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A Cell-Specific Enhancer of the Mouse α1-Antitrypsin Gene Has Multiple Functional Regions and Corresponding Protein-Binding Sites

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We have previously described the isolation and characterization of genomic clones corresponding to the mouse α1-antitrypsin gene (Krauter et al., DNA 5:29–36, 1986). In this report, we have analyzed the DNA sequences upstream of the RNA start site that direct hepatoma cell-specific expression of this gene when incorporated into recombinant plasmids. The 160 nucleotides 5′ to the cap site direct low-level expression in hepatoma cells, and sequences between −520 and −160 bp upstream of the RNA start site functioned as a cell-specific enhancer of expression both with the α1-antitrypsin promoter and when combined with a functional β-globin promoter. Within the enhancer region, three binding sites for proteins present in hepatoma nuclear extracts were identified. The location of each site was positioned, using both methylation protection and methylation interference experiments. Each protein-binding site correlated with a functionally important region necessary for full enhancer activity. These experiments demonstrated a complex arrangement of regulatory elements comprising the α1-antitrypsin enhancer. Significant qualitative differences exist between the findings presented here and the *cis*-acting elements operative in regulating expression of the human α1-antitrypsin gene (Ciliberto et al., Cell 41:531–540, 1985; De Simone et al., EMBO J. 6:2759–2766, 1987).

For many years the most valuable attribute of cellular differentiation to the biochemist interested in the problem was the differential production of proteins, each of which could be measured (27). During the past few years, it has become increasingly clear that the differential distribution of proteins is based to a large extent on differential gene transcription in different cell types (11). For example, hepatocytes make many enzymes and secreted proteins that are not made in other cells, and many of the genes for these products are transcribed exclusively or mainly in liver (8, 14, 54). The basis for this coordinated transcriptional control within the same tissue remains unknown.

With the techniques of recombinant DNA and molecular genetics, coupled with the ability to reintroduce mutant genes into differentiated cell lines or animals, at least some of the sequences that participate in differential transcription are being located for both genes active in liver and those active in other organs (2, 3, 7–9, 13, 15, 17, 19, 21, 22, 25, 28, 29, 31, 32, 41, 46, 48–50, 53, 63, 64, 67). Detailed information on at least a reasonable sample of genes expressed predominantly in the liver will be needed to determine the extent to which necessary transcription factors function in a coordinated manner on the different genes. To that end, we and others are studying a variety of rodent and human genes that fit the pattern of liver-specific transcription (2, 3, 5, 7–9, 13–15, 17, 21, 22, 25, 28, 29, 32, 46, 49, 53, 54, 67). One such gene is α1-antitrypsin (α1-AT), a protein which is produced by hepatocytes and to a lesser extent by macrophages (15, 51). We previously described the isolation of cDNA and genomic clones corresponding to this gene in mice (14, 35). In this paper, we report the sequences required for the cell-specific expression of transfected α1-AT constructs in the human hepatoma cell line Hep-G2. These same sequences are not operative in directing expression in HeLa cells.

An important pattern has begun to emerge in the regulatory regions of various viral genes and some cellular genes (30, 60, 69). Multiple protein-binding sites for presumed transcription factors have been observed; there are repeated sites for one type of factor as well as several sites for different factors (1, 3, 8, 10, 12, 26, 34, 36–39, 41, 52, 57, 59, 61, 62, 66). The binding of several factors to their recognition sequences is capable of potentiating transcription in vitro (18, 38, 45, 65). We likewise find that a number of different DNA regions required for α1-AT expression (presumed *cis*-acting elements) are present in the sequences between 200 and 475 nucleotides upstream of the mouse α1-AT RNA start site. This region contains multiple elements capable of exhibiting hepatoma-specific enhancer function, while the 160 nucleotides nearest the cap site contain information for hepatoma-specific transcription at low levels. Each of at least three regions within the enhancer is shown to bind specific nuclear proteins, using the gel retardation or gel shift assay. Chemical footprinting assays carried out with dimethyl sulfate (DMS) in the presence or absence of proteins from hepatoma cells were used to delineate the residues involved in the DNA-protein interaction. These results form the basis for assays that should lead to the purification of factors required for the cell-specific transcription of α1-AT and ultimately to comparison with the factors needed for the liver-specific expression of other genes. The ultimate goal is to establish the function of these nuclear proteins in cell-specific transcription in vitro.

MATERIALS AND METHODS

Sequencing of the α1-AT promoter and enhancer. Various restriction fragments located between the *Eco*RI site at −523 base pairs (bp) and the downstream *Bgl*II site at +490 bp

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were subcloned into either gemini 1 or 2 (Promega Biotech, Madison, Wis.) after isolation from low-melting-point agarose gels (Bethesda Research Laboratories, Gaithersburg, Md.). Both strands of individual plasmids were sequenced by using two different primers made to within the Sp6 and T7 promoters of the plasmid vectors, using the dideoxy chain-terminating sequencing method (56).

Localization of the α1-AT first exon and RNA start site. Previous experiments had tentatively localized the first exon to a region downstream of the *Bgl*II site at +490 bp (6, 35). A 25-bp oligonucleotide made to within this region failed to hybridize to mouse liver poly(A)$^+$ RNA isolated by the method described by Chirgwin et al. (4). A series of restriction fragments spanning this region and various regions upstream (extending to −523 bp) were subcloned into gemini 1. Uniformly labeled antisense RNA was made from each subclone, using either Sp6 or T7 polymerase and [α-^{32}P]UTP (3,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) as described previously (8, 44). The labeled RNA probes (1 ng) were hybridized to 1 μg of liver poly(A)$^+$ RNA for 6 to 12 h at various temperatures in 20 μl of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–400 mM NaCl–1 mM EDTA containing 80% formamide. At the end of the incubation, each sample was chilled on ice and 60 U of T2 RNase (Bethesda Research Laboratories) was added per ml. The T2 RNase-protected hybrids were analyzed on denaturing polyacrylamide gels. By using a series of restriction fragments, the first exon was localized to a 160-nucleotide region which was sequenced as described above.

The location of the major transcriptional start site was determined by primer extension of a 26-bp synthetic oligomer (Fig. 1, left panel; indicated by colons). The oligomer was made to the region thought to correspond to the 3′ portion of the first exon based on a sequence comparison to the human gene (5). The oligomer was 5′-end labeled (43) with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP (7,000 Ci/mmol; New England Nuclear Corp.). The oligomer (1 ng) was hybridized at both 60 and 65°C to 2.5 μg of liver poly(A)$^+$ RNA for 12 h in 40 mM PIPES–400 mM NaCl–1 mM EDTA. The hybrids were chilled and precipitated with 3 volumes of ethanol. The products were suspended in 50 μl of reverse transcriptase buffer (42) containing 40 U of RNAsin (Promega Biotech) and extended with 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.). The primer extension was carried out for 2 h at 42°C, and the products were sized on a denaturing polyacrylamide gel (43).

