Widespread expression of human α_1 -antitrypsin in transgenic mice revealed by in situ hybridization

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In situ hybridization is a powerful means of identifying sites of gene expression. We used this technique to examine the spatial and developmental control of transcription of the human α_1 -antitrypsin (α_1 AT) gene in transgenic mice carrying this gene and extensive 5'- and 3'-flanking sequences. In addition to expression in yolk sac and liver, human α_1 AT RNA was detected in gut, stomach, pancreas, nasal epithelium, pharynx, bronchi, spinal ganglia, and ossifying cartilage of transgenic fetuses at 14.5 days post coitum (dpc). In transgenic adults, expression was no longer found in the pancreas but was found in the kidney and salivary gland. In each tissue, expression was confined to a specific cell population. This pattern of α_1 AT expression was found to correlate with that seen in several fetal and adult human tissues. These results suggest a wider role of α_1 AT in human physiology and development than previously suspected, and they demonstrated the potential value of this approach in delineating the physiological role of human proteins. Expression of the endogenous α_1 AT gene in mice was confined to a limited, but overlapping, set of tissues, suggesting that the *cis*-acting DNA sequences that regulate the expression of the human and mouse genes interact differently with transcription factors present in mouse cells.

[**Key Words:** α_1 -Antitrypsin; transgenic mice; in situ hybridization; tissue-specific expression; developmental regulation]

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α_1 -Antitrypsin (α_1 AT), also known as α_1 -protease inhibitor (Pi), is the most abundant protease inhibitor in human plasma. Its major known physiological role is to protect alveolar connective tissue from degradation by elastase secreted by neutrophils populating the lower respiratory tract (Travis and Salvesen 1983). α_1 AT is secreted chiefly by hepatocytes, although the lower levels of synthesis by alveolar macrophages and peripheral blood monocytes may be necessary for local protease homeostasis in the lung and at sites of inflammation and infection (Gautier et al. 1977; Eriksson et al. 1978; Isaacson et al. 1981; Van Furth et al. 1983; Perlmutter et al. 1985). Synthesis in the brain (Dziegielewska et al. 1986) and the fetal yolk sac (Gitlin and Pericelli 1970) has also been reported, but the extent and significance of extrahepatic gene expression has remained largely unexplored.

Many genetic variants have been described for the single Pi locus (Cox 1981; Fagerhol and Cox 1981). Of these, the most clinically important is PiZ, characterized by apparently normal synthesis but impaired secretion of the protein, so that homozygotes show reduced plasma α_1 AT levels. Serum α_1 AT deficiency results in a predisposition to early-onset pulmonary emphysema,

probably due to the upset of the normal protease-anti-protease balance in the lung (Laurell and Eriksson 1963; Janoff et al. 1977, 1979). Less frequently, PiZ homozygotes develop liver cirrhosis or hepatoma (Sharp et al. 1969; Eriksson et al. 1986). The pathogenesis of the liver disease is not understood but could be related to the accumulation of excess protein in the liver (Cox et al. 1986). The PiZ allele is also tenuously associated with kidney disease (Miller and Kushner 1969; Moroz et al. 1976; Fagerhol and Cox 1981), gastrointestinal ulcers (Andre et al. 1974), pancreatitis (Novis et al. 1975), islet cell hyperplasia (Ray and Zumwalt 1986), panniculitis (Viraben et al. 1986), and arthritis (Cox and Huber 1976; Teh et al. 1985; Sanders et al. 1986); any molecular basis for these suggested links has yet to be found.

Transgenic mice provide a convenient means for studying gene expression and its regulation. Several laboratories have succeeded in achieving efficient expression of the human α_1 AT gene in transgenic mice (Kelsey et al. 1987; Ruther et al. 1987; Sifers et al. 1987). Nuclease protection assays revealed human α_1 AT mRNA not only in the liver but also in a limited number of extrahepatic sites, such as kidney and gut, in these transgenic mice. This latter finding, while unexpected, was subsequently found to reflect the normal distribution of transcripts in human tissues, suggesting species-specific differences in the control of α_1 AT expression (Kelsey et al. 1987).

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We sought to extend these studies by carrying out a detailed examination of human α_1 AT expression in transgenic mice by *in situ* hybridization. This technique allows detection of gene expression in small samples such as embryonic tissues and minor cell subpopulations of larger tissues not detectable by other RNA analyses. It also has some advantages over immunohistochemical techniques when the gene product is a secreted protein, as it is in this case. Thus, we hoped to identify all sites of human α_1 AT expression and the specific cell types responsible, to approach the question of the physiological and pathological significance of α_1 AT. We also sought to describe the temporal and spatial expression pattern of the transgene to compare its developmental regulation with that of the endogenous human and mouse genes.

We report here that human α_1 AT is expressed in a far wider variety of tissues than previously suspected, suggesting diverse roles for the protein. The pattern of expression of the transgene resembles that of the endogenous gene in humans, rather than that of the mouse gene, in both tissue specificity and developmental profile.

