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Research Article

alpha 1-Antitrypsin (alpha 1 AT) is plasma glycoprotein that constitutes the principle inhibitor of neutrophil elastase in tissue fluids. It has been considered a prototype for liver-derived acute phase proteins in that its concentration in plasma increases three- to fourfold during the host response to inflammation/tissue injury. However, recent studies have shown that alpha 1 AT is expressed in several types of extrahepatic cells, including mononuclear phagocytes and enterocytes, and that there are distinct transcriptional units used in hepatocytes and at least one extra-hepatic cell type, blood monocytes. In this study, we have used a combination of ribonuclease protection assays, primer elongation analysis, and transcriptional run-on assays to further characterize mechanisms of basal and modulated alpha 1 AT gene expression in hepatocytes, enterocytes, and macrophages. The hepatoma cell line HepG2, intestinal epithelial cell line Caco2, and primary cultures of human peripheral blood monocytes were used as examples of the cell types. The results indicate that there are three macrophage-specific transcriptional initiation sites upstream from a single hepatocyte-specific transcriptional initiation site. Macrophages use these sites during basal and modulated expression. Hepatoma cells use the hepatocyte-specific transcriptional initiation site during basal and modulated expression but also switch on transcription from the upstream macrophage transcriptional initiation sites during modulation by the acute phase mediator interleukin 6 (IL-6). Caco2 cells use the hepatocyte-specific transcriptional […]

Constitutive and Modulated Expression of the Human α_1 Antitrypsin Gene

Different Transcriptional Initiation Sites Used in Three Different Cell Types

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Abstract

α_1-Antitrypsin (α_1 AT) is plasma glycoprotein that constitutes the principle inhibitor of neutrophil elastase in tissue fluids. It has been considered a prototype for liver-derived acute phase proteins in that its concentration in plasma increases three- to fourfold during the host response to inflammation/tissue injury. However, recent studies have shown that α_1 AT is expressed in several types of extrahepatic cells, including mononuclear phagocytes and enterocytes, and that there are distinct transcriptional units used in hepatocytes and at least one extrahepatic cell type, blood monocytes. In this study, we have used a combination of ribonuclease protection assays, primer elongation analysis, and transcriptional run-on assays to further characterize mechanisms of basal and modulated α_1 AT gene expression in hepatocytes, enterocytes, and macrophages. The hepatoma cell line HepG2, intestinal epithelial cell line Caco2, and primary cultures of human peripheral blood monocytes were used as examples of the cell types. The results indicate that there are three macrophage-specific transcriptional initiation sites upstream from a single hepatocyte-specific transcriptional initiation site. Macrophages use these sites during basal and modulated expression. Hepatoma cells use the hepatocyte-specific transcriptional initiation site during basal and modulated expression but also switch on transcription from the upstream macrophage transcriptional initiation sites during modulation by the acute phase mediator interleukin 6 (IL-6). Caco2 cells use the hepatocyte-specific transcriptional initiation site during basal expression. There is a marked increase in the use of this site and an increase in the rate of transcriptional elongation of α_1 AT mRNA during differentiation of Caco2 cells from crypt-type to villous-type enterocytes. Caco2 cells also switch on transcription from the upstream macrophage transcriptional initiation sites during modulation by IL-6. These results provide further evidence that there are differences in the mechanisms of constitutive and regulated expression of the α_1 AT gene in at least three different cell types, HepG2-derived hepatocytes, Caco2-derived enterocytes and mononuclear phagocytes. (*J. Clin. Invest.* 1992. 89:1214–1222.) Key words: acute phase proteins • hepatocytes • mononuclear phagocytes • enterocytes

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Introduction

α_1 Antitrypsin (α_1 AT)[1] is the archetype of a family of plasma proteins that are called serpins because many of the members are serine protease inhibitors. As shown by the consequences of deficiencies in specific serpins, these proteins play important roles in control of blood-borne limited proteolytic cascade systems (complement, coagulation, fibrinolysis), in control of connective tissue turnover, in limiting tissue destruction and initiating tissue repair at sites of injury/inflammation (reviewed in 1, 2). Many of the serpins are also called "acute phase reactants" in that there are marked increases in their plasma concentrations during the host response to inflammation (reviewed in 3).

For these reasons, there has been a great interest in understanding the mechanisms that determine the net concentrations of serpins in tissues and body fluids. There is still relatively little information about the clearance and catabolism of serpins but more is known about biosynthesis both during basal and regulated states. For α_1 AT, liver is the predominant site of synthesis (4, 5). In this site, synthesis of α_1 AT is modulated by the acute phase mediator IL-6 (6–8). α_1 AT is also synthesized in mononuclear phagocytes (9, 10). In these cells its synthesis is modulated by bacterial lipopolysaccharide (LPS) and IL-6 (8, 11). In both hepatocytes and macrophages synthesis of α_1 AT is modulated by α_1 AT-protease complexes as a part of a feed-forward servomechanism (reviewed in 2). Studies in transgenic mice have suggested that α_1 AT is also synthesized in other extrahepatic cell types including renal tubular epithelial cells, intestinal epithelial cells, nonparietal cells of the gastric mucosa, and pancreatic islet cells among others (12–14). In fact, we have shown that α_1 AT is synthesized in a human enterocyte-like cell line and that its expression in this cell line increases markedly during differentiation from crypt-like to villous-like enterocytes (15).

