3794–3799 *Nucleic Acids Research, 1998, Vol. 26, No. 16*

Divergent expression of α_1-protease inhibitor genes in mouse and human

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Received April 6, 1998; Revised and Accepted June 17, 1998

DDBJ/EMBL/GenBank accession nos+

ABSTRACT

The α_1-protease inhibitor proteins of laboratory mice are homologous in sequence and function to human α_1-antitrypsin and are encoded by a highly conserved multigene family comprised of five members. In humans, the inhibitor is expressed in liver and in macrophages and decreased expression or inhibitory activity is associated with a deficiency syndrome which can result in emphysema and liver disease in affected individuals. It has been proposed that macrophage expression may be an important component of the function of human α_1-antitrypsin. Clearly, it is desirable to develop a mouse model of this deficiency syndrome, however, efforts to do this have been largely unsuccessful. In this paper, we report that aside from the issues of potentially redundant gene function, the mouse may not be a suitable animal for such studies, because there is no significant expression of murine α_1-protease inhibitor in the macrophages of mice. This difference between the species appears to result from an absence of a functional macrophage-specific promoter in mice.

INTRODUCTION

The α_1-protease inhibitor (α_1-PI) genes of mice comprise a five-member multigene family. The human analog, α_1-antitrypsin (α_1-AT), is a major plasma protein whose primary physiological target is neutrophil elastase. Reduced plasma levels of α_1-AT due to homozygous deletion or point mutation leads to a severe form of early-onset emphysema in affected adults (1–7). Whether α_1-AT deficiency emphysema can be considered a paradigm for the development of emphysema in 'normal' adults, however, remains an important unanswered question. In order to explore the feasibility of establishing a mouse model of human α_1-AT deficiency syndrome, we have undertaken a detailed study of the function and expression of α_1-PI inhibitor in murine macrophages. Although both human and mouse inhibitors are predominantly expressed in liver and are present at high serum concentrations, human alveolar macrophage-derived α_1-AT may significantly contribute to the inhibition of elastase within lung parenchymal tissue (8–10).

It has been shown that human cells use a dual promoter mechanism to express α_1-AT in a tissue-specific manner in both hepatocytes and macrophages, albeit at a 70-fold lower level in macrophages (11). We have previously conducted an analysis of the mouse promoter in hepatic and non-hepatic cells (12). Based on analysis of transfected reporter constructs, expression of the α_1-PI genes is under complex control by several well-known liver-specific and general transcriptional factors. However, in both cultured cells and in over 31 independent transgenic constructs, including as much as 10 kb of DNA sequence upstream of the transcriptional start site, we were unable to obtain high levels of gene transcription (T.Harris, PhD thesis). We speculate that the genes in the mouse contain promoter proximal as well as distal locus controlling elements required for high level expression (13).

The studies presented in this report show that not only do murine macrophages express α_1-PI at extremely low levels (~10^{-5} the level in liver), but that mice do not utilize a macrophage-specific promoter mechanism. The low level of expression combined with the absence of a macrophage-specific promoter may preclude an appreciable physiological role for expression of α_1-PI in murine macrophages. Mice appear to have evolved a different mechanism for elastase inhibition and as such may not provide an optimal animal model system for the human deficiency syndrome.

MATERIALS AND METHODS

Cell culture and probes

Mouse macrophage cell line J774 was obtained from Dr Barry Bloom (Albert Einstein College, New York, NY) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 10% NCTC-109, penicillin (50 U/ml), streptomycin (50 U/ml) and 1% non-essential amino acids. Conditioned medium was a gift of Dr Gretchen Darlington and was prepared from medium used to culture EJ bladder carcinoma cells grown for 48 h, which consisted of conditioned medium from HepATP-SK cells which included 3 ng/ml phorbol myristate acetate (PMA) and 10 µg/ml lipopolysaccharide (LPS). The J774 medium is thought to contain interleukin (IL)-1, PMA, LPS and IL-6. Mouse peritoneal macrophages were isolated from ascites fluid after injection of thioglycolate (1 ml i.p.; Difco). Full-length α_1-PI-I (2) and ribosomal protein L30 (4,16) are previously described cDNA clones. pATΔNco5 was derived from a genomic clone which included α_1-PI-I and corresponds to a 650 bp restriction fragment which spans exon 1 (2,12).

