypsin (AAT) is expressed in the liver, and a 318 bp fragment immediately flanking the CAP site of the gene was found to be sufficient to drive the expression of a reporter gene (CAT) specifically in hepatoma cells. The enhancing activity however, was orientation-dependent. The DNA fragment was separated into a distal region and a proximal region. A "core enhancer" sequence GTGGTTTC is present within the distal region and is capable of activity enhancement in both orientations when complemented by the proximal region in the sense orientation. The results strongly suggest that there are multiple cis-acting elements in the human AAT gene that confer cell specificity for its expression. Nuclear proteins prepared from the hepatoma cells bound specifically to the proximal region in a band-shifting assay that was resistant to competition by the globin promoter DNA. Foot-printing analysis showed a protected domain within the proximal region that contains a nearly perfect palindromic sequence TGGTTAATATTCACCA, which may be important in the regulation of AAT expression in the liver.

INTRODUCTION

α_1 -antitrypsin (AAT) is synthesized in the liver and is the major protease inhibitor in plasma. Although the protein exhibits a wide spectrum of substrate specificity (1), its major physiological function is to inhibit neutrophil elastase in the lung (2). At present, more than 30 genetic variants of AAT have been identified (3,4). Two variants of AAT which commonly occur in individuals of Northern European descent are designated PiS and PiZ (Pi-proteinase inhibitor). Homozygosity for the PiS allele results in partial AAT deficiency in plasma (3,5). Individuals homozygous for the PiZ allele suffer from severe deficiency and are predisposed to development of chronic lung emphysema (2,6).

We have previously reported the cloning and sequencing of a full-length cDNA of human AAT (7). The sequencing data enabled us to predict that a substitution of Glu to Lys at position 342 in the PiZ protein was the result of a G to A transition in the corresponding position in the AAT gene. This

prediction was confirmed recently by cloning and sequence characterization of the Z allele by us and others (8,9). The original observation led to the development of specific oligonucleotides for detection of the Z allele in genomic DNA of carrier and affected individuals for the prenatal diagnosis of AAT deficiency (10,11). The entire genomic DNA sequence of the human AAT gene has also been reported (12). It contains five exons and four introns and codes for 418 amino acids including a 24-residue signal peptide.

Regulation of the tissue-specific expression of the AAT gene has not been intensively studied. Ciliberto et al. (13) reported that the 5' flanking sequence of the human AAT gene was able to confer cell-specific expression of a heterologous reporter gene in hepatoma cells. We and others recently reported the introduction of the entire human AAT gene into the germ line of mice (9,14) and found that the human gene was expressed in a tissue-specific manner. In this communication we report that the 5' flanking sequence of the human AAT gene contains multiple regulatory elements that confer cell specificity for its expression. In addition, we demonstrate the presence of nuclear proteins in hepatoma cells which specifically interact with the 5' cis-regulatory sequences of the human AAT gene.

MATERIALS AND METHODS

Enzymes and reagents

Restriction endonucleases, T4 nucleotide kinase, T4 ligase, DNA polymerase (Klenow fragment) and reverse transcriptase (Maloney Murine Leukemia Virus) were purchased from New England Biolabs or Boehringer Mannheim and used according to the suppliers' specifications. ^{14}C -Chloramphenicol was purchased from New England Nuclear. Acetyl CoA (Na Salt) was purchased from Sigma. O-Nitro-phenylgalactoside (ONPG) was purchased from Aldrich. Poly (dI-dC) was purchased from Pharmacia Fine Chemicals. All other reagents used were reagent grade.

Construction of vectors

A 1.2 kb BamHI fragment containing 5' flanking sequence (-1200/-31) of the human AAT gene was prepared by restriction digestion of cloned genomic DNA (9). This fragment was used for subsequent restriction digestion to obtain shorter DNA fragments. The restriction fragments were isolated by low-melting agarose gel electrophoresis and cloned into the BglII site of vector pA₁₀CAT₂ (15). The orientation of the cloned inserts was determined by restriction mapping and confirmed by hybridization with specific oligonucleotides.