Construction of α1-AT expression vectors. The manipulations used in the cloning of the various restriction fragments were performed by methods described by Maniatis et al. (42). The α1-AT minigenes were constructed by first making a parent plasmid containing the required downstream sequences. A *Bam*HI to *Sst*I restriction fragment isolated from the genomic clone λH (35) was first subcloned into gemini 2 (Promega Biotech). This construct contained ~500 bp of the 3′ portion of the third intron and extended ~1 kilobase (kb) downstream of the fifth exon. The promoter deletions (see below) were cloned into the *Eco*RI and *Bam*HI sites of the above vector, using a convenient *Bgl*II site at +490 bp which resulted in the indicated *Bgl*II/*Bam*HI fusion (see Fig. 2A). Deletions within the 5′-flanking region of the α1-AT promoter were generated by first linearizing the *Eco*RI (at position −523 bp)-to-*Bgl*II (+490 bp) gemini 1 construct at the *Eco*RI site. Successive 5′-to-3′ deletions were made by digestion with BAL 31 exonuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by the methods described by Legerski et al. (40). The digested ends were made double stranded with the Klenow fragment of *Escherichia coli* (Bethesda Research Laboratories). *Hin*dIII linkers (Pharmacia Biochemicals) were ligated to the blunt ends with T4 DNA ligase (Bethesda Research Laboratories). Subsequent digestion with *Hin*dIII and *Bgl*II produced a nested set of fragments which were then subcloned into the parent minigene vector. Each promoter deletion was sequenced as described above to verify the 5′ endpoint of the construct.

The α1-AT/β-globin enhancer constructs were generated by using the β-globin–E1B fusion vector described previously (8, 21). This plasmid contains a functional β-globin promoter (including 341 bp of 5′-flanking sequence) and the entire first exon (143 bp) and second exon (206 bp) fused to a restriction fragment from the adenovirus E1B gene that contains a splice acceptor, as well as the sequences required to direct polyadenylation. Various regions upstream of the α1-AT promoter were cloned into an artificially introduced gemini 2 polylinker and the *Hin*dIII site located 341 bp upstream of the β-globin RNA start site. The α1-AT sequences used (see Fig. 3A) were generated from restriction fragments previously subcloned into gemini 1, from the promoter deletion constructs, or from a combination of each. Again, each construct was sequenced to verify the presence of the indicated sequences.

Transient assays and T2 RNase analysis. Human hepatoma cells (Hep-G2 [33]) were grown and passaged in Hams F12 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum (Whittacker M.A. Bioproducts), 25 μg of gentamicin sulfate per ml (Schering Pharmaceutical Corp., Bloomfield, N.J.), 0.1 mM minimum essential medium nonessential amino acids (GIBCO Laboratories), and 0.5 U of insulin per ml (Eli Lilly & Co., Indianapolis, Ind.). HepG2 cells were transfected at 50% confluency in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The plating of the cells and the formation of the calcium-phosphate DNA coprecipitate were as described previously (8). HeLa cell monolayers were maintained and transfected in the Dulbecco modified Eagle medium described above.

Initial experiments were undertaken to optimize the transfection efficiency as a function of increasing DNA concentration. Optimal expression was consistently observed with 50 μg of the test plasmid and 5 μg of the control vector. The α1-AT minigene constructs (50 μg) were cotransfected with the simian virus 40 (SV40) enhancer-driven β-globin expression vector (5 μg) into both Hep-G2 and HeLa cells in parallel. The SV40–β-globin construct served as an internal control for transfection efficiency. The coprecipitate was left on the cells for 12 h, at which time the cells were washed with phosphate-buffered saline and fresh medium was added. Total cytoplasmic RNA was isolated 36 h later, using the Nonidet P-40 lysis method (42). The level of expression obtained from each construct was determined by T2 RNase digestion of hybrids formed between RNA (25 μg) isolated from transfected cells and uniformly labeled antisense RNA probes specific to the α1-AT exon 1 and the β-globin exons 1 and 2 (8). The protected hybrids were analyzed by electrophoresis through an 8% polyacrylamide gel in the presence of 8 M urea followed by autoradiography of the dried gels. Since the transfection efficiency varied between different cell samples, the α1-AT signals were normalized by comparison with the β-globin expression in each transfected cell culture.

Gel retardation (gel shift) analysis. Nuclear extracts were

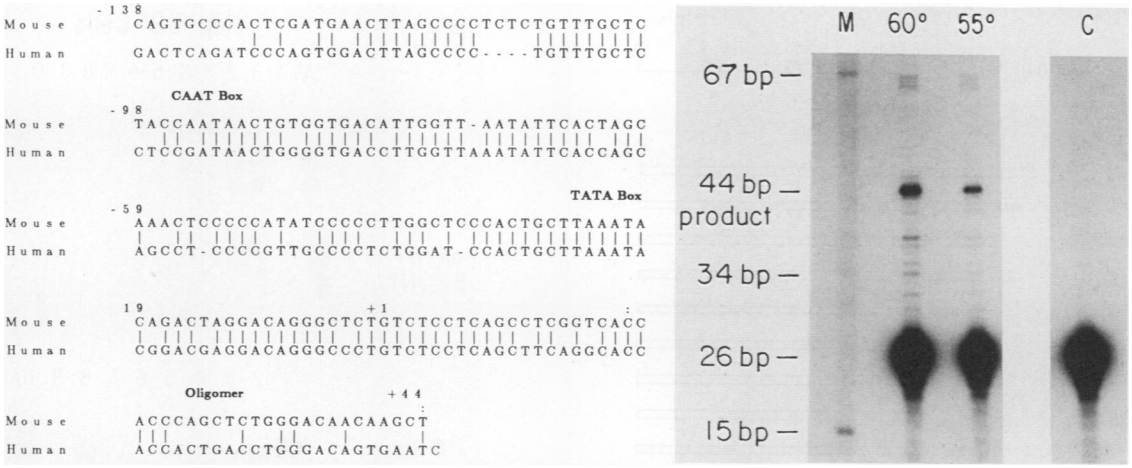


FIG. 1. DNA sequence comparison between the mouse and human α1-AT promoter region and first exon. (left) The human α1-AT (5) and mouse sequence starting 138 nucleotides upstream of the RNA start site were compared. The vertical lines represent nucleotide identities, and the dashes represent regions where gaps were introduced to maximize the alignment. The locations of the CCAAT and TATA boxes for the mouse sequence are indicated. The cap site for both genes is located at +1. There is a 78.9% identity with the 154-bp overlap. (right) A 26-bp oligomer (bases between the colons, left panel) was synthesized for use in a primer extension experiment. A primer extension was carried out after hybridization of the kinased oligomer to liver poly(A)$^+$ RNA at two different temperatures (see Materials and Methods). Lane C is the primer extension obtained with yeast RNA as a control template. The primer-extended products (44 bp) were sized on a 15% denaturing polyacrylamide gel along with labeled molecular weight markers. The size of the extended product was the same as that obtained when a uniformly labeled RNA probe was used to measure the first exon.

prepared from cultured cells by the method of Dignam et al. (16), using a final NaCl concentration of 0.8 M. At this salt concentration, lysis of the nuclei occurred. The chromatin was removed by centrifugation at 50,000 rpm in an SW56 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 1 h. The supernatant from this final spin was dialyzed against 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid), pH 7.6–100 mM KCl–0.1 mM EDTA–1 mM dithiothreitol. Protein concentrations were determined by the dye binding method (Bio-Rad Laboratories, Richmond, Calif.). Typically, 30 mg of protein was obtained from 4×10^8 Hep-G2 cells.