Several *cis*-acting elements and *trans*-acting factors that direct the expression of the α_1 AT gene in hepatocytes have been identified and characterized. Tissue-specific expression of α_1 AT in human hepatoma HepG2 cells is directed by structural elements within a 137-nucleotide region upstream of the hepatocyte transcriptional initiation site within exon IC (16–19). The *cis*-acting elements within this region bear homology with elements upstream of other genes expressed in hepatocytes including albumin, fibrinogen, and haptoglobin. There is a consensus sequence for the nuclear transcription factor AP-1 (20) ~ 300 nucleotides upstream from the hepatocyte transcription initiation site. There are also two sequences similar to the proximal portion of the IL-6 response element (21) ~ 200 nucleotides upstream from the hepatocyte transcription initiation site. A sequence ~ 200 nucleotide sequences upstream from the macrophage transcription initiation site (22) bears even

1. *Abbreviations used in this paper:* AT, antitrypsin

greater homology with the IL-6 consensus sequence. There are at least two distinct *trans*-acting factors involved in binding to the upstream flanking regions and therein determining the constitutive expression of the α_1 AT gene. One of these factors LF-B1 (also called HNF-1, APF, HP1) is a 90-kD glycoprotein that has structural similarities to Drosophila homeobox-encoding genes and which binds to an element at residues −84 to −70 of the upstream flanking region of the α_1 AT gene (17). This factor plays a major role in hepatocyte-specific transcription of albumin, α-fetoprotein, fibrinogen, transthyretin, pyruvate kinase and the pre-S1 gene of the hepatitis B virus. Transcription of the liver-specific and IL-6–inducible, C-reactive, protein gene is also regulated by HNF-1 (23). A second *trans*-acting protein, LF-A1, binds to an element at residues −125 to −100 of the α_1 AT gene (19). A 68-kD nuclear protein termed HNF-2, probably corresponding to LF-A1, has recently been purified (24). There is also evidence for several other nuclear proteins in liver that bind to the upstream flanking region of the α_1 AT gene but the functional significance of these other proteins has not yet been extensively investigated.

There is relatively little information about *cis*-acting elements and *trans*-acting factors, which direct the expression of the α_1 AT gene in extrahepatocytic cell types. However there is some evidence that different elements and nuclear proteins are involved, especially since the initiation site for transcription of the α_1 AT gene in mononuclear phagocytes is located ~ 2,000 nucleotides upstream from the hepatocyte transcriptional initiation site (13, 22). In this study we have characterized in detail the transcriptional initiation sites used for basal expression in hepatocytes as well as in extrahepatic cell types, enterocytes, and mononuclear phagocytes. In addition we characterized the transcriptional initiation site used during modulated expression of the α_1 AT gene in each cell type: e.g., increased α_1 AT gene expression in HepG2 cells mediated by IL-6; increased α_1 AT gene expression in Caco2 cells during differentiation or mediated by IL-6; increased α_1 AT gene expression in mononuclear phagocytes as mediated by LPS or IL-6.

Methods

Materials. Ribonuclease A was purchased from Sigma Chemical Co. (St. Louis, MO), ribonuclease T1 from Bethesda Research Laboratories (Gaithersburg, MD), and DNAse I, and ribonuclease inhibitor were purchased from Promega Biotec (Madison, WI). *E.coli* serotype 0111:B4 lipopolysaccharide preparations were purchased from Sigma Chemical Co. Baculovirus-derived human IL-6 was kindly provided by Dr. P. B. Sehgal, Rockefeller University, New York. [^{32}P]dCTP was purchased from Amersham Corp. (Arlington Heights, IL), [^{32}P]CTP and [^{32}P]UTP were purchased from DuPont-New England Nuclear (Boston MA), and [^{32}P]ATP was purchased from ICN Radiochemicals (Costa Mesa, CA). DNA probes included a hepatocyte α_1 AT cDNA probe (25) inserted into the pGEM4z plasmid (Promega Biotec), a macrophage α_1 AT cDNA L17.3 (22), a 2.2 kb EcoRI-EcoRI α_1 AT genomic clone, pEE3, used to determine the sequence around the macrophage α_1 AT cap site (22), an albumin cDNA clone (26) provided by Dr. D. Kurnit, Harvard Medical School, Boston, MA, and a polyubiquitin cDNA clone (27) provided by Dr. M. Schlesinger, Washington University School of Medicine, St. Louis, MO. Human intestinal RNA was provided by Dr. D. H. Alpers, and Dr. B. Wice, Washington University School of Medicine, St. Louis, MO.

Cell culture. Primary cultures of human peripheral blood monocytes were established according to previously described methods (11). Maintenance of HepG2 and Caco2 cells has also been previously described (8, 15).

RNA blot analysis. Total cellular RNA was isolated from cell lines and surgical specimens by guanidine isothiocyanate extraction and ethanol precipitation (28). RNA was then subjected to agarose-formaldehyde electrophoresis and transferred to nitrocellulose filters (29) for hybridization with ^{32}P-labeled DNA probes.

Ribonuclease protection assays. Specific DNA probes were subcloned into the pGEM4z plasmid and a previously described protocol used (30). After linearization ^{32}P-labeled RNA probes complementary to sense strands were synthesized using linearized plasmid (5 μg), [^{32}P]-CTP (50 μCi) and T7 or SP6 RNA polymerase. From this reaction mixture, 1/1,000 of the final product was allowed to hybridize overnight to 10 μg total cellular RNA in 50% formamide at 55°C. RNAse digestion was performed at room temperature with RNAse A and T1 and the protected fragments separated on 6% polyacrylamide gels. The size of each protected fragment was determined by its migration relative to a dideoxy sequencing reaction.

Primer extension analysis. Oligonucleotides were end-labeled with [^{32}P]ATP, allowed to hybridize overnight with 20 μg total cellular RNA and subjected to primer extension as previously described (31). The exon IA oligo was complementary to the sequence of the DNA region around the macrophage specific α_1 AT cap site (22) from base +110 to +140 (5-GGAGGTTGCCGCCCCTCCAACCTGGAATTC-3′). The exon IB oligo was complementary to the sequence of the macrophage α_1 AT cDNA (22) from base +230 to +260 (5′-ATTTACTGAGT-CACCCCAAAATGCCTGATG-3′). The exon IC oligo was complementary to the sequence of hepatocyte α_1 AT cDNA (25) from base +28 to +58 (5′-AGGCAGCAGCACAGGCCTGCCAGCAGGAG-GATG-3′). The length of each primer extension product was determined by its migration relative to a dideoxy sequencing reaction on 6% polyacrylamide gels.