Cell transfection and enzyme assays

Human hepatoma cells, HepG₂ and PLC/PRF/5, were cultured in MEM-10% fetal calf serum (FCS). NIH 3T3 cells were cultured in high glucose DMEM-10% FCS. Cells were plated out at a density of 5×10^5 cells per 10 mm dish 24 hours before transfection. 15 μ g of each test DNA construct and 15 μ g of pRSVZ (a plasmid containing the *E. coli* β -galactosidase gene controlled by the LTR of Rous sarcoma virus, ref. 16) were used to transfect cells in each dish by the calcium phosphate co-precipitation method (17). The pRSVZ vector was used throughout transfection experiments to monitor the transfection efficiency. 48 hours after transfection, cells were harvested by scraping and sonicated in 400 μ l of 0.25 M Tris-HCl buffer (pH 7.5). Cell debris were removed by centrifugation and the supernatant was used for enzyme assays.

For the β -galactosidase assay, 25 μ l of cell extract was added to 675 μ l of Z buffer (0.1 M NaPO₄, 10 mM KCl, 1mM MgSO₄, 50 mM β -mercaptoethanol, pH 7.0) containing 0.8 mg ONPG. The reaction was allowed to proceed for 1 hour at 37 °C and terminated by the addition of 0.5 ml 1M Na₂CO₃. The absorbance at 420 nm was then determined and used to normalize transfection efficiency. CAT activity assay was carried out essentially as described by Gorman et al. (18) except that the cell extract was heated for 5 minutes at 60 °C (19) prior to the addition of substrates. Reaction products were extracted with 0.6 ml ethyl acetate and chromatographed on silica gel plates with chloroform: methanol (19:1) as developing solvent. After exposure to X-ray film, the products were scraped from the silica gel and quantitated by liquid scintillation counting.

Isolation of RNA and primer extension reaction

Total RNA was isolated from transfected PLC/PRF/5 cells by the hot phenol/SDS method and purified by precipitation in LiCl. Primer extension was carried out according to the procedure of Devine et al. (20). The primer used was a 30-base oligonucleotide complementary to the mRNA coding for the 6th to the 15th amino acids of the CAT protein (21). The primer was 5' end-labeled by T4 polynucleotide kinase and γ -³²P-ATP to a specific activity of 1.33×10^6 cpm/pmole. Total RNA (50 μ g) was hybridized with the primer (2×10^6 cpm) at 55 °C for 5 hrs in 50 μ l of 10 mM pipes buffer (pH 6.4) containing 0.4M NaCl. The RNA-primer hybrid was ethanol precipitated and redissolved in 50 μ l of the extension buffer (50mM Tris-HCl, pH8.0, 10mM DTT, 6mM MgCl₂, 2.5 μ g actinomycin D, 2.5mM of each of the 4 deocytiribonucleotide triphosphates, 80mM KCl and 400 units of reverse

Fig. 1. (A) Construction of 5'AAT/CAT vectors for the analysis of CAT gene expression. The 1.2kb BamHI fragment of the 5' flanking region of the human AAT gene was cloned into the BglII site of pA₁₀CAT₂ vector in the sense (+) or antisense (-) orientations. **(B)** Detection of CAT activity in PLC/PRF/5 and NIH3T3 cells transfected with various constructs. 15μg of DNA was used to transfect PLC/PRF/5 and NIH3T3 cells by CaPO₄ coprecipitation method. CAT activity was assayed as described in "MATERIALS AND METHODS". Lanes 1 and 6, mock transfection; Lanes 2 and 7, pA₁₀CAT₂; Lanes 3 and 8, pSV₂CAT; Lanes 4 and 9, construct 1 (+); Lanes 5 and 10, construct 1 (-).

transcriptase). The reaction was allowed to proceed at 37 °C for 2 hrs. The extension product was then phenol extracted, ethanol precipitated, dissolved in 6μl of alkaline sequencing dye and analyzed in a 6% polyacrylamide/7M urea gel.