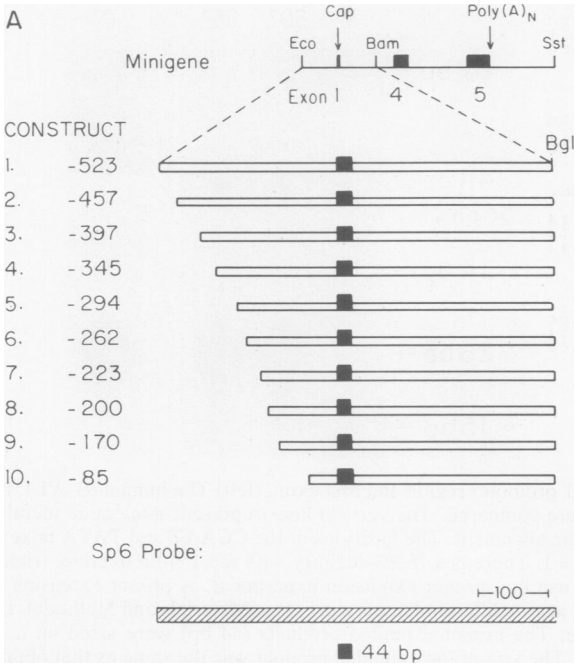
Uniquely 3'-end-labeled probes were generated from various restriction fragments by filling in with all four ^{32}P-labeled deoxynucleotides, digesting with a second restriction enzyme, and isolating the fragment from a low-melting-point agarose gel. The gel shift experiments were performed by first incubating the labeled probe (1 to 2 ng) with 5 to 10 μg of nuclear extract as previously described (9, 20, 24, 34). After 30 min at room temperature, 40% of the reaction was electrophoresed on a 6% native polyacrylamide gel in 22 mM Tris, pH 7.4–22 mM boric acid–5 μM EDTA. The gel was run at 4°C for 3 to 4 h, during which the electrophoresis buffer was recirculated. The gels were dried and autoradiographed at room temperature to visualize the specific DNA-protein interactions. Generally, a 50- to 100-fold molar excess of the homologous unlabeled probe was used for the competitions.

Methylation protection and interference mapping of the protein-binding sites. The methylation protection experiment (9, 23) involved incubating 20 ng of the high-specific-activity, 3'-end-labeled probes with 100 μg of Hep-G2 nuclear extract in a 400-μl volume, using the conditions described for the gel shift assay. After 30 min at room temperature, the reaction was treated with 2 μl of DMS for 3 to 5 min. The mixture was subsequently loaded on a preparative native polyacrylamide (6%) gel as described above. Specific bands were localized by exposure of the gel for 2 h at 4°C. The shifted bands as well as the unbound probe were excised and eluted from the acrylamide gel by shaking overnight at 37°C in 500 mM NH_4 acetate–300 mM sodium acetate–10 mM Tris, pH 8.0–1 mM EDTA–0.1% sodium dodecyl sulfate. The eluted material was extracted with phenol-chloroform and ethanol precipitated after the addition of 50 μg of glycogen (Sigma Chemical Co., St. Louis, Mo.) as carrier. Residual acrylamide was removed by a further purification of the labeled DNA through an Elutip-d column as described by the manufacturer (Schleicher & Schuell, Keene, N.H.). The eluted DNA was then cleaved with piperidine (43), and the products were analyzed on a denaturing acrylamide gel which varied in concentration from 10 to 15% depending on the size of the probe used. Each lane of the resultant autoradiograph was scanned with a laser densitometer to determine which residues were protected and which were only partially protected. The corresponding G and G+A sequencing ladders (43) were run on the same gel to demarcate the location within the sequence where protein interaction was occurring.

The methylation interference experiments (59) were performed essentially as described above except that the restriction fragment probes were partially methylated with DMS (43) prior to incubation with extract. All subsequent manipulations were performed on a preparative scale as described for the methylation protection experiments.

RESULTS

α1-AT cap site mapping and promoter sequence. The restriction maps of the genomic clones containing the mouse α1-AT gene and the positions of the downstream exons within these clones have been described previously (35). The coordinates of the first exon were localized by using a series of genomic subclones located between 5 and 7 kb upstream of the second exon. Various restriction fragments spanning this region were cloned into the vector gemini 1. Labeled antisense RNA was generated from each subclone and hybridized to liver poly(A)$^+$ RNA as outlined (see Materials



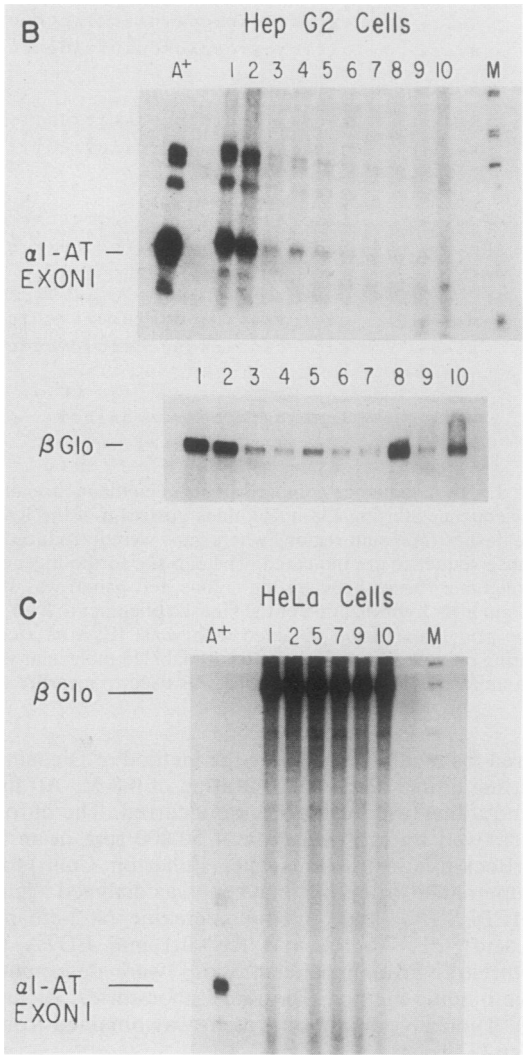


FIG. 2. Transient assays of the 5′ promoter deletion minigene constructs in Hep-G2 and HeLa cells. (A) Structures of the minigenes used to analyze the 5′-flanking regions of α1-AT in regulating expression from its own promoter after transfection of both Hep-G2 and HeLa cells. Sequential 5′ deletions from the *Eco*RI site located at −523 were generated with BAL 31 exonuclease. The downstream portion of the minigene contained both the fourth and fifth exons as well as the information required to direct polyadenylation. (B) Transient assays from Hep G2 cells transfected with the minigene fusion constructs. A 50-μg portion of each α1-AT minigene construct was cotransfected with 5 μg of an SV40 enhancer-driven β-globin expression vector (A) into the human hepatoma cell line Hep-G2. Total cytoplasmic RNA was harvested 30 to 36 h after removal of the calcium-phosphate coprecipitate. The amount of mRNA generated from each construct was measured after hybridizing 25 μg of RNA to two uniformly labeled RNA probes: probe 1 spanned the first exon of the α1-AT gene; probe 2 spanned both the first and second exons of the β-globin transfection control. The T2 RNase-resistant products were sized on an 8% denaturing polyacrylamide gel followed by autoradiography of the dried gel, as shown in the upper part. The sizes of the protected products were the same as those obtained by using mouse liver poly(A)$^+$ RNA. The band corresponding to the β-globin second exon is shown underneath and was used as an internal control for transfection efficiency. (C) Transient assays of the transfected minigenes in HeLa cells. The indicated constructs were transfected and analyzed as described for panel B. The first exon of the β-globin transfection control is evident at the top of the photograph. No expression from the α1-AT minigenes was apparent even after prolonged exposure of the gel.