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RNA detection

Northern analysis and RNase protection assays were performed as previously described (12,22). Quantitative PCR reactions were performed essentially as described by Gilliland *et al*. (18). cDNA from macrophage or liver mRNA was prepared using AMV reverse transcriptase (Life Sciences, FL). Briefly, 5–10 µg total RNA was heated to 65°C for 5 min in H_2O. RNA was added to a cDNA/PCR reaction mixture which contained 50 mM KCl, 20 mM Tris–HCl, pH 8.4, 2.5 mM $MgCl_2$, 1 mM each dGTP, dCTP, dATP and TTP, 1 U/µl RNasin (Promega, Madison, WI), 100 pmol random hexamer primers and 40–50 U AMV reverse transcriptase. Fixed amounts of cDNA products from this reaction were mixed with increasing quantities of a genomic DNA fragment of α_1-PI-I which included exon 4, intron 4 and exon 5. The fragment mixture was subjected to PCR using 20 pmol each primers corresponding to highly conserved oligonucleotides in exons 4 and 5 (forward primer GGAATCAACAGAAGGAGA-ATGCT, reverse primer GTAAGCTTCATTTATGTGTGGGAT-CTAC) and including 50 µCi [^{32}P]dCTP in cDNA/PCR reaction buffer. Cycle times were 94°C for 60 s, 55°C for 90 s and 72°C for 150 s for 30 cycles. Samples were subjected to gel electrophoresis on 1.5% low melting point agarose gels and bands corresponding to genomic DNA standard and cDNA sample excised and radioactivity determined by scintillation counting. The ratio c.p.m. control:c.p.m. sample was normalized for the size of the PCR product and then plotted. Where the ratio was 1, the concentrations of sample and control are equal.

Anchored PCR

Anchored PCR was performed as previously described (7,14). cDNA was synthesized using an oligonucleotide primer corresponding to sequences within the exon 2 protein encoding region (GTTTGTAGCAATCTC). cDNA was separated from free primer by precipitation with 10 mM spermine, washed with 70% ethanol, resuspended in terminal deoxynucletidyl transferase (TDT) buffer (2 mM $CoCl_2$, 1 mM GTP, 0.5 U/ml TDT) and incubated at 37°C for 60 min. G-Tailed cDNA was precipitated by addition of 2 M NH_4COOH and 2 vol ethanol. cDNA was used as template in a PCR reaction with 20 pmol nested exon 2 amplimer (GGCCTGCCA-GAAGCAGTAGA), 3 pmol 'anchor-C' amplimer (GCGGCCGC-ATGCGAATTCTGCAGCCCCCCCCCCCC) and 24 pmol anchor amplimer (GCGGCCGCATGCGAATTCTGCAGC), cDNA/PCR buffer, 2 U Taq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA). The conditions of amplification were 94°C for 60 s, 50 (first 5 cycles) or 55°C for 90 s (remaining 30 cycles) and 72°C for 120 s. Products of the reaction were subjected to gel electrophoresis and bands were excised, the gel fragment melted and subjected to a second round of PCR with 20 pmol anchor amplimer and 20 pmol nested exon 2 oligonucleotide (ATGCTCAGTGGG-GAGAAGATGA). The products were again subjected to gel electrophoresis on 8% non-denaturing polyacrylamide gels, bands eluted, treated with the the Klenow fragment of DNA polymerase I to repair 'ragged' ends and then subcloned into the bi-directional sequencing vector SP72 (Promega). Following transformation into bacteria, colonies were screened by filter hybridization with a probe corresponding to exon 1. Fifty positive colonies were selected and subjected to DNA sequence analysis as previously described (23).

Identification of gene-specific transcripts

Slot blots of PCR products and hybridization of gene-specific oligonucleotides to the blots to identify specific genes were exactly as described previously (2,3).

RESULTS

DNA sequence comparison among α_1-AT genes

The tissue-specific transcription of the single human α_1-AT gene is controlled by two independent promoters. Liver expression initiates at a position ~7 kb upstream of the translational start site, while macrophage transcription starts ~9 kb upstream (8,11, 14,15). Comparison of the mouse and human gene sequences in the region of putative macrophage transcriptional initiation, however, shows little significant homology between the two species. The 5′ upstream regions extending ~4 kb upstream of mouse genomic clones pliv3B and pliv3N (12), which correspond to the α_1-PI-I and α_1-PI-II genes (2,3), were subjected to DNA sequence analysis. Figure 1A shows the results of a DNA sequence comparison between the two mouse genes using a graphic matrix analysis. The degree of identity between these two DNA sequences is >93%. This level of identity in a non-transcribed region is striking and suggests that the duplication event which generated this multigene family from a single primordial gene either occurred quite recently or reflects a gene correction mechanism (2).