DNA-protein mobility shift assay

Crude nuclear extracts were prepared from PLC/PRF/5 cells and HeLa cells according to the modified method of Dignam et al. (22) as described by Pastorcic et al. (23). The restriction fragments used in the binding assay were generated from the 5' flanking sequence of the human AAT gene and consisted of a BglII/BamHI (-348 to -31) fragment and two smaller fragments derived from it, BglII/HpaII (-348 to -150) and HpaII/BamHI (-150 to -31). The mobility shift assay was performed according to the method of Sagami et al. (24). Briefly, [³²P] end-labeled DNA fragment was incubated with nuclear protein and various amounts of unlabeled poly (dI-dC) at room temperature for 10 minutes in a 10 µl reaction mixture containing 10 mM Hepes (pH 7.9), 100 mM KCl, 1mM DTT, 0.05 mM EDTA, 2.5 mM Mg Cl₂, 6% glycerol and 2% Ficoll (Pharmacia Fine Chemicals). The resulting complexes were then separated from protein-free DNA on a 5% polyacrylamide gel. The retardation of the mobility of DNA fragments was examined by exposure of the gel to XAR-5 X-ray film with Cronex intensifying screens (Dupont).

DNase I foot-printing reactions

DNase I foot-printing was performed as described by Galas and Schmitz (25) and modified by Pastorcic et al. (23). A 20 µl reaction mixture containing 18 mM Hepes (pH 7.9), 90 mM KCl, 4.5 mM MgCl₂, 0.2 mM EDTA, 1.8 mM DTT, 18% glycerol, 1 µg of poly (dI-dC) and 1-3 ng of [³²P] end-labeled HpaII/HinfI (-150 to +46) fragment was incubated with PLC/PRF/5 or HeLa nuclear extract at room temperature for 10 minutes. Pancreatic DNase I (Worthington, DPFF) was then added to a final concentration of 1-8 µg/ml and incubation was continued for 2 minutes at room temperature. The reaction was stopped by adding 100 µl of a solution containing 10 mM EDTA, 0.1% SDS, 5 µg proteinase K and 1.5 µg of pBR322 DNA. The mixture was incubated for an additional 15 minutes and then treated at 90 °C for 3 minutes. Finally, the samples were extracted with phenol/chloroform, precipitated by alcohol and fractionated in an 8% polyacrylamide/7M urea sequencing gel.

RESULTS

The 5' flanking region of the AAT gene confers hepatoma-specific expression of the CAT gene.

In order to identify potential cis-regulatory elements possibly present in the 5' flanking sequence of the human AAT gene, a 1.2 kb 5' flanking DNA fragment was cloned 5' to the CAT gene in the enhancer-less

Construct	5' AAT Fragment	% Enhancement of CAT Activity			
		PLC/PRF/5		NIH 3T3	
		+	-	+	-
1	5' Δ -1200 -31	100	13	1	<1
2	-732	128	9	<1	4
3	-348	63	11	4	2
4	-171	23	7	<1	1
5	-131	22	3	<1	<1
6	-77	18	11	<1	2
7	3' Δ -732 -77	39	12	6	5
8	-150	34	5	9	5
9	-230	29	17	5	4
10	-348	9	7	1	2

+, Sense orientation
 -, Anti-sense orientation

Fig. 2. Enhancement of CAT gene expression by the 5' flanking fragments of the human AAT gene. Restriction fragments were cloned in both orientations into the BglII site of pA₁₀CAT₂ vector as in Fig. 1 (A). Transfection of cells and quantitation of CAT activity were carried out as described in detail in "MATERIALS AND METHODS". The basal CAT activity due to pA₁₀CAT₂ vector was subtracted from each transfection experiment. The data represents the average value of three independent experiments and the activities are expressed relative to construct 1(+) in Fig. 1 after normalization for transfection efficiency.

expression vector, pA₁₀CAT₂ in both orientations (Fig. 1A). The constructs were introduced into human hepatoma cell, PLC/PRF/5 and NIH 3T3 cells. As shown in Fig. 1B, expression of the CAT gene was significantly enhanced in PLC/PRF/5 cells (lane 4) but not in NIH 3T3 cells (lane 9), while pSV₂CAT was active in both cell types (lanes 3 and 8). However, the enhancement of CAT gene expression in the hepatoma cells was not effective when the fragment was in the reverse orientation (lane 5), a result consistent with the observation reported previously (13). The orientation-dependent enhancement of CAT gene expression was also observed in another human hepatoma cell line, HepG₂ (data not shown). Since eukaryotic enhancers function relatively independent of distance and orientation, the results suggest that the 5' flanking sequence of the human AAT gene may contain another regulatory element(s) in addition to an enhancer for cell-specific expression.