and Methods). T2 RNase digestion and electrophoretic analysis of the resultant hybrids revealed protection of a 44-bp nucleotide fragment that mapped to a site between 6 and 7 kb upstream of exon 2. The 160-nucleotide restriction fragment spanning the region of this presumed first exon was sequenced. A 25-nucleotide oligomer was constructed after comparison of the mouse genomic sequence with the previously published human sequence (5). After hybridization to mouse liver poly(A)$^+$ RNA, primer extension of the 5′-end-labeled probe yielded a major extended product of 44 nucleotides (Fig. 1B). The size of the primer-extended product and that of the T2 RNase-resistant fragment were the same, confirming the first-exon boundaries. These experiments positioned the mRNA start site of this gene approximately 1 kb upstream of an earlier estimate not based on primer extensions and RNase T2 digestions (6, 35). The second exon lies 6.5 kb downstream of the first exon/intron junction. When RNA from other cells (e.g., HeLa or yeast RNA) was used in the hybridization and extension, no major bands were seen. With liver mRNA, several minor bands which corresponded in size to 65 to 70 nucleotides were evident. These products presumably result from a lower level of expression from an upstream RNA start site possibly within the same gene. This seems likely since the minor transcripts were also present when transfected constructs containing only 160 nucleotides of upstream α1-AT sequence were used to monitor specific expression in transient assays (Fig. 2B). There is a second TATA box located at position −70 bp relative to the indicated cap site that might be utilized less effectively than the major start site.

A comparison was made of the mouse sequences proximal to the RNA start site and the previously published human α1-AT sequence in the same region (5). Within the 135 bp of the start site, the sequences were about 80% identical, presumably reflecting the importance of these residues in the regulated expression of these two genes. The 4-bp insertion within the mouse sequence starting at position −108 bp interrupts a region which has been shown to be important to

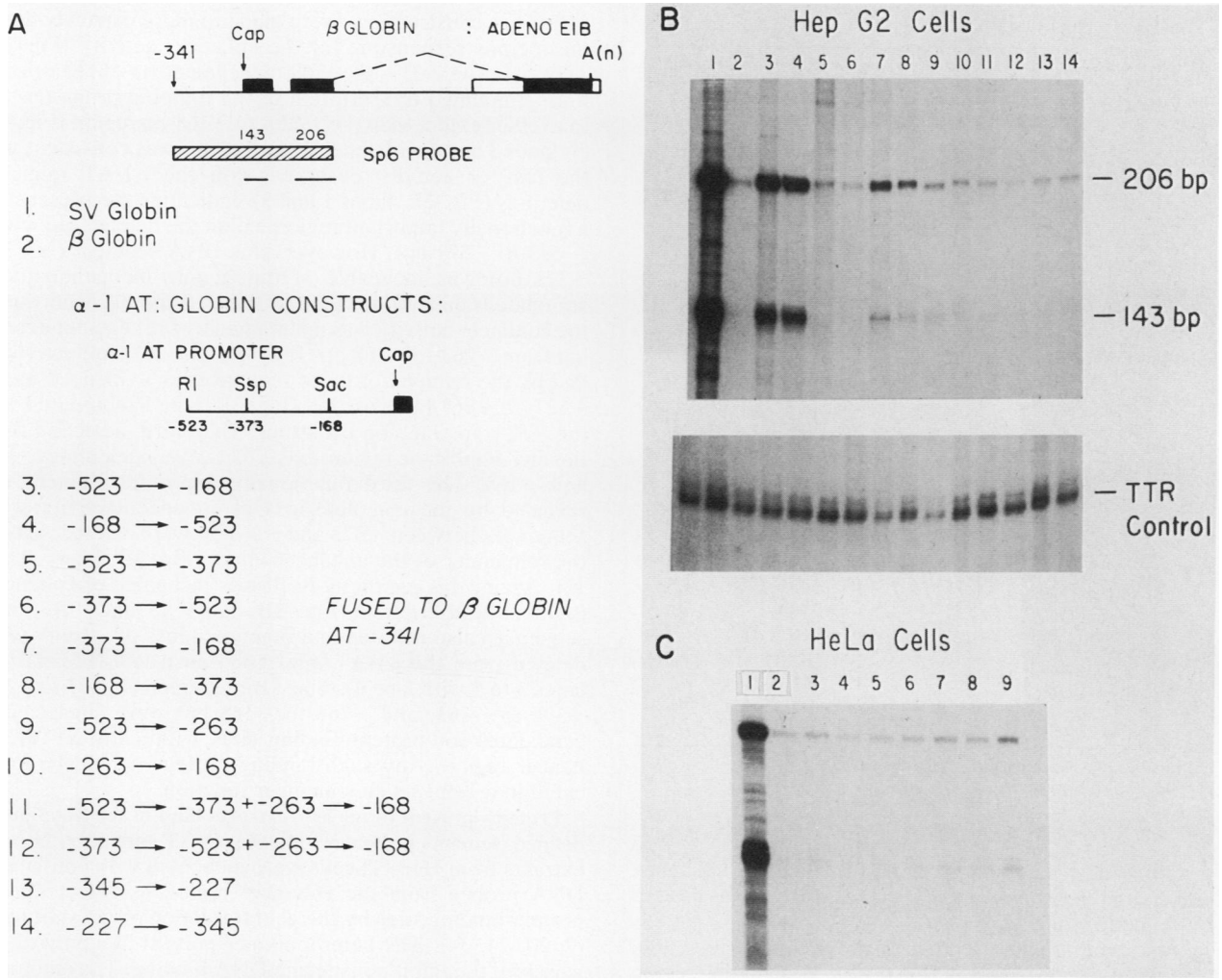


FIG. 3. Transient assays of the α1-AT/β-globin fusion vectors in HepG2 and HeLa cells. (A) The indicated upstream regions of the α1-AT gene were cloned into the functional β-globin expression vector (line 1) 341 bp upstream of the cap site. The indicated probe was used to measure expression of each construct from the β-globin promoter, protecting bands of 143 and 206 bp after T2 RNase digestion. The locations of the α1-AT sequences and their relative orientations are indicated. The β-globin promoter construct with and without the SV40 enhancer was transfected in parallel for comparative purposes. (B) The transfections and subsequent analysis were performed as described in the legend to Fig. 2. An enhancer-containing transthyretin expression vector (8) was used to monitor the transfection efficiency for each construct. The probe used to score expression from this plasmid spanned the first exon of the transthyretin gene. The T2 RNase-resistant products were visualized after electrophoresis on an 8 M urea–polyacrylamide (6%) gel after autoradiography. Each lane of the autoradiograph was scanned to determine the ability of the inserted α1-AT sequences to enhance expression of the β-globin promoter (summarized in Table 1). Sequences from −523 to −168 bp of α1-AT were required for maximal enhancer activity. (C) HeLa cell transfections were done exactly as the Hep-G2 experiments except that a functional α-globin expression plasmid was cotransfected to monitor the transfection efficiency (data not shown). In each case, no expression above that obtained with the β-globin promoter alone was observed.

the human promoter-proximal enhancer (15). That is, mutations within this region abolish the ability of the human −137- to −37-bp sequences to enhance expression of the SV40 promoter in transient assays and may reflect one of the differences in the upstream elements of these genes (see Discussion). Significant sequence divergence was noticeable within the first exon and became greater further downstream (data available in genbank).