Isolation of nuclei and transcriptional elongation assays. Caco2 cells were scraped into phosphate-buffered saline with a rubber policeman and then pelleted by centrifugation. Cells were homogenized in a loosely fitting Dounce homogenizer in chilled 0.32 M sucrose, 3 mM $MgCl_2$, 1 mM Hepes (pH 6.8) at a concentration of ~ 1 g of cells/ml. This homogenate was subjected to sucrose density gradient centrifugation as previously described (32). For transcriptional elongation assays, the nuclei were thawed and resuspended in 200 μl 18% glycerol, 10 mM Tris (pH 7.6), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1% beta-mercaptoethanol, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM GTP and 1 mCi/ml [^{32}P]-UTP. After a 15 min incubation at 37°C, nuclei were digested with RNAse-free DNAse I for 5 min at 37°C. Nuclear RNA was then isolated exactly as described above. Radiolabeled nuclear RNA was hybridized at 37°C for 72 h with DNA blots. DNA blots were prepared by subjecting appropriately digested plasmid DNA constructs to nondenaturing agarose gel electrophoresis. Gels were subjected to alkali denaturation and neutralization and then the DNA was transferred to nitrocellulose filters. Filters were prehybridized at 37°C for 24 h. After hybridization the filters were washed in 2× SSC/0.1% SDS at room temperature for 10 min and then at 56°C for 10 min. Results were quantified by densitometric scanning of autoradiograms on a laser densitometer (2222 Ultrascan XL; LKB Instruments, Inc., Houston, TX).

Results

Different transcriptional initiation sites for α_1 AT mRNA in HepG2 cells, Caco2 cells and mononuclear phagocytes. Total cellular RNA was isolated from control and LPS-activated monocytes, undifferentiated and differentiated Caco2 cells, control and IL-6–activated HepG2 cells and subjected to RNAse protection assays with three different DNA probes (Fig. 1). Probe A is a 299–nucleotide SmaI–EcoRI fragment from the genomic DNA clone pEE3 and spans the previously described macrophage-specific transcriptional initiation site. Probe B is a 192 nucleotide EcoRI–PstI fragment from cDNA clone L17.3 and spans the 5′-terminus of exon IB. Probe C is a

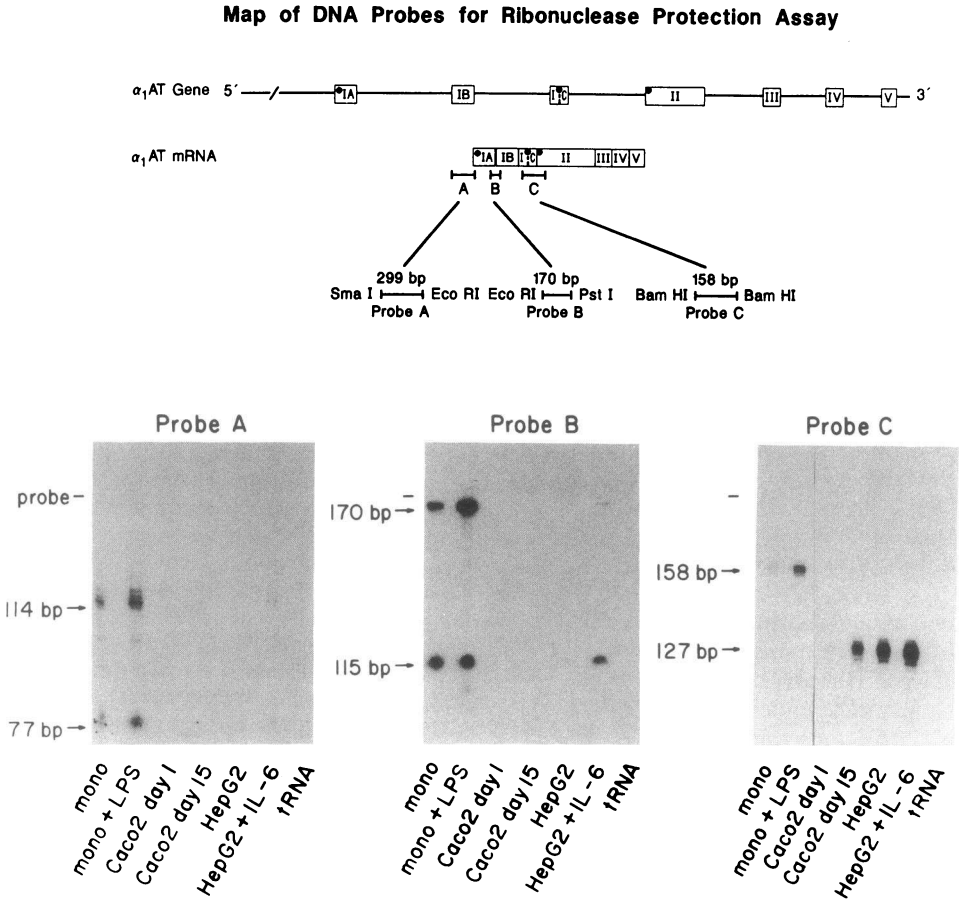

Figure 1. Ribonuclease protection assays to map transcriptional initiation sites for α_1 AT gene expression in different cell types. After 24 h in culture peripheral blood monocytes were incubated for 24 h in serum-free control medium or medium supplemented with LPS (100 ng/ml) and then total cellular RNA was isolated. HepG2 cells were incubated for 24 h in serum-free control medium or medium supplemented with recombinant human IL-6 (100 ng/ml) and then total cellular RNA was isolated. Caco2 cells were subcultured into 30-mm tissue culture wells bearing collagen-coated nitrocellulose filters (Costar Transwell, Cambridge, MA). Total cellular RNA was isolated when the monolayers reached confluence (d 1) or 15 d after reaching confluence (d 15). In each case 10 μg of total cellular RNA was used to hybridize to the appropriate ^{32}P-labelled antisense cRNA probe and ribonuclease protection assays followed the protocol described in Methods. Probe A is a 299-base pair SmaI-EcoRI fragment from a genomic clone pEE3 used to provide the sequence of the DNA region around the macrophage-specific α_1 AT cap site (22). It also has 39 bases of vector sequence. Probe B is a 170-base pair EcoRI-PstI fragment from the 5′-terminus of macrophage cDNA clone L17.3 (22). It also has 24 bases of vector sequence. Probe C is a 158-base pair BamHI–BamHI fragment derived from L17.3 and spanning the hepatocyte-specific α_1 AT cap site. It also has 60 bases of vector sequence. A map of the probes is shown at the top and the relative electrophoretic migration of the probe, which was different for each of the three autoradiograms, is shown at the left margin. The size of each protected fragment, as determined by its migration relative to a sequencing ladder, is indicated by arrows at the left margin of each panel. The autoradiogram with probe C was exposed to its gel for only 2 h as compared to 6 h for those with probes A and B.