Defining the boundaries of the cis-regulatory elements

To further define the boundaries of DNA sequence necessary for enhancing CAT gene expression in hepatoma cells, partial deletion mutants were constructed and expression was analyzed in PLC/PRF/5 and NIH 3T3 cells. As shown in Fig. 2, 5' deletion to -732 bp resulted in a slight but consistent increase of CAT gene expression in PLC/PRF/5 cells. Further deletion to -348 bp caused some reduction in activity, while deletion to -171 bp and beyond resulted in a drastic decrease in CAT gene expression. No significant enhancement of CAT gene expression was observed when these 5' deleted constructs were used to transfect NIH 3T3 cells, indicating that the enhancing effect exerted by the 5' sequence was cell-specific. The enhancement of CAT gene expression in hepatoma cells was consistently orientation-dependent. Reversing the orientation of the AAT gene fragment substantially decreased the enhancing effect in all constructs tested.

In an attempt to narrow down the sequence necessary for enhancing CAT expression, the -732/-31 fragment in construct #2 in Fig. 2 which best enhanced CAT expression was analyzed by 3' deletion. Deleting 46 bp from the 3' end to the -77 position resulted in a greater than two-third loss of activity in PLC/PRF/5 cells (construct #7). Further deletion from the 3' end to the -230 position produced only gradual loss of enhancing activity. Subsequent deletion to the -348 position however, abolished most of the enhancing activity (construct #10). Similar to the 5' deletion experiment, constructs with fragments cloned in reverse orientation also showed low CAT gene expression in PLC/PRF/5 cells. Interestingly, consistent low levels of CAT activity were detectable in NIH 3T3 cells transfected with the 3' deletion constructs up to the -230 position (constructs #7-9).

Identification of distal and proximal cis-regulatory element(s)

The results strongly suggested the presence of an enhancer element(s) in the 5' flanking sequence of the human AAT gene, presumably located between positions -732 to -171. However, the orientation-dependence of CAT gene expression deviates from the behavior of classical enhancer elements. It has been shown that some viral enhancers share the "core enhancer" sequence GTGG^{AAA}_{TTT} (15,26). Similar core elements have also been found in fragments with enhancer activity from the human and rat insulin genes (27) and other liver-specific genes (28,29). We searched for the core enhancer sequence in the 5' flanking region of the human AAT gene and found a homologous sequence GTGGTTTC located at the -225 to -217 position. To test whether this DNA fragment would enhance CAT gene expression in both orientations, we inserted

the BglII/HpaII (-348 to -150) restriction fragment further 5' to the recombinant containing the -171 to -31 fragment (Fig. 3). The results showed that the 5' distal fragment was indeed capable of complementing the proximal fragment for expression in the hepatoma cells in both orientations (constructs #11+ and 11-). Thus, a component within the BglII/HpaII fragment fulfills the criteria of an enhancer, namely it enhances transcription relatively independent of orientation and distance. Furthermore, since the entire DNA fragment (-348 to -31) showed orientation dependency (Fig. 3, constructs 3+ and 3-), it suggests that orientation dependency is a function of cis-acting element(s) within the proximal region of the 5' flanking AAT gene sequence.

Determination of transcription start sites of the CAT messenger RNA

To verify that the cell type-specific enhancement of CAT expression by the cis-regulatory element(s) of the AAT gene is due to increased efficiency in the utilization of SV40 promoter, we determined the authenticity of transcriptional start sites by performing primer extension analyses. CAT mRNA was isolated from PLC/PRF/5 cells transfected with various recombinant