Transient expression in HepG2 and HeLa cells from α1-AT promoter deletions. A series of recombinant plasmids were prepared to analyze the role of the upstream sequences in directing expression of the α1-AT gene. Two separate series of constructs were prepared and tested for cell-specific expression by transfection into Hep-G2 and HeLa cells. First, a set of deletions within the 5′-flanking sequences was prepared. Either convenient restriction sites were utilized or BAL 31 exonuclease digestions were carried out, and these were joined in the first intron with a segment of α1-AT DNA containing a part of the third intron, the fourth and fifth exons, and the polyadenylation site (Fig. 2A), thus creating a "minigene." The second set of constructs utilized upstream α1-AT sequences fused to a mouse β-globin reporter construct (Fig. 3A) to determine the capacity of the α1-AT sequences in activating a heterologous promoter. After transfection, expression was assayed by protection of a uniformly labeled antisense RNA probe specific for either the first 44 nucleotides of the α1-AT primary transcript or the first (143 bp) and second (206 bp) β-globin exons (Fig. 2A and 3A). In all experiments, cotransfection with a control plasmid, either an SV40 enhancer-driven β-globin vector or

TABLE 1. Quantitation of the HepG2 transfection data

Deletions/constructs	No.	Construct	Fold induction
5′ α1-AT BAL 31 deletions[a]	1	−523	26.5
	2	−457	14.0
	3	−397	3.6
	4	−345	4.0
	5	−294	3.8
	6	−262	1.0
	7	−224	1.0
	8	−200	0.9
	9	−170	1.0
	10	−85	
α1-AT β-globin constructs[b]	1	β-Globin	1.0
	2	Simian virus globin	~75.0
	3	−523 to −168	13.0
	4	−168 to −523	9.1
	5	−523 to −373	1.0
	6	−373 to −523	1.0
	7	−373 to −168	3.7
	8	−168 to −373	2.5
	9	−523 to −263	1.5
	10	−263 to −168	1.5
	11	−523 to −373+ −263 to −168	1.0
	12	−373 to −523+ −263 to −168	1.0
	13	−345 to −227	1.0
	14	−277 to −345	1.0

[a] The minigene constructs used are illustrated in Fig. 2A.
[b] The indicated fusion vectors as depicted in Fig. 3A.

an α-globin construct, was used to normalize the efficiency of transfection. A second antisense labeled RNA was used to score the control plasmids.

The first experiments used deletions leaving 1,850, 1,300, 720, and 523 nucleotides of the α1-AT 5′-flanking region joined within the first intron to the minigene. Each of these plasmids gave an equivalent signal in Hep-G2 cells, and none were expressed in HeLa cells. BAL 31 exonuclease deletions from −523 bp towards the cap site were then used (Fig. 2). Two regions were required for maximal expression. The first was located between 523 and 397 nucleotides (Fig. 2B; cf. lanes 1 and 2 with lanes 3 to 5) and the second was between 294 and 262 nucleotides (Fig. 2B; cf. lanes 3 to 5 with lanes 6 to 9) upstream of the RNA start site. Minimal expression was then maintained until the deletion that removed sequences between 170 and 85 nucleotides upstream of the cap site (Fig. 2B, lanes 9 and 10). Again, none of the deletions gave a signal upon transfection of HeLa cells (Fig. 2C). This set of experiments defined a promoter region capable of directing low-level hepatoma-specific transcription plus upstream elements that were required for maximal expression (summarized in Table 1).

To test whether the more distant upstream sequences could function as an enhancer, the −523- to −168-bp restriction fragment was attached in either orientation (at −341 bp) to a mouse β-globin promoter (diagrammed in Fig. 3A). The results of transfection experiments showed a 10- to 15-fold enhancement of β-globin transcription when this region was present in either orientation (Fig. 3B; cf. lane 2 with lanes 3 and 4). This enhancement only occurred in HepG2 cells and not in HeLa cells (cf. Fig. 3B with 3C). Thus, both a cell-specific enhancer and a minimal cell-specific promoter are found in the α1-AT 5′-flanking region.

Further constructions were made to more narrowly define the regions responsible for the enhancer activity (Fig. 3B; Table 1). The −373- to −168-bp segment (in either orientation) stimulated transcription of the β-globin promoter, but to a lesser extent than the −523- to −168-bp region (Fig. 3B; cf. lanes 3 and 4 with lanes 7 and 8). This was consistent with the loss of activity observed with the α1-AT minigene deletions (Fig. 2B, lanes 1 and 3), indicating the presence of a functionally important region within the deleted sequences (−523 to −373 bp). However, this DNA segment (−523 to −373 bp) was incapable of functioning independently to stimulate transcription (Fig. 3B, lanes 5 and 6). A portion of the enhancer activity was maintained by the segment extending from −263 to −168 bp (Fig. 3B, lane 10), and activity is lost by the removal of these sequences as evidenced by the −523- to −263-bp construct (Fig. 3B, lane 9) compared with the −523- to −168-bp construct. Therefore, a second functionally important region exists between nucleotides −263 and −168. The third functionally important element was revealed by the complete loss of enhancer activity when sequences between −373 and −263 bp were deleted, leaving the remainder of the enhancer intact (Fig. 3B, lanes 11 and 12). Again, this region by itself was incapable of enhancing β-globin transcription (Fig. 3B, lanes 13 and 14). These sequences also resulted in a functional loss of activity when deleted from the α1-AT minigene constructs (Fig. 2B; cf. lanes 3 to 5 with lane 6). Thus, three regions (−523 to −397, −397 to −263, and −263 to −168 bp) were suggested as candidates for protein-binding sites within the α1-AT enhancer region. Any such binding proteins would be implicated in a cell-specific enhancer function.

Proteins present in nuclear extracts bind to the functionally defined elements present within the α1-AT enhancer. Nuclear extracts from Hep-G2 cells were incubated with end-labeled DNA probes from the enhancer region to detect nuclear protein-binding sites by the gel retardation or gel shift assay (9, 20, 24, 34). The heteropolymer poly(dI-dC):poly(dI-dC) was used to inhibit nonspecific DNA binding. The amount of nonspecific competitor required to eliminate nonspecific binding was titrated for each labeled fragment used. Specific unlabeled DNA fragments from the enhancer region were included as competitors to test the specificity of binding. The products of the binding reaction were subsequently analyzed on a native polyacrylamide gel, and the slower migrating (presumably higher-molecular-weight) bands, representing protein-bound DNA, were visualized by autoradiography of the dried gel. Probe 1, which extended from −523 to −373 bp (Fig. 4, top), showed the presence of two shifted bands which were not eliminated by incubation with a nonspecific oligonucleotide but were eliminated by a 50-fold molar excess of the corresponding unlabeled fragment (indicated by the arrows in Fig. 4, bottom). The higher-molecular-weight doublet did not compete with the homologous fragment and must therefore represent a nonspecific interaction. The second probe used (−373 to −168 bp) exhibited a slightly more complex pattern of shifted bands. Two sets of shifted bands were evident, each of which was inhibited by an excess of unlabeled homologous fragment (fragment 3, −344 to −168 bp; Fig. 4, bottom). A second fragment from the α1-AT enhancer (labeled fragment 4; −262 to −168 bp) inhibited the faster migrating gel shift bands independently of the more slowly migrating bands (Fig. 4, bottom right). This result indicated the possibility of two binding sites within the −344- to −168-bp region of the enhancer. The first site is located between −344 and −262 bp, while the second site lies further downstream (between −262 and −168 bp). In