158-nucleotide BamHI-BamHI fragment from cDNA clone L17.3 and spans the previously described hepatocyte-specific transcriptional initiation site (22).

Probe A protects two fragments in monocytes and therefore indicates that there are two transcriptional initiation sites with exon IA. This confirms our previous results (22). There is a modest increase in the levels of these fragments induced by LPS. The same two fragments were also identified in control and LPS-activated human bronchoalveolar macrophages (data not shown). These fragments are specific as shown by their absence in tRNA, undifferentiated (day 1) and differentiated (day 15) Caco2 cells and control HepG2 cells. These two fragments are present at low levels in HepG2 cells that have been incubated with IL-6. This is more easily seen on longer autoradiographic exposure of this gel as well as in gels from four other experiments (data not shown).

Probe B also protects two fragments in monocytes. The larger fragment is fully protected, and therefore represents the two transcripts that have started upstream from exon IA. The smaller fragment must therefore represent a unique transcriptional initiation site for α_1 AT mRNA upstream from exon IB. Based on these studies and primer extension analysis (see below) we have mapped this initiation site to within several nucleotides of position −1892 relative to the hepatocyte transcriptional initiation site (according to the sequence of Long et al. [33]). There is an increase in the level of each of these fragments during modulation by LPS. These fragments are not detected in undifferentiated or differentiated Caco2 cells and are not detected in control HepG2 cells but are induced in HepG2 cells treated with IL-6.

Probe C, which overlaps the so-called hepatocyte transcription initiation site in exon IC, protects one fragment in monocytes. This fragment is fully protected, and therefore, represents the transcripts which have started upstream in exons IA and IB. There is an increase in the level of this fragment induced by LPS. A smaller fragment is present in Caco2 cells and HepG2 cells. This, therefore, represents use of the hepatocyte-specific transcriptional initiation site within exon IC. There is a marked increase in the level of this fragment during differentiation of Caco2 cells. There is a lesser increase in the level of this fragment in IL-6–treated HepG2 cells. These results indicate that there are three macrophage-specific transcriptional initiation sites and one hepatocyte-specific initiation site. There is an increase in the use of the cell-specific transcriptional initiation

sites for each cell type, monocytes and hepatocytes, during modulated expression. There is also a switch to induce transcription from the upstream macrophage-specific initiation sites in HepG2 cells activated by IL-6. Caco2 cells use the hepatocyte transcriptional initiation site excusively for basal expression as well as during the marked increase in expression that accompanies their differentiation.

We next examined the effect of a single mediator, IL-6, on α_1 AT mRNA transcriptional initiation sites in the three different cell types (Fig. 2). For this analysis we used probes B and C in ribonuclease protection assays. The results indicate that probe B protects the same two fragments in monocytes, HepG2 cells and differentiated Caco2 cells after modulation by IL-6. Probe C protects one large fragment in monocytes and a smaller fragment in HepG2 cells and differentiated Caco2 cells after modulation by IL-6. These results indicate that mononuclear phagocytes use only the upstream transcriptional initiation sites during basal expression or expression modulated by LPS (Fig. 1) or IL-6 (Fig. 2). Hepatocytes and enterocytes use the downstream transcriptional initiation site during basal and modulated expression but also switch on transcription at lower levels from the upstream initiation sites during expression modulated by IL-6.

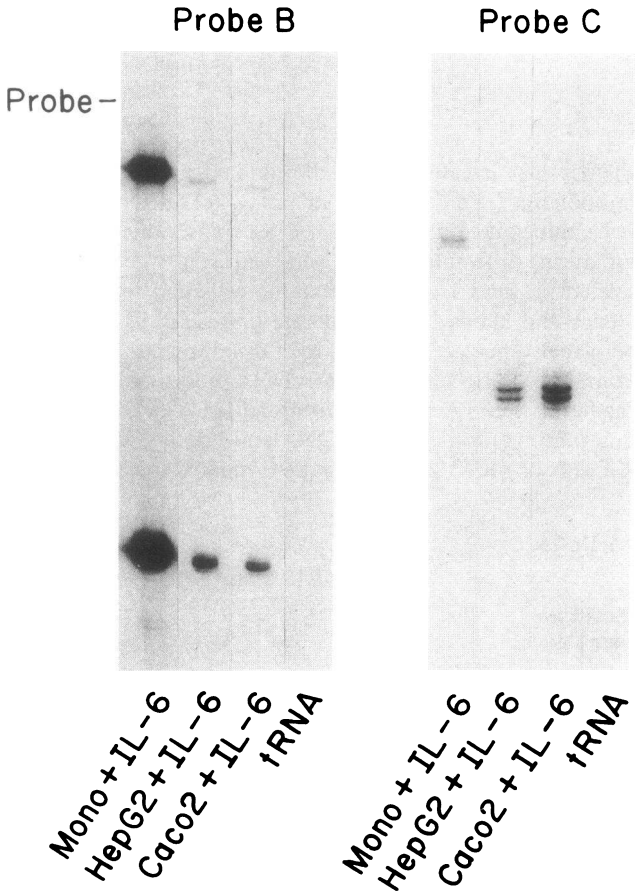

Figure 2. Mapping of transcriptional initiation sites for α_1 AT gene expression in different cell types after IL-6 activation. After 24 h in culture peripheral blood monocytes were incubated for 24 h in serum-free control medium or medium supplemented with IL-6 (100 ng/ml). HepG2 cells and Caco2 cells, the latter after 15 d of postconfluence, were also incubated for 24 h in serum-free control medium or medium supplemented with IL-6 (100 ng/ml). In each case total cellular RNA was then isolated and subjected to ribonuclease protection assay with probes B and C exactly as described in the legend to Fig. 1. tRNA was used as a negative control. The relative electrophoretic migration of each probe is indicated at the left margins.