Figure 1B shows a similar comparison between murine α_1-PI-I (from –3600 to +40 bp) and human α_1-AT (from –2000 to +90 bp). We have previously shown a high degree of similarity between the human and murine genes in exon 1 to –133 bp upstream (12). This sequence identity is represented by the distinct line in the upper right-hand corner of the matrix. Similarity between the sequences upstream of this region, however, is not apparent. This observation is consistent with previous findings which emphasize the differences in regulatory control between the two species, despite the relatively high degree of similarity in protein coding regions (12). When the human macrophage promoter sequence was directly compared with the mouse sequences between –2100 and –1500 bp, no significant identity was detected (data not shown). The lack of interspecies homology in the region of the macrophage promoter combined with the similarity of DNA sequences near the liver-specific promoter is consistent with an absence of a macrophage promoter in the mouse α_1-PI upstream region.

Levels of expression of α_1-protease inhibitor genes in macrophage

In humans, a 2 kb macrophage-specific α_1-AT transcript can be detected via northern analysis and is easily distinguishable from the 1.7 kb liver mRNA. To determine whether murine macrophage express α_1-PI, total RNA from mouse peritoneal macrophages (thioglycolate stimulated) and the murine macrophage cell line J774 (16,17) was subjected to northern analysis (Fig. 2). RNA was isolated from J774 cells grown in either medium alone [Fig. 2, lane 774(–)] or treated with conditioned medium containing IL-1, IL-2, PMA and LPS [Fig. 2, lane J774(+)]. This was used to determine whether the expression of α_1-PI could be stimulated by addition of inflammatory factors *in vitro*. It is clear that mRNA expression in liver is several orders of magnitude greater than that in macrophage cells (compare the Liver 20 Minute lane with the Liver 19 Day lane in Fig. 2 and note the differences in gel loading). Also, the size of the barely detectable mRNA in macrophages (Fig. 2, Mouse

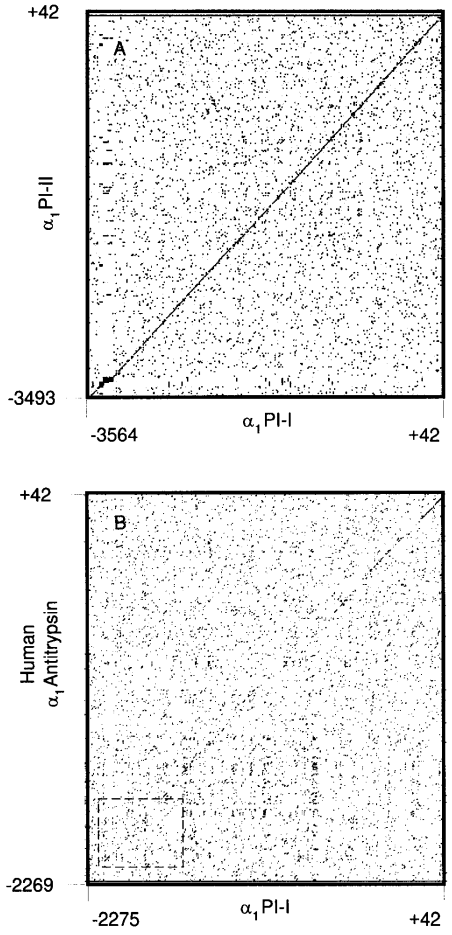

Figure 1. Graphic matrix analysis of protease inhibitor upstream DNA sequences. (**A**) DNA sequences from position –3564 to +42 bp of the murine α_1-PI-I and –3493 to +42 bp of the α_1-PI-II genes, where +1 represents the transcriptional start sites, were subjected to graphic matrix analysis using SIP (24). The >90% identity of DNA sequences over these intervals is shown by the diagonal pattern of dots seen as a line from lower left to upper right. (**B**) Comparison of the regions –2275 to +42 bp of the murine α_1-PI-I gene and –2269 to +42 bp of the human α_1-AT genes, where +1 is the transcriptional start site. The pattern of dots shows the high degree of identity in the region from approximately –500 to +40 of both genes, while upstream of this segment, identity cannot be readily discerned. The dotted box in the lower left of the figure is the location of the human promoter region.