Fig. 3. Demonstration of an enhancer element and additional distinct cis-regulatory elements in the 5' flanking sequence of the human AAT gene. The construction of 3 (+), 3 (-), 4 (+) and 4 (-) vectors is described in the legend to Fig. 2. Vectors 11 (+) and 11 (-) were constructed by cloning a BglII/HpaII restriction fragment (-348 to -150) of the 5' flanking sequence of human AAT gene into the Sali site of pA₁₀CAT₂, which is located 89 bp 5' to the BglII site in the vector where shorter 5' flanking region of the AAT gene has been inserted. Transfection of PLC/PRF/5 cells and assay of CAT activity are as described in "MATERIALS AND METHODS". CAT activity is expressed relative to construct 1 (+). Thick line, 5' flanking sequence of human AAT gene; thin line, sequence of pA₁₀CAT₂.

constructs. The primer used was a 30-base oligonucleotide complementary to the mRNA coding for the 6th to the 15th amino acids of the CAT protein (21). For comparison purposes, RNA isolated from cells transfected with pSV₂CAT was used as a control for transcription initiation from the SV40 promoter. As shown in Fig. 4, lane 2, two major extension products of 143 and 142 bases long were observed in pSV₂CAT-transfected cells. These products correspond to the correct initiation sites at the G residues located at 21, 22 base pairs 3' to the SV40 TATA box (30). Similarly, RNA from cells transfected with various recombinant constructs containing the 5' sequence of the AAT gene

Fig. 4. Primer extension for the determination of transcription initiation site of the CAT gene in hepatoma cells transfected with various CAT constructs. PLC/PRF/5 cells were transfected with 30 μ g of plasmid DNA. 48 hrs. after transfection total RNA was isolated. 50 μ g of RNA was hybridized to a 30- base primer (5'end-labelled, 2x10⁶ cpm) at 55 °C for 5 hrs. in 10mM Hepes buffer, pH 6.4 containing 0.4M NaCl. The primer extension reaction is described in detail in "MATERIALS AND METHODS". The extension products were analyzed in 6% polyacrylamide/7M urea gel. Lanes 1 to 6, primer extension products from 1) mock transfection; 2) pSV₂CAT; 3) construct 1(+); 4) construct 3(+); 5) construct 11(+); 6) construct 11(-), respectively. Lane 7, molecular weight marker, pBR322 digested with HpaII.

showed identical primer extension products (Fig. 4, lanes 3-6). Thus, enhancement of transcription for the CAT constructs by various AAT flanking sequences in the hepatoma cells was properly initiated from the SV40 CAP site.

Detection of hepatoma nuclear proteins that interact with the cis-regulatory elements

Our results above indicate that there is an enhancer-like element(s) within positions -348 to -150, plus additional cis-regulatory element(s) crucial for efficient transcription in hepatoma cells within positions -171 to -31. It is expected that specific interaction between these elements and trans-acting proteins might result in preferential expression of certain genes in a tissue-specific manner. We therefore carried out band shifting assays to determine if nuclear proteins from hepatoma cells would interact with the 5' flanking sequence of the AAT gene. We chose to label the BglII/BamHI (-348 to -31) fragment as the probe since it contains most of the cis-regulatory elements. Incubation of the probe with nuclear proteins from hepatoma cells resulted in a prominent band with reduced electrophoretic mobility, which could not be competed by a 16,000 fold excess of non-specific DNA competitor poly (dI-dC) (data not shown).

To further localize the region to which hepatoma nuclear proteins bind, we generated two smaller probes: a 199 bp BglII/HpaII (-348 to -150) probe and a 120 bp HpaII/BamHI (-150 to -31) probe (Fig. 5). A band-shifting assay was performed in the presence or absence of a DNA fragment containing the 5' end of the chicken β -globin gene (-793 to +75). It was observed that binding of nuclear proteins to the 199 bp distal probe could be competed by the β -globin gene fragment. The 120 bp proximal probe however, produced two shifted bands resistant to competition by an 80 fold molar excess of the β -globin DNA fragment (Fig. 5).