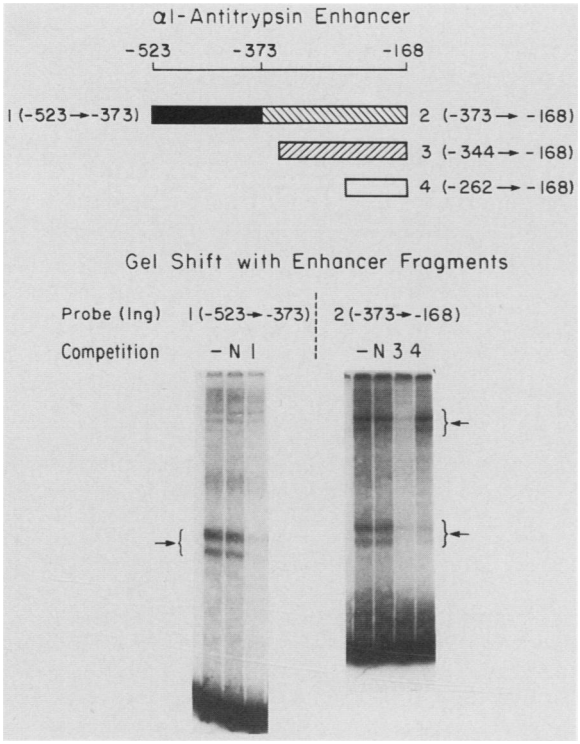


FIG. 4. Gel retardation or gel shift assay with fragments spanning the α1-AT enhancer. (Top) Entire α1-AT enhancer region (−523 to −168 bp). A convenient *Ssp*I site located at −373 bp was used to divide this sequence into two fragments, which were independently used to detect nuclear proteins present in Hep-G2 extracts which interact with sequences within the enhancer. Fragments 3 and 4 were used in competition experiments as depicted in the lower panel. (Bottom) Probe 1 or 2 was incubated with 6 μg of the nuclear extract for 30 min with or without (−) the indicated competitors. The products of the binding reaction were separated on a 6% nondenaturing polyacrylamide gel and subjected to autoradiography (see Materials and Methods). The arrows in each panel indicate the presence of shifted bands which were eliminated by either the homologous fragment or one of the deleted fragments.

all of the gel shift assays of fragments from this region, multiple shifted bands corresponding to an apparant single binding site were observed (i.e., the closely migrating doublets). As discussed below, these doublets footprint in identical regions of each probe used. They may result from the interaction of a second protein which complexes with the primary protein binding the DNA. Alternatively, it may indicate that the DNA-binding protein exists in more than one form either as a result of a posttranslational modification or as a consequence of limited proteolysis which occurred during preparation of the extract.

Methylation protection mapping of the binding sites located within the α1-AT enhancer. The nucleotides recognized by specific DNA-binding proteins present in crude nuclear extracts can be determined with DMS protection experiments (9, 23). A uniquely end-labeled probe is incubated with extract to allow protein binding to occur. After 30 min, DMS is added to specifically methylate G residues in the DNA that are not protected by protein. By increasing the time of incubation with the reagent, partial methylation of unprotected A residues will also occur. The reaction was performed on a preparative scale with several DNA fragments spanning the α1-AT enhancer region (see Materials

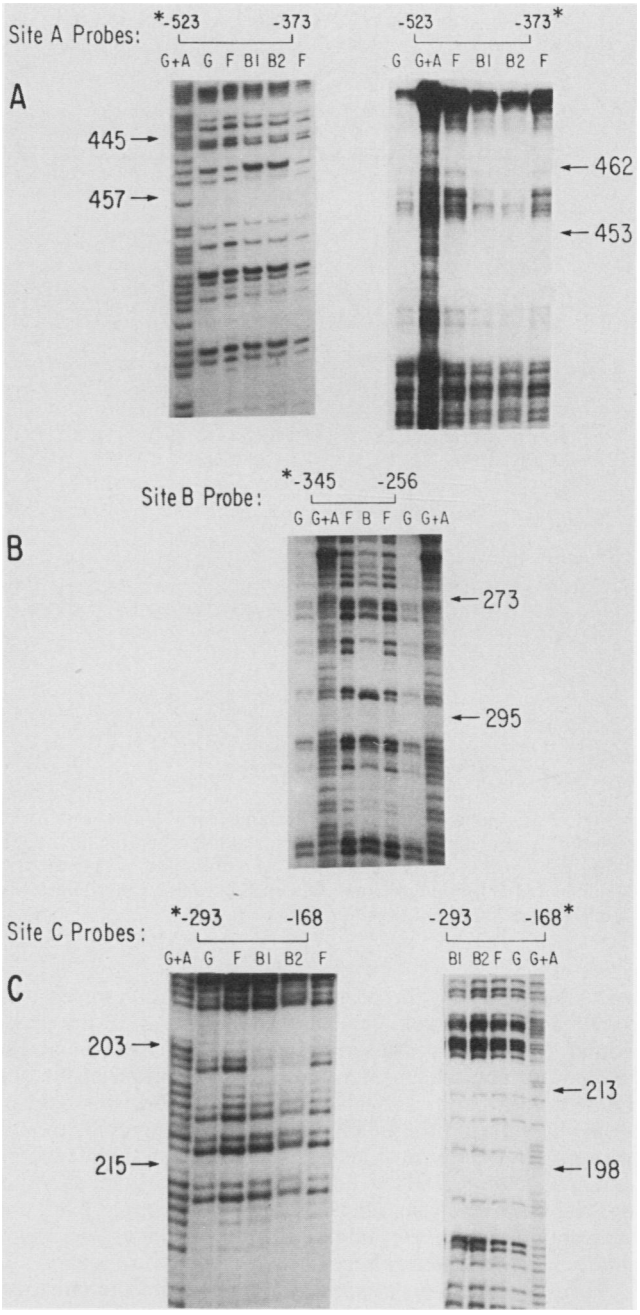


FIG. 5. Protection of three regions within the α1-AT enhancer by proteins present in nuclear extracts. The map positions of the labeled probes used for each experiment are indicated above each panel. The specific 3′ end labeled with each probe is indicated by an asterisk. The probes were incubated with nuclear extract as described for Fig. 4 and then treated with DMS for 3 to 5 min. The samples were immediately subjected to electrophoresis through a nondenaturing polyacrylamide gel. The protein-bound DNA (B1, B2) was isolated as was the free probe (F). The eluted DNA was deproteinized, subjected to piperidine cleavage, and analyzed on a denaturing polyacrylamide gel. Appropriate sequencing ladders (i.e., G, G+A) were run in parallel on the same gel to position the regions protected. Each lane of the gel was scanned and compared with results obtained with the unbound (F) probe. The regions protected by protein binding are indicated by the arrows, and specific residues are shown in the sequence presented in Fig. 6.