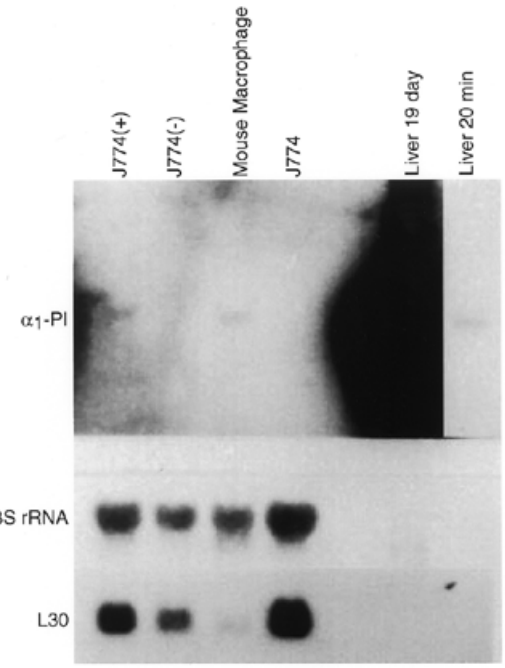

Figure 2. Northern analysis of α_1-PI expression in mouse macrophages. Twenty five micrograms of cytoplasmic RNA from J774 treated with growth medium [J774(+)], 25 µg cytoplasmic RNA from cell line J774 [J774(–)], 25 µg cytoplasmic RNA from mouse intraperitoneal macrophages, 25 µg cytoplasmic RNA from J774 cultures which had been passaged within 24 h of harvesting and 5 µg total cellular mRNA from mouse liver was subjected to denaturing agarose gel electrophoresis, blotted to nylon membrane, then hybridized to a [^{32}P]dCTP-labeled α_1-PI-I cDNA probe. The probe recognizes all five α_1-PI gene products. The position of migration of full-length liver α_1-PI mRNA is shown. Two exposures of 19 days and 20 min are shown for the liver mRNA. The macrophage RNA-containing samples were exposed for 19 days. Loading controls were 28S rRNA and ribosomal protein L30. The rapidly proliferating J774 cells show high levels of L30, while in non-proliferating liver no accumulation of L30 mRNA is seen.

Macrophage) was indistinguishable from the liver transcript. No evidence of a larger α_1-PI mRNA could be detected by northern analysis, RNase protection or RACE PCR (see below).

While it appears that expression of α_1-PI mRNA in J774 is induced by the 'conditioned medium' [compare Fig. 2, lanes J774(+) and J774(–)], it seems likely that this is due to an overall stimulation of mRNA synthesis by the medium rather than a gene-specific stimulation, because a housekeeping gene, ribosomal protein L30, also shows a significant increase in stimulated cells [Fig. 2, lanes J774(+) and J774(–)]. Although equal amounts of total cellular RNA are loaded in each lane, the level of expression of the ribosomal protein encoding mRNA increases in proportion to the level of α_1-PI mRNA. Thus the apparent 'induction' of α_1-PI expression in the enhanced medium probably reflects an overall metabolic stimulation of J774 cells. Correcting for the effects of the intensifying screen, the loading differences and the radioactive decay of the signal over a 19 day exposure, we calculate from the northern hybridization data that the macrophage α_1-PI was expressed at only 2×10^{-5} times the level found in liver.

To confirm and extend the observations obtained by northern analysis, the relative levels of α_1-PI expression in macrophages and liver were examined using the quantitative PCR method described by Gilliland *et al.* (18). With this method, a competition for available specific oligomers between an internal standard and the test α_1-PI mRNA is used to measure the amount of a particular transcript in a given RNA sample. In these experiments, the internal standard was a plasmid construct containing an α_1-PI genomic segment spanning intron 4, including exons 4 and 5. The test cDNA was generated from either liver mRNA or macrophage mRNA using a primer in exon 5. Upon mixing these templates, a single set of amplimers in exons 4 and 5 produces easily distinguishable products (see Fig. 3). Comparison of the ratio of products produced in a set of reactions in which an increasing amount of the standard template is mixed with a fixed amount of cDNA provides a direct measure of the concentration of the cDNA (i.e. when the molar ratio is 1:1 the concentration of the cDNA equals the concentration of the standard). By this method, the molar ratio of α_1-PI mRNA molecules in macrophages compared with liver is 1.5×10^{-5} (Fig. 3). Assuming that there are ~10 000 α_1-PI molecules/cell in liver (19), this suggests that fewer than one in 10 macrophage cells express this