Our interpretation of these results was consistent with results of previous primer extension analysis (22) as well as additional primer extension studies performed here: two primer extension products were detected in monocytes with the exon IA oligo; three primer extension products were detected in monocytes with the exon IB oligo; and one primer extension product was detected in hepatocytes and enterocytes by the exon IC oligo (data not shown). Our interpretation of these results was also confirmed by results of RNA blot analysis (Fig. 3). Using probe A, which spans the macrophage-specific transcriptional start sites within exon IA a single ~ 2.0 kb RNA is detected in monocytes but not in Caco2 cells or HepG2 cells. There is a modest increase in the level of this mRNA in monocytes incubated with LPS and the magnitude of this increase is similar to that detected by ribonuclease protection assay. Using probe D, a macrophage cDNA probe (22) which extends from the 3′ terminus of probe A into the 3′ untranslated region of the α_1 AT mRNA, a 1.6 kb mRNA is detected in Caco2 cells and HepG2 cells. There is a modest increase in the level of this mRNA in HepG2 cells incubated with IL-6. Two mRNAs that are larger in apparent molecular mass, 1.8 and 2.0 kb, are detected in LPS-treated monocytes. Both of these mRNAs are also seen in control monocytes with longer autoradiographic exposure times (data not shown). Using probe E, a hepatocyte cDNA probe (25), which extends from the hepatocyte transcription initiation site to the 3′-terminus of the α_1 AT coding sequence, the results are almost identical to those of probe D. A 1.6 kb mRNA is detected in Caco2 cells and HepG2 cells. There is a marked increase in the level of this mRNA in Caco2 cells between days 1 and 15 in culture (data not shown, see reference 15), and a modest increase in IL-6-treated HepG2 cells. Two mRNAs of 1.8 and 2.0 kb are detected in monocytes. There is an increase in the level of these mRNAs in LPS-activated monocytes.

Taken together the results of ribonuclease protection assays, primer extension analysis and RNA blot analysis are schematically summarized in Fig. 4. Start of transcription within exon IC results in a 1.6 kb mRNA, called α_1 AT mRNA 1. This mRNA species is found in Caco2 cells and HepG2 cells during basal and modulated expression but is not found in monocytes. Start of transcription within exon IA results in two mRNA species called α_1 AT mRNAs 2 and 3. Based on ribonuclease protection assays these two mRNA species differ by ~ 40 bp at the extreme 5′ terminus although they are not resolved by RNA blot analysis; i.e., there is one broad band with an apparent molecular mass of 2.0 kb in Fig. 3. Finally, start of transcription within exon IB results in a 1.8-kb α_1 AT mRNA 4. α_1 AT mRNAs 2, 3, and 4 are found in monocytes and their levels increase in LPS- and IL-6–activated monocytes. These three mRNA species are also induced in HepG2 cells and differentiated Caco2 cells by IL-6. The level of these three mRNA species in IL-6–activated HepG2 cells and IL-6–activated Caco2 cells just reaches the limits of sensitivity of ribonuclease protection assays (Figs. 1 and 2) but does not reach the limits of sensitivity of RNA blot analysis (Fig. 3).

The increase in α_1 AT gene expression during differentiation of Caco2 cells involves a transcriptional mechanism and is reflected by the tissue distribution of α_1 AT mRNA. In a pre-

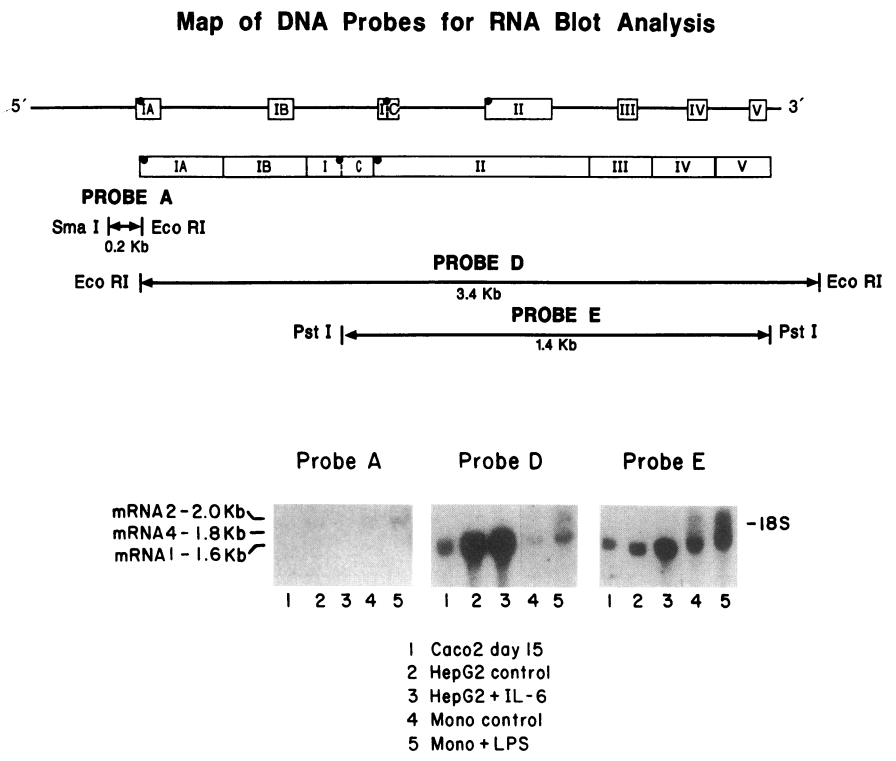

Figure 3. RNA blot analysis to determine the relative electrophoretic migration of α_1 AT mRNA in different cell types. Total cellular RNA was isolated exactly as described in the legend for Fig. 1 and 15 μgs was subjected to RNA blot analysis as described in Methods. The relative electrophoretic migration of ethidium bromide-stained 18S ribosomal RNA is indicated at the right margin and that of α_1 AT mRNA species at the left margin. The relative amounts of α_1 AT mRNA in monocytes cannot be compared to that of Caco2 cells or HepG2 cells since longer autoradiographic exposure was necessary for the blot with probe A and for lanes 4 and 5 with probes D and E. A map of the probes is shown at the top of the figure.