Defining the nuclear protein binding domains within the cis-acting element(s)

A DNase I foot-printing assay was then performed to determine the boundaries of protein-DNA interaction at the nucleotide level. A HpaII/HinfI restriction fragment (-150 to +46) of the AAT gene was used as probe in order to obtain better resolution at the 3' boundary. As shown in Fig. 6, digestion of the probe with DNase I in the presence of nuclear proteins produced a major protected region spanning nucleotides -78 to -52. Examining the nucleotide sequence revealed that the protected region contained a nearly perfect palindromic domain, TGGTTAATATTCACCA, between nucleotides -56 and -71. In addition, a region spanning nucleotide sequence -118 to -98 was also

Fig. 5. Binding of hepatoma nuclear proteins to different regions of the 5' flanking sequence of the AAT gene and competition by globin promoter sequence. 5 μ g nuclear proteins and 8 μ g of poly (dI-dC) were incubated with end-labelled probe (0.5 ng 10^5 cpm/ng) in the presence or absence of an 80 fold molar excess of chicken β -globin promoter DNA fragment (-793 to +75). The probe used for the first three lanes was a 199 bp BglII/HpaII fragment and that used for the last three lanes was the 120 bp HpaII/BamHI fragment, as shown in the bottom part of the figure.

found to be partially protected by the hepatoma nuclear proteins.

To test whether the protected DNA sequence in the foot-printing assay was also responsible for the observed band-shifting, we carried out a band-shifting assay using a specific synthetic oligonucleotide (a 27-base oligonucleotide from -52 to -78) and a non-specific oligonucleotide (random sequence) of the same length as competitor DNAs. As shown in Fig. 7, binding of nuclear proteins from hepatoma cells to the 196-base probe (-150 to +46) could be competed by the specific oligonucleotide but not by the non-specific oligonucleotide, suggesting that the DNA sequence protected by hepatoma nuclear protein(s) was indeed the sequence that caused the band-shifting.

DISCUSSION

Recent studies have established that cis-regulatory elements in the 5'

Fig. 6. DNase I foot-printing of the hepatoma nuclear proteins-DNA complex. The 5' end-labelled AAT gene fragment used for footprinting is diagrammed at the bottom of the figure. The probe used was the coding strand and labelled at the 5' end. One nanogram of the probe (10^3 cpm) was incubated with 0 or 20 μ g of hepatoma nuclear proteins in the presence of 1 μ g of poly (dI-dC) at room temperature for 10 minutes. Following DNase I digestions for 2 minutes the reaction was terminated and the samples were applied to a 8% polyacrylamide/7M urea sequencing gel as described in "MATERIALS AND METHODS".

flanking region of eukaryotic genes play a pivotal role in tissue-specific gene expression. Two cis-regulatory elements, TATA box and the upstream promoter element are present in most, but not all mammalian genes. These

Fig. 7. Band-shifting assay showing binding of hepatoma nuclear proteins to the labelled probe (-150 to +46) in the presence of specific or non-specific DNA competitor. The assay conditions were the same as described in the legend to Fig 5. except that 900ng of competitor DNA was used. The specific DNA competitor was a synthetic double-stranded DNA spanning the region from -78 to -52 of the probe, while the non-specific competitor DNA was a random sequence, double-stranded DNA of the same length.

elements are generally located within 120 bp upstream from the transcription start site. Their functions are thought to ensure precise and efficient initiation of transcription. In addition, enhancer elements have also been found in the 5' flanking region of many eukaryotic genes at various locations, (27, 28, 31), although their presence in the intronic sequence (32, 33) and 3' flanking sequence have also been well documented (34). The precise mechanism concerning how these cis-regulatory elements act to control the expression of certain genes in specific cell types or at different stages during development is not fully understood. Recent evidence, however, suggests that specific gene expression is probably regulated by the combinatorial effects of a variety of trans-acting factors with multiple cis-regulatory elements (28, 31, 35, 36).

Fig. 8. A schematic representation of a model depicting various cis-regulatory elements in the 5' flanking region of the human AAT gene. GC: GC rich sequences; E: putative enhancer; PE: proximal element; EBP: enhancer binding protein(s); HNF, hepatic nuclear factor(s). Dashed arrows indicate the interaction between putative trans-acting factors and the cis-acting elements.