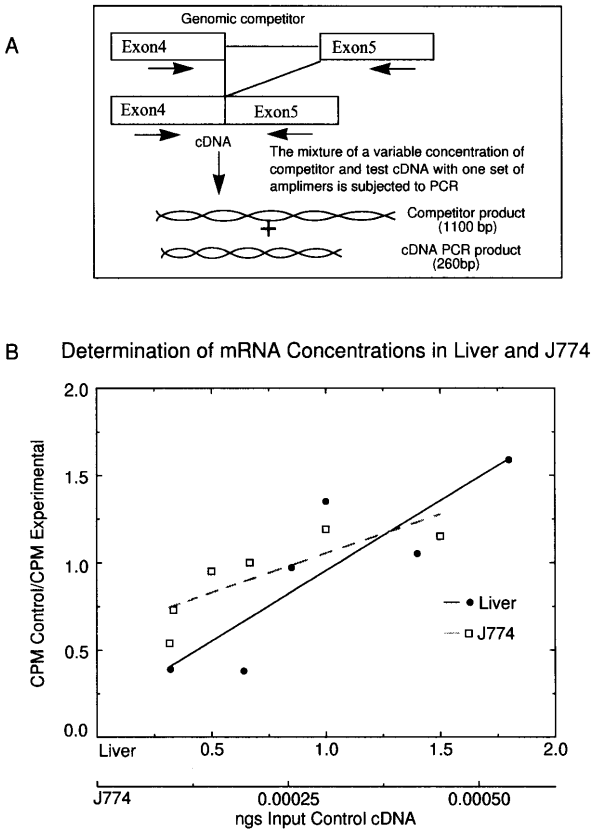

Figure 3. Quantitative PCR analysis of α_1-PI expression. Competitive RT–PCR was used to quantitate levels of expression of α_1-PI mRNA expression in liver and in J774 cells. (**A**) The general strategy. A variable amount of competitor genomic DNA fragment was mixed with a fixed amount of cDNA derived from either liver or J774 cells. When equimolar amounts of control and cDNA are included in the reaction, the ratio of products will be one.

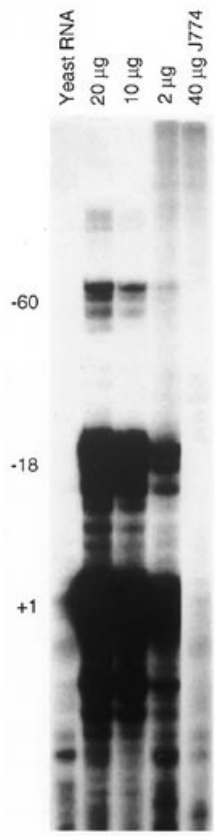

Figure 4. 5′-End mapping of α_1-PI expression by RNase protection. Nuclease protection was used to determine the transcriptional start sites utilized in liver and in cell line J774. Decreasing amounts of mouse liver total cellular RNA were hybridized with a [^{32}P]UTP-labeled riboprobe fragment spanning the segment from –525 to +75, which includes the entire exon 1 and extends 525 bp upstream. Following hybridization and RNase I plus T1 treatment samples were subjected to gel electrophoresis on 8% polyacrylamide DNA sequencing gels. Arrows in the figure show putative start sites at +1, –18 and –60 bases upstream. No transcript is detected by this technique from either macrophage or J774 cells.

mRNA at steady-state. This is in contrast to levels in human macrophages, which produce nearly 10% of the level in liver (11). These results confirm the estimate derived from the northern analysis and clearly establish the low level of α_1-PI expression in murine macrophage.

Expression of α_1-PI genes in macrophages is driven by the liver promoter

The low level of expression of the α_1-PI genes in macrophages could either be due to low levels of transcription from a macrophage-specific promoter or ‘leaky’ transcription from the liver-specific promoter. To differentiate between these possibilities, we used nuclease protection to determine the location of α_1-PI transcriptional initiation sites used in mouse liver. These were then compared with the results with initiation sites active in macrophages, as determined by the more sensitive technique of anchored PCR (see below; 7,14).