vious study we have shown that there is an increase in steady state levels of α_1 AT mRNA in Caco2 cells during the time interval from day 1 to day 16 after the cells have become confluent, a time interval that is associated with differentiation from crypt-like to villous-like enterocytes by both morphological and biochemical criteria (15). Ribonuclease protection assays in the current study have confirmed these previous results and have demonstrated that the hepatocyte-specific transcriptional initiation site is used for basal expression as well as for the marked increase in expression of the α_1 AT gene during differentiation of Caco2 cells (Fig. 1). In order to determine whether this increase was caused by an increase in rate of transcription and/or a decrease in rate of α_1 AT mRNA turnover we examined the rate of α_1 AT mRNA transcriptional elongation during differentiation of Caco2 cells (Fig. 5). Nuclei were isolated on days 1 and 12, after the cells had reached confluence and allowed to incorporate [^{32}P]UTP. The resulting radiolabeled nuclear RNA was hybridized to an α_1 AT cDNA probe as well as to several control cDNA probes on DNA blots. The results demonstrate hybridization to α_1 AT cDNA but not to negative control albumin cDNA and not to either plasmid pGEM4z or pKT218. There is also a marked increase in spe-

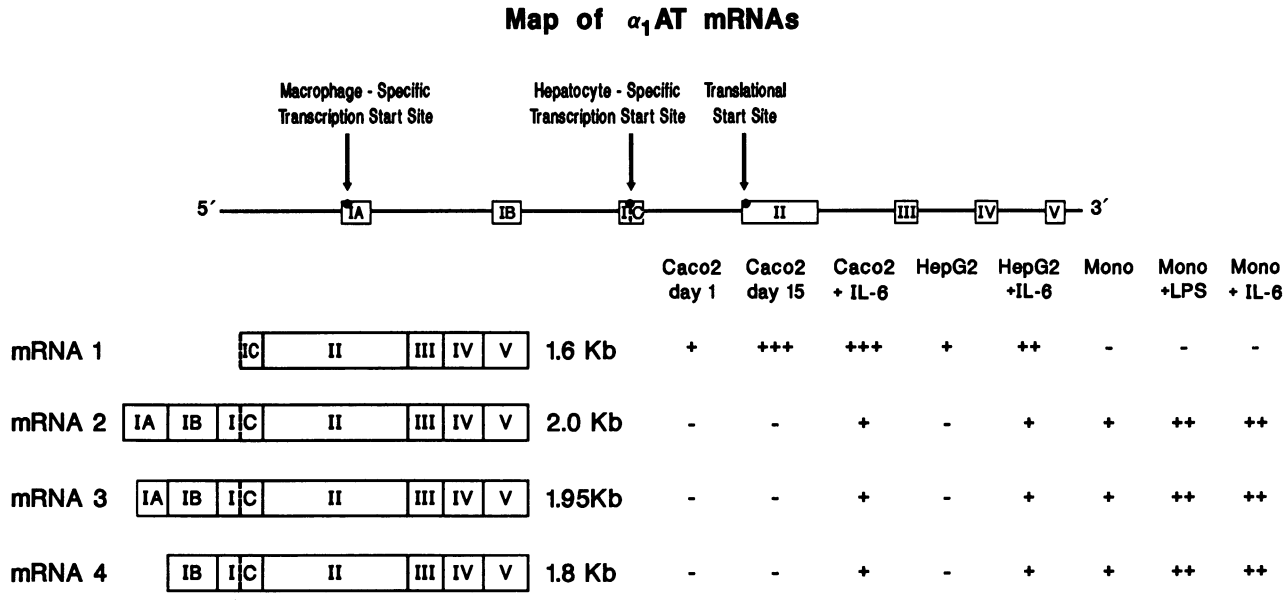

Figure 4. Schematic representation of α_1 AT mRNAs. See text for description.