The 5' flanking sequence of the human AAT gene has been studied by Ciliberto et al. (13) using 5' deletion analysis. They were also able to demonstrate that orientation-dependent enhancement of CAT gene expression in Hep3B was controlled within 700 bp of the 5' flanking sequence. We have confirmed these observations in another hepatoma cell line, PLC/PRF/5 and further characterized this region to define distinct cis-acting elements and to detect the corresponding trans-acting proteins in hepatoma nuclear extract.

We have previously reported that transgenic mice bearing genomic sequence of the human AAT gene plus approximately 2 kb of 5' and 3' flanking sequence are able to express the human gene in a tissue-specific manner (9). Recently, we found that the 700 bp (-732/-31) 5' sequence of the human AAT gene is sufficient to confer tissue-specific expression of the CAT gene in transgenic mice (Shen, et al. unpublished results). Thus, this region appears to contain all the necessary genetic elements for tissue-specific gene expression *in vivo*. The identification of nuclear proteins in hepatoma cells that specifically interact with a palindromic domain in this 5' flanking fragment of the human AAT gene at a region crucial for transcription has posed the possibility that the interaction might be responsible, at least in part, for cell-specific expression of the gene *in vivo*. We have shown that binding of hepatoma nuclear proteins to this region could be competed by a synthetic oligonucleotide containing the protected DNA sequence but not by the promoter of a heterologous gene or a synthetic oligonucleotide of random sequence (Fig. 5 and 7). In this respect, nuclear proteins from HeLa cells,

although bound to the 196 bp probe, produced a banding pattern which was distinct from that produced by the hepatoma nuclear proteins and could not be competed by the specific oligonucleotide (data not shown). Based on these observations, a model depicting various cis-regulatory elements in the 5' flanking region of the human AAT gene is presented (Fig. 8).

There is a GC-rich sequence located in the -732 to -348 region of the human AAT gene (GGGCGG, -402 to -398). The GC-rich sequence could be a potential binding site for the transcription factor SP1 (37), and may account for the observation that its deletion reduces enhancing ability by about 50%. Secondly the majority of the enhancing activity appears to reside upstream of the -150 position. We have shown that the BglII/HpaII (-348 to -150) fragment which contains the "core enhancer" sequence was able to enhance CAT gene expression in either orientation. It has thus provided strong evidence that the 5' flanking sequence of the AAT gene does contain an enhancer element(s) which may interact with enhancer binding proteins (EBP) in the cell nucleus (39-44). The demonstration that transcription initiation sites of the CAT gene were not altered by the introduction of the 5' AAT sequence suggests that enhanced CAT expression is due to an increase in transcription efficiency, presumably through the interaction between enhancer-binding proteins and the 5' sequence of the AAT gene.

Since the pA₁₀CAT₂ vector contains a SV40 viral promoter which functions efficiently with its own enhancer in hepatoma cells, the inability of the 5' AAT flanking sequence to function in reverse orientation implies that cis-regulatory element(s) other than the upstream "putative" enhancer is important in controlling cell-specific expression. Our finding that removal of sequence between -77 and -31 bp from the -732/-31 fragment greatly impeded its enhancing activity demonstrates that sequence within or proximal to this region are necessary for maximal activity. Since this region possessed very little enhancing activity by itself, its effect is probably on the transcriptional efficiency in hepatoma cells. We thus propose that there are cis-acting element(s) within this region designated PE for "proximal elements" that may play a critical role in tissue-specific expression of the AAT gene.

The demonstration that nuclear proteins from hepatoma cells bound specifically to the palindromic sequence between -52 and -78 bp further supports the contention that this region might be important in controlling tissue-specific gene expression. It has recently been shown that the 5' flanking sequence of the mouse alpha-fetoprotein (AFP) gene contains two

inverted repeat sequences between -54 and -94 bp (38). Furthermore, the region between -52 and -82 is absolutely essential for AFP transcriptional activity and for cell-specific expression of the AFP gene in HepG₂ cells (31). Neither AFP gene nor AAT gene contains an apparent CAAT box or GC-rich sequence between -80 and -100 upstream of their CAP sites. Whether the presence of a palindromic sequence in these two liver genes is fortuitous, or represents important elements for interaction with hepatic nuclear factors (HNF) in order to achieve tissue-specific gene expression remains to be explored.

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