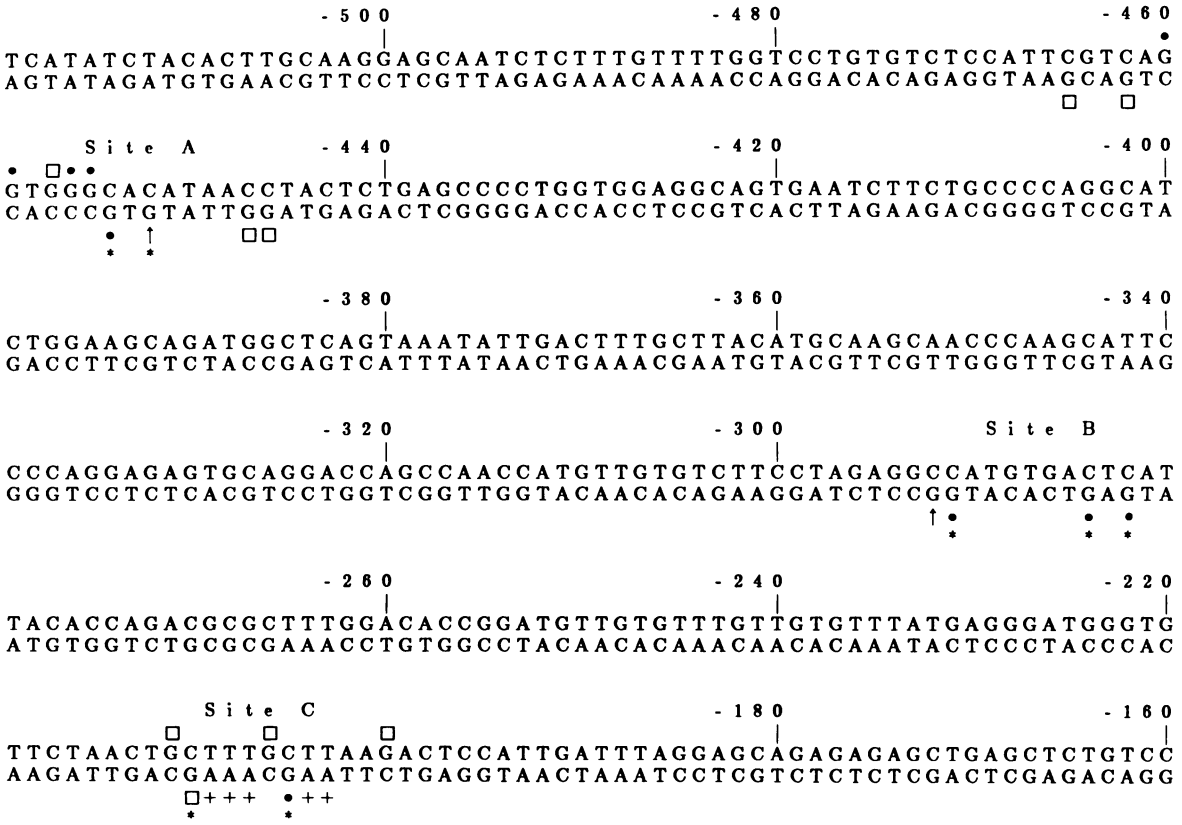


FIG. 6. Sequence of the α1-AT enhancer and locations of the three binding sites within the region. Both strands of the entire enhancer region were sequenced by the dideoxy chain-terminating method (see Materials and Methods). The positions of binding sites A, B, and C are indicated. Each site was localized by a combination of both methylation protection and interference techniques (Fig. 5 and 7). Symbols used to delineate the binding regions: ●, G protection; □, partial protection; ↑, hypermethylation; +, A protection; *, residues defined as contact points by methylation interference experiments.

and Methods), and the products were electrophoresed on a native acrylamide gel. This allowed separation of the probe bound to proteins present in the shifted bands and the unbound probe which migrated near the bottom of the gel. Each shifted band, as well as the free probe, was excised from the gel, and the labeled DNA was recovered, treated with piperidine (to introduce breaks at the methylated G's), and analyzed on a DNA sequencing gel (43). Comparison of the cleavage reactions obtained with each shifted band and that obtained with the unbound probe indicates the region involved in protein binding.

Various restriction fragments (Fig. 5) from the enhancer region were uniquely end labeled, as indicated (*), for use in this analysis. Each probe was incubated with 6 μg of a Hep-G2 nuclear extract in the presence of sufficient amounts of poly(dI-dC):poly(dI-dC) to inhibit nonspecific DNA binding. Portions of the resultant sequencing gels showing regions of protection within each DNA region are presented between the arrows (Fig. 5). Sequencing ladders were run in parallel on the same gel to determine the precise location of affected residues. Each lane of the individual gels was scanned with a laser densitometer to differentiate between full and partial protection of individual guanylate bases.

The position of each protected base and the location of the three elements within the enhancer are presented in Fig. 6. Sequences spanning each protein-binding site were shown in earlier experiments (Fig. 2 and 3; Table 1) to be required for full enhancer activity. In addition, sequences containing each binding site were incapable of enhancer activity alone (summarized in Table 1).

Identification by methylation interference experiments of specific guanylate residues involved in protein binding. In addition to methylation protection by protein-DNA interaction, individual residues involved in protein binding can be identified by prior partial methylation of the DNA by DMS. If the G residue is required for protein contact, then its methylation will disrupt the binding of the protein, thus eliminating this DNA molecule from the protein-bound fraction. ^{32}P-end-labeled restriction fragments spanning the A, B, and C boxes (Fig. 6) were partially methylated with DMS (see Materials and Methods) and exposed to Hep-G2 nuclear extracts, and protein-bound and free products were recovered after separation on nondenaturing acrylamide gels. The recovered free and protein-bound bands were then cleaved with piperidine and analyzed on sequencing gels to determine whether particular G residues were absent from the sequence ladder generated from the protein-bound band (Fig. 7). The residues determined by this technique to be important for binding within each site are illustrated in Fig. 6 by the asterisks. Within site A, two residues were affected. One residue was lacking in the sequence ladder and at one site an exaggerated band was present, indicating that methylation of this base favored protein binding. Within sites B and C, only methylated bases which reduced the effective binding affinity of the respective protein(s) were observed. In all three regions, the methylation interference results