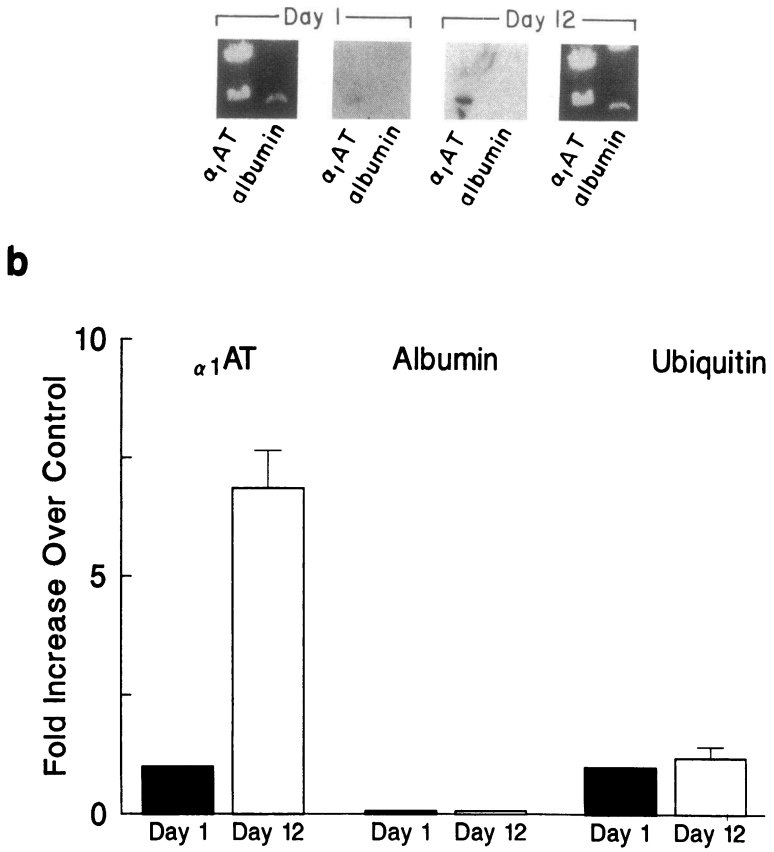

Figure 5. Transcriptional elongation assays to determine the basis for increased α_1 AT mRNA levels during differentiation of Caco2 cells. (*a*) Nuclei were isolated from Caco2 cells on the day the cells reached confluence (*day 1*) and 12 d after reaching confluence (*day 12*). Transcriptional run-on assays were carried out as described in Methods. Radiolabeled nuclear RNA was hybridized to DNA blots as described in Methods. DNA blots were prepared by subjecting PstI digests of α_1 AT cDNA (4 μg), albumin cDNA (4 μg) and polyubiquitin cDNA [4 μg (data not shown)] to nondenaturing gel electrophoresis. Ethiduim bromide staining of each gel, shown in the outside panels, demonstrates ~ 3.2 kb plasmid and ~ 1.4 kb insert for α_1 AT and ~ 4.0 kb plasmid and ~ 1.2 kb insert for albumin. DNA from these gels was denatured in 1 N NaOH, neutralized, transferred to nitrocellulose filters and the resulting filters used for hybridization. (*b*) Results of densitometric scanning of autoradiograms from three separate blots for each probe. Results are reported as fold increase as compared to the value for day 1, which was arbitrarily designated 1.0 U. Each result is reported in mean ±1.0 SD. There was no dectectable albumin signal in any of the blots.

cific α_1 AT mRNA transcriptional elongation by day 12 in culture (Fig. 5 *a*). Results of three separate experiments indicate that there is a 6.7-±1.3-fold increase in α_1 AT mRNA transcription and that the increase is specific, in that there is no change in a control, ubiquitin mRNA transcription (Fig. 5 *b*). Thus the increase in α_1 AT mRNA levels during differentiation of Caco2 cells is due, at least in large part, to an increase in α_1 AT gene transcription.

Finally, we examined the possibility that this increase in α_1 AT mRNA transcription during differentiation of Caco2 cells into villous-like enterocytes was reflected by the distribution of α_1 AT gene expression within human intestine. Total cellular RNA from human jejunum and colon was subjected to ribonuclease protection assays (Fig. 6) with probe C from Fig. 1. Probe C protects a large fragment in monocytes. This fragment is almost the same length as the probe and therefore represents transcript, or transcripts, which have started upstream in exons IA and IB (see also Figs. 1 and 2). Probe C protects a smaller fragment, therefore representing use of the hepatocyte transcriptional initiation site, in Caco2 cells and jejunum but does not protect any fragment in the colon. These data provide additional evidence that there is transcription of the α_1 AT gene in the intestinal epithelium, that the same promoter is used for transcription of the α_1 AT gene in human adult jejunal epithelium as is used in a human intestinal epithelial cell line, and that the tissue distribution of α_1 AT transcripts corresponds to an increase in the rate of α_1 AT mRNA transcriptional elongation during differentiation from crypt-like to villous-like enterocytes.

Discussion

These data provide further evidence for differences in the mechanisms responsible for basal expression of the α_1 AT gene in different cell types and show that there are also differences in the mechanisms responsible for upregulation of α_1 AT gene expression in these cells. Blood monocytes and tissue macrophages use three unique upstream transcriptional initiation sites for basal expression of this gene. These same cell type-specific transcriptional initiation sites are used for increased expression of the α_1 AT gene during activation by LPS or IL-6. Hepatoma-derived hepatocytes use one downstream transcriptional initiation site for basal expression and for the upregulated expression of α_1 AT mediated by IL-6. However, under the influence of IL-6, hepatocytes also initiate α_1 AT mRNA transcripts from the upstream macrophage-specific initiation sites. Results of this study also provide further evidence that Caco2-derived enterocytes express the α_1 AT gene and initiate α_1 AT mRNA transcripts from the downstream hepatocyte-specific initiation site.

Several previous studies have shown that alternative upstream promoters were used for α_1 AT transcription in macrophages (13, 22). Perlino et al. also noted two short upstream open reading frames with initiation and termination codons

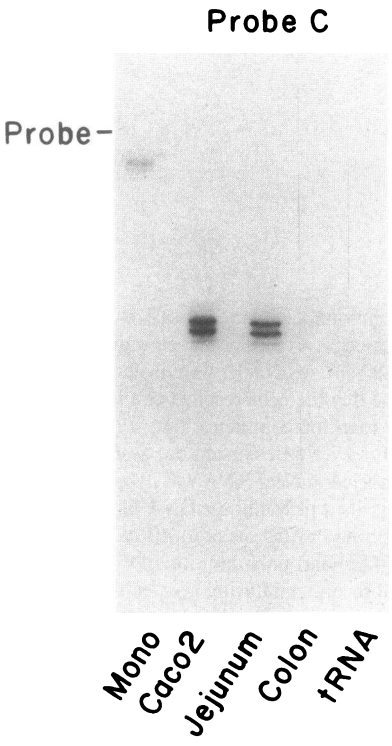

Figure 6. Ribonuclease protection assays to determine the distribution of α_1 AT mRNA transcripts in human intestine. Total cellular RNA (10 μgs) from LPS-activated monocytes, Caco2 cells after 15 d in culture, human jejunum, human colon and tRNA was subjected to ribonuclease protection assay with probe C exactly as described in the legend to Fig. 1.