Previous studies have defined the major α_1-PI start site in liver (20). We used a nuclease protection assay with a fragment spanning this start site and including ~600 nt upstream, as a probe to confirm the location of this site. Figure 4 shows the results of a protection experiment using both liver RNA and macrophage RNA. In liver, we readily detect three initiation sites which are located at positions 1, –18 and –60 and which are expressed at a ratio of ~500:50:1 (Fig. 4, lanes 2–4, compare bands 1, –18 and –60). However, using RNase protection, we were unable to detect the 5′-end of α_1-PI transcripts in macrophages (Fig. 4, lane 5). Even using a full-length α_1-PI riboprobe, we were unable to detect transcripts in macrophages using this method (data not shown). This suggests that the level of expression of α_1-PI in macrophages is extremely low and is consistent with the northern analysis. We therefore used anchored PCR to map the start site of macrophage-derived transcripts.

α_1-PI cDNA synthesized from J774 mRNA using a primer in exon 2 of the α_1-PI mRNA within the amino acid coding region was treated with TDT to add a homopolymer tail [oligo(dG)] to its 3′-end. This tailed end-specific cDNA was then used in a PCR reaction with nested amplimers and with an ‘anchoring’ amplimer consisting of a specific 20mer linked to oligo(dC) (7,14). Several anchored PCR products were generated with these amplimers, purified by gel electrophoresis, subcloned into plasmid vectors and then subjected to DNA sequence analysis. Nine of the 12 positive clones were found to have sequences beginning at +3 of the mature liver mRNA, while the remaining three had start sites corresponding to –18 relative to the previously defined start site (data not shown). No transcripts from the –60 start site could be found,

however, we would only anticipate that one of 500 transcripts might include this start site if the ratio of initiation sites in macrophages is similar to that in liver.

These data are consistent with the hypothesis that transcription in mouse macrophages initiates at the liver promoter. No evidence for a transcript utilizing a distal upstream start site like that seen in human macrophages was found. Thus, not only do murine macrophages utilize the liver promoter to drive the expression of α_1-PI, but the macrophage mRNA is apparently transcribed from both major and minor liver-specific initiation sites.

All five α_1-PI genes are expressed by macrophages

We have previously demonstrated that five closely related α_1-PI genes are co-expressed in mouse liver (2,3). To determine whether the low level of α_1-PI mRNA in macrophage cells was a result of expression of one α_1-PI gene or all five, we used a PCR-based analysis which we have described previously (3). RT–PCR was performed on macrophage mRNA with amplimers which flank the polymorphic reactive center in exon 5, near Met353 (2,3,18). The PCR products of this reaction were then probed with gene-specific oligonucleotides which we have previously shown to distinguish among the five genes under stringent hybridization conditions. Figure 5 (column labeled Mouse Intraperitoneal Macrophage) shows that, by this analysis, all five α_1-PI genes are expressed in peritoneal macrophages and J774 cells. Positive controls demonstrating the specificity of the oligonucleotide probes as well as the ability of the method to identify all five transcripts in mouse liver mRNA are shown. In addition, controls using either no RNA or human mRNAs were negative. The weak positive signal on the HepG2 PCR product reflects weak cross-reaction with the probe due to partial homology between the human and mouse α_1-PI-V probe. These comparisons are not quantitative, because the PCR conditions are not designed to synthesize PCR products in proportion to the template concentration. Therefore, this experiment does not permit a determination of the relative amounts of each mRNA. The extreme sensitivity of PCR to detect α_1-PI transcripts is demonstrated by the observation that non-stimulated J774 cells produce all five α_1-PI transcripts when assayed by PCR, but northern analysis, with long exposure times and a low background, fails to detect any α_1-PI mRNA (Fig. 2).