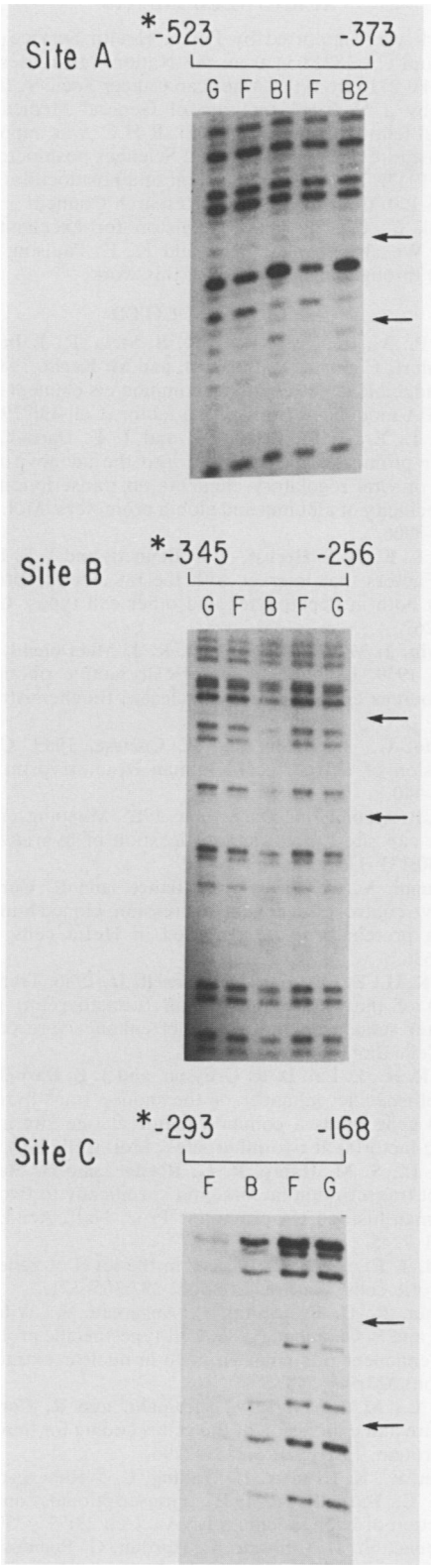


FIG. 7. Delineation of residues involved in protein binding by methylation interference. End-labeled probes spanning binding sites A, B, and C were treated with DMS before the binding reactions were carried out. The methylated probes were incubated with the Hep-G2 nuclear extract, using the same conditions used for the gel shift experiments. Fractionation of the free and protein-bound DNA was essentially as described for the methylation protection experiments (Fig. 5). Lanes marked F were free probe, and B, B1, and B2 were gel-shifted bands. The cleaved products were separated on a denaturing acrylamide gel and visualized by autoradiography. The arrows indicate regions in which residues important for protein interaction were found. The individual residues affected are indicated in Fig. 6.

pointed mainly to the same residues protected in the methylation protection experiments.

DISCUSSION

These experiments have demonstrated a very complex arrangement of regulatory elements within a 300-nucleotide stretch (−480 to −280 bp) upstream of the mouse α1-AT transcription unit. While the 160 nucleotides immediately upstream of the cap site are sufficient for a minimal cell-specific transcription, the entire region (up to −520 bp) boosts expression in the human hepatoma cell line (Hep-G2) about 20- to 30-fold. No further transcriptional stimulation was afforded by any other DNA segments either extending up to 2 kb 5′ from the RNA start site or within the long first intron. Neither the 5′-proximal sequences nor the enhancer sequences of the α1-AT gene function in HeLa cells, a human cell line of epithelial origin. Thus, a hepatoma-specific factor or factors must exist that are responsible for transcriptional activation of this gene.

The cell-type-specific enhancer region contains multiple sites for protein interaction based on several criteria. Deletion analysis of the enhancer region produced at least three separate DNA elements required for function, each of which was capable of binding nuclear proteins as tested by the gel retardation or gel shift assay. Within each of these regions, a small 10- to 20-nucleotide stretch was defined as important for protein binding based on both methylation protection and methylation interference experiments. With nuclear proteins present, certain G residues were protected from DMS methylation by protein interaction, while if DMS was first used to methylate the probe prior to protein binding, certain G's did not appear in gel shift bands, indicating their importance in DNA-protein complex formation. These experiments provide evidence of protein contact points in each region that had been shown capable of participating in enhancer activity. Deletion of the three segments involved in protein binding (A, B, and C; Fig. 6) produced in each case a much smaller boost of hepatoma-specific transcription than did the complete enhancer. This observation substantiates by a functional criterion the importance to full enhancer activity of the presence of each protein-binding site. Work in progress has defined an additional protein-binding site located just downstream of the CCAAT box (located at −80 bp) which may contribute to the observed cell specificity of the promoter.

It is of interest that the factors in human cells (Hep-G2 cells) recognize the mouse α1-AT sequences, a fact that is not surprising considering the conservation of sequence between the mouse and human genes up to −137 bp, where sequence in both species is available. However, substantial qualitative differences exist between the two species in terms of the enhancer elements operative in regulating their expression. Promoter deletions within the human α1-AT constructs indicate the presence of three regions important for maximal expression in Hep-G2 transient assays. However, unlike what we report here for the mouse gene, which

requires 500 bases upstream of the cap site for maximal expression, only 261 bp of 5'-flanking sequence is necessary for full activity of the human α1-AT promoter. Two upstream regions of the human α1-AT gene (−488 to −356 and −356 to −210 bp) act as independent enhancers for expression of the heterologous SV40 promoter (15). However, these sequences fail to enhance expression of the wild-type promoter, implying a possible functional redundancy of the elements operative in regulating expression of the human α1-AT promoter. Moreover, the upstream enhancers are not cell specific as they activate expression of the heterologous SV40 promoter in both Hep-G2 and HeLa cells. A minimal tissue-specific element has been identified between nucleotides −137 and −37 of the human gene that activates transcription of the SV40 promoter only in Hep-G2 cells. This element may be responsible for mediating the cell specificity of the human α1-AT promoter. As described in this report, the mouse α1-AT enhancer is composed of multiple elements, all of which are required for full enhancer activity and which function only in Hep-G2 cells. Each element is independently incapable of enhancer activity when used to drive a functional β-globin promoter. Since the human and mouse genes are structurally similar, the observed differences between the enhancers of these two genes were surprising. The differences in cell specificity and function may be a consequence of the specific promoter used to evaluate enhancer-driven expression. Alternatively, the observed differences may be a property of the human gene which is different, but contains a similar array of upstream regulatory elements. When sequence data upstream of −137 bp of the human gene become available, a comparison between the two DNAs in the functionally defined regions can be made.

Why are there so many binding sites in this gene, each of which appears to be required for maximal function in Hep-G2 cells? In viral genes, e.g., the SV40 early transcription unit, there appear to be at least four or five different types of binding sites and repetitions of several subclasses (30, 37, 45, 66, 69). Some of these sites appear to be used only in some cell types (12, 47, 55, 58, 68). Thus, the virus potentially gains flexibility by being able to have its DNA transcribed in cells with different factor distributions. But for a liver-specific transcription unit, it seems that several sites might have accumulated in the course of evolution to maximize RNA synthesis. Just as with α1-AT, the liver-specific transthyretin (TTR) gene has multiple enhancer-binding sites each of which contributes to raising the transcription rate from a cell-specific promoter region (8, 9). For mouse albumin, there is one segment located between −10 and −12 kb (not finely mapped with respect to protein-binding sites) that has a 10-fold enhancing effect (53). There is also evidence in this gene of an additional requirement for sequences between −150 bp and the RNA start site for cell-specific expression of this gene (3, 29). The mouse α-fetoprotein gene has multiple enhancers that seem to differ from those present in either the mouse α1-AT or TTR because, like the upstream human α1-AT sequence, they are active in both Hep-G2 and HeLa cells (25). However, these regions of α-fetoprotein are responsible for high rates of transcription in the mouse liver when introduced into transgenic animals (28). In all these cases, one reasonable interpretation is that active transcription complexes may be established by promoter-proximal sequences but that more distal sequences contribute to an increased rate of transcription and that at least in some cases there are cell-specific factors that activate the enhancer function.

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