that are presumably included in macrophage α_1 AT transcripts. Several eukaryotic genes have short sequences with open-reading frames, but only a few have multiple short upstream open-reading frames. In two cases, the yeast GCN4 gene (34) and a cytomegalovirus β gene (35), such sequences have been shown to govern translation. GCN4 encodes a nuclear transcription factor necessary for the expression of amino acid biosynthetic enzymes in response to amino acid starvation. Removal of the initiation codons of four short open-reading frames upstream from the GCN4 gene by deletions or point mutations leads to constitutive derepression of GCN4 by allowing translation (34, 36). It is now thought that these structures can repress GCN4 expression under nonstarvation conditions by acting as initiation sites during ribosomal scanning, allowing for only inefficient reinitiation at the downstream internal initiation codon. During amino acid starvation, two *trans*acting nuclear proteins, GCD1 and GCN2, interfere with the interaction of the ribosomes and the upstream initiation sites, allowing for more efficient initiation at the "true" internal initiation codon. Our current studies were, in part, stimulated by the analogy between GCN4 and α_1 AT with respect to these upstream structures but also by the fact that the increase in α_1 AT gene expression in macrophages mediated by the prototype macrophage inflammatory activator LPS involves a marked increase in the efficiency of translation of α_1 AT mRNA (37): LPS mediates a marked increase in synthesis of α_1 AT (5- to 10-fold) in blood monocytes and bronchoalveolar macrophages but much less impressive increase in steady state levels of α_1 AT mRNA (1.5- to 2.5-fold); moreover, there is a 4- to 5-fold increase in cell-free synthesis of α_1 AT directed by RNA from LPS-activated monocytes as compared to that from control monocytes. With the regulation of GCN4 during amino acid starvation in mind, we therefore predicted that the translational effect of LPS would be associated with an increase in α_1 AT mRNA transcripts initiated at the downstream hepatocyte-specific initiation site. A direct examination of this prediction in the current study shows that there is absolutely no initiation at the hepatocyte α_1 AT promoter in LPS-activated monocytes. There are several possible alternative explanations for the translational effect of LPS on α_1 AT gene expression. For example, it is possible that a "slowing" of ribosomal scanning in some cases, as might occur during initiation and termination at upstream codons, has an enhancing effect on recognition of the downstream initiation codon and thereby facilitates translation (38, 39). Specific RNA-protein interactions and changes in RNA folding are alternative possible explanations for the translational effect of LPS on α_1 AT gene expression. In fact, formation of stem loop structures in the upstream flanking region is now thought to be an important prerequisite for translational regulation of the transferrin receptor gene in response to iron (40, 41) and that of the ornithine decarboxylase gene in response to hormones (42). We have subjected the macrophage α_1 AT mRNAs to analysis by a computer program for folding of RNA molecules (43). This analysis shows that the macrophage α_1 AT mRNAs form very stable secondary structures with at least four stem-loop configurations but does not provide an obvious explanation for the translational effect of LPS on macrophage α_1 AT gene expression.

In this study we also mapped the transcriptional initiation sites for basal and modulated expression of the α_1 AT gene in human hepatoma cells. We were particularly interested in the effect of IL-6 which is known to mediate an $\sim$ 2.5-to 3.5-fold increase in α_1 AT mRNA levels and in synthesis of α_1 AT (6, 8). In this case there was a modest increase in the levels of α_1 AT mRNA transcripts initiated at the cell type-specific initiation site but also an induction of transcripts that originated at the upstream initiation sites. It is unlikely that these longer transcripts decrease the overall translation of α_1 AT mRNA in hepatocytes because they represent a relatively minor fraction of the net α_1 AT transcripts in the cell and because results of previous experiments have suggested that the increase in α_1 AT mRNA levels, detected as the 1.6 kb mRNA species on RNA blot analysis, roughly corresponds in magnitude to the increase in synthesis of α_1 AT mediated by IL-6 (8). It has recently become recognized that the effect of IL-6 on the other acute phase genes in HepG2 cells is dependent on the binding of a transcription factor, IL-6DBP, also called H-APF-2 and NF-IL-6, to a consensus sequence AGTGANGNAA (21, 23, 44, 45). There is no perfect match in the upstream untranslated region of the α_1 AT gene for this consensus sequence but several similar sequences AGTGGAA at nucleotides -195 to -189 and AGTGAG at nucleotides -169 to -164 from the hepatocyte α_1 AT cap site (according to the sequence of Long et al. [33]) may account for the relatively modest increase in transcription initiated at the hepatocyte promoter in response to IL-6. Another sequence AGAGCTGAAA at nucleotides -178 to -169 from the macrophage α_1 AT cap site (22) may account for induction of transcripts at the upstream macrophage-specific initiation sites in response to IL-6.

Because our previous data had indicated that IL-6 has the same effect on synthesis of α_1 AT in mononuclear phagocytes as in HepG2 cells (8), it was also of great interest to us that ribonuclease protection assays demonstrated use of the same macrophage-specific transcriptional initiation sites in IL-6–activated monocytes as in LPS-activated and control monocytes.

These data suggest that there is greater restriction on promoter usage in mononuclear phagocytes than in hepatocytes although it is still possible that other, as yet unstudied, mediators can induce transcription from the downstream hepatocyte promoter in mononuclear phagocytes.

Results of the current experiments also extend our previous observations about expression of the α_1 AT gene in enterocytes. In a human intestinal epithelial cell line, Caco2, the hepatocyte α_1 AT promoter is used for basal expression. There is a marked increase in α_1 AT mRNA levels and a corresponding increase in synthesis and secretion of α_1 AT during differentiation of this cell line into a villous-like enterocyte (15). This increase is, at least in large part, explained by an increase in the rate of transcription and resulting transcripts originate exclusively from the hepatocyte promoter. The distribution of these α_1 AT transcripts in human jejunum but not in human colon may reflect the effect on this gene of differentiation towards the villous cell lineage as contrasted to the crypt cell lineage. This differentiation-dependent increase in Caco2 cell α_1 AT gene expression could also represent a recapitulation of late fetal development as has been suggested for the expression of α-fetoprotein in this cell line (46). Finally expression of α_1 AT in differentiated Caco2 cells may be further modulated by soluble mediators such as IL-6. In this case there is an increase in transcription from the downstream hepatocyte promoter but IL-6–activated Caco2 cells also induce transcripts originating from the upstream initiation sites in a manner similar to that of IL-6–activated HepG2 cells.

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