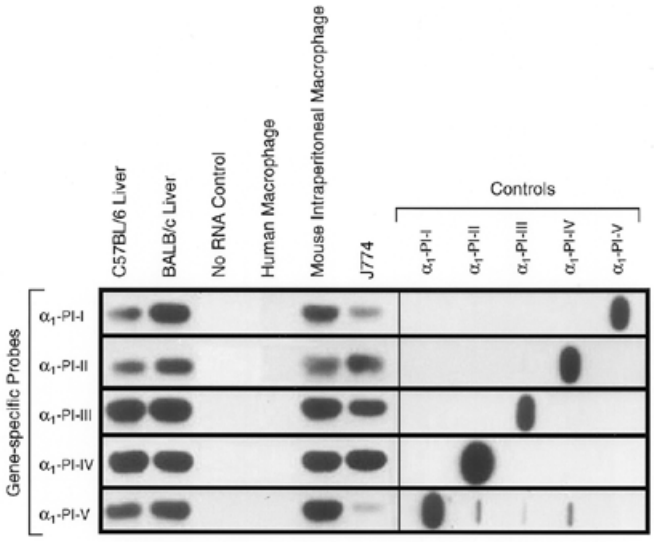

Figure 5. Gene-specific oligonucleotide detection of PCR products. RT–PCR was performed on total RNA derived from the indicated cell types and subjected to agarose gel electrophoresis. After producing five duplicate blots, the filters were probed with gene-specific oligonucleotides under stringent conditions in order to assay for the presence of specific transcripts as previously described (2). The control is a blot of cDNA clones of each of the five α_1-PI mRNA expressed in liver.

DISCUSSION

Our results show that there is a clear difference in the pattern of expression of murine α_1-PI and human α_1-AT in macrophages. In humans, α_1-AT is expressed from a tissue-specific promoter at relatively high abundance in macrophages, while in mice there is insignificant accumulation of α_1-PI mRNA in macrophages. The nearly undetectable α_1-PI mRNA which is expressed in mouse macrophages appears to be driven by the liver promoter elements. Indeed, our DNA sequence analysis of the immediate upstream and far upstream regions of a mouse α_1-PI gene reveals no evidence of homology with the single human gene in the region where macrophage transcription is expected to initiate. Moreover, in previous work we have been unable to detect expression of either α_1-PI or reporter gene constructs driven by up to 10 kb of upstream mouse sequence in non-hepatic cell lines or in any tissue of 31 independent transgenic mouse lines (14; T.Harris, PhD thesis). It could be argued that further tests for a functional promoter in macrophages could be carried out using transfection approaches in macrophage cells. However, such experiments would be at best difficult to relate to the *in vivo* observation that macrophages simply do not express appreciable levels of α_1-PI. For example, failure to observe expression in macrophages with any particular construct could be either a confirmation of our *in vivo* observations or that we have failed to include the appropriate signals. Similarly, the finding of expression of a particular construct in transfected macrophage-derived cells, which we have clearly shown do not express the endogenous gene, cannot be interpreted.

Using extremely sensitive PCR techniques, we can find evidence for RNA transcripts of the five mouse genes in macrophages. However, the levels of these transcripts are consistent with no more that one cell in 10 expressing only a single copy of the mRNA. We speculate that the low level expression is the result of 'leaky' control of the liver promoter, which occurs with all five α_1-PI genes. It is unlikely that expression is the result of DNA contamination, since PCR of these RNA samples with primer pairs spanning an intron (see Fig. 3) shows no product which includes intronic sequences. Since our experiments utilize PCR to detect transcripts, we cannot rule out the possibility that at least a part of this expression is the result of read-through transcription of the genes and does not actually give rise to functional mRNAs. However, the observation of a faint signal by northern analysis after extended exposure suggests that at least a portion of the transcripts are spliced to produce mRNAs of the size seen in liver. It seems highly improbable that such low levels of expression could have physiological significance with respect to protease inhibition.

The ability to selectively modify mouse genes and thereby generate mouse models of human disease is clearly an attractive technology to study mechanisms of pathology and to explore potential therapies. For gene deficiencies like α_1-PI, where both somatic gene therapy (21) and drug therapies are already underway, a mouse model would be particularly attractive.

However, it is clear that in the establishment of models, it is critical to demonstrate that the physiology of the process under study is similar or identical to humans. Our data demonstrate that the expression pattern of mouse α_1-PI differs in a fundamental way from human expression in the tissues which are believed to be critical to the human disease. Thus it may be necessary to choose other animal models for this syndrome. Alternatively, the failure of α_1-PI to be expressed in mouse macrophages may reflect the fact that expression in human macrophages may have less importance in the normal function of the inhibitor than has been hypothesized. Transgenic techniques which could bring about macrophage expression of the gene in mice may provide a route to test whether macrophage expression affords any greater protection from inflammation in lung than normal levels of circulating α_1-PI.

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health grant CA39553 (to K.S.K.) and Cancer Center grant CA13330. In addition, we acknowledge the generous gift of conditioned medium from Dr Gretchen Darlington and helpful discussions with Dr Kate Montgomery.

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