Results

Human α_1 AT expression in transgenic mice

To enable the sites of expression of human α_1 AT to be surveyed thoroughly using a relatively small number of sections, *in situ* hybridization was carried out initially with whole 14.5 days post coitum (dpc) transgenic mouse embryos, cut at various depths in both the sagittal and transverse planes. This stage of mouse embryogenesis is sufficiently late for all the major organs to be present but sufficiently early for whole body sections to be easily prepared and handled. Hybridization was seen in the yolk sac, apparently confined to the endodermal component (Fig. 1a). Several organs of the digestive system showed hybridization, in particular the liver, gut, and pancreas; weaker hybridization signal was seen in the stomach (Fig. 1b), while none was seen in the esophagus. The signal was confined to the endodermal components of the gut and stomach, and extended along the entire length of the gut including the colon.

In the respiratory tract, hybridization was seen in the epithelium lining the nasal sinus (Fig. 1c) and in the pharynx, from the posterior end of the palate to the branching of the esophagus and trachea (Fig. 1d). Transverse and serial parasagittal sections showed no expression in the trachea (data not shown), but expression was clearly seen in the epithelia of the bronchi and bronchioles (Fig. 1e).

Hybridization was observed in various regions of cartilage, specifically those known to be undergoing primary ossification at this stage of embryogenesis (Rugh 1968; Theiler 1972). These included the frontal, nasal (Fig. 1c), basioccipital, basisphenoid (Fig. 1d), mandibular, clavicular, rib (Fig. 1e,f) and vertebral cartilage (Fig. 1g), and cartilage of the long bones. Some of these areas of cartilage were examined by alizarin red staining; cal-

cium deposits were found in all cases, confirming that α_1 AT expression was occurring at sites of ossification.

Weak but definite hybridization was seen in spinal ganglia (Fig. 1g). No hybridization was seen in the metanephros, which gives rise to the functional kidney, or in the brain, spinal cord, heart, or gonads.

The above observations were consistent for several different embryos of two independent transgenic lines (16 and 39; Kelsey et al. 1987). Hybridization was specific to sections hybridized with antisense RNA probe; sections hybridized with sense RNA control probe showed no signal above background (Fig. 1). The same was true of all experiments subsequently described, so negative controls are omitted from Figures 2–4 for the sake of brevity.

Based on the above and previous observations of human α_1 AT mRNA distribution (Kelsey et al. 1987; Ruther et al. 1987; Sifers et al. 1987), we examined several organs in adult transgenic mice by *in situ* hybridization. This allowed identification of the specific population of cells in which the gene is expressed in fully developed and functional organs.

As expected, strong hybridization was seen in adult liver, specifically in the hepatocytes rather than the cells of the portal tracts (Fig. 2a). The histology of hybridized sections did not allow us to identify the Küpffer cells. The signal intensity did not appear to vary across the lobules.

Expression of human α_1 AT in transgenic mouse gut has been reported previously (Kelsey et al. 1987; Ruther et al. 1987; Sifers et al. 1987). In the present study, we observed that expression in the ileum was localized to the epithelial cells of the villi and to cells at the bases of the crypts (Fig. 2b). On the basis of their position and histochemical staining with phloxine and tartrazine (data not shown), the latter are most likely to be Paneth cells. The relative intensities of the hybridization signals in the villi and Paneth cells varied in sections from different regions of the small intestine.

In the stomach, very strong hybridization was observed in the glands of the fundic mucosa (Fig. 2c), with weaker signal observed in the pyloric region, and none at all on the nonglandular side. No hybridization was seen in the esophagus or pancreas of adult transgenic mice. Salivary glands were examined due to their functional relationship to pancreas; hybridization was moderate in the parotid gland and only slight elsewhere (Fig. 2d).

Human α_1 AT RNA was not detected in the trachea, however, hybridization signal was observed in bronchial and bronchiolar epithelia in sections of adult lung (Fig. 2e). The signal in the alveoli was only slightly above background. The kidney of adult transgenic mice showed a particularly striking pattern of α_1 AT expression. Strong hybridization in the outer cortex was seen, in juxtaglomerular tubular structures that appeared to be proximal convoluted tubules (Fig. 3b). Very intense hybridization signal was scattered radially in the medulla, becoming dense in the medullary pyramid (Fig. 3c), presumably due to human α_1 AT expression in the collecting ducts. Stronger hybridization was seen in the kidneys of a line 39 male than in those of a line 16 fe-

Figure 1. (See facing page for legend.)

Figure 2. Human α_1 AT RNA in transgenic adult mouse tissues. Bright-field photomicrographs of hybridized tissue sections are shown with the corresponding dark-field images below. Samples from both lines 16 and 39 are included. (a) Liver, showing an area of hepatocytes (H) surrounding a portal tract (PT). (b) Transverse section of ileum, showing villi (V), crypts (C), and surrounding muscle bilayer (M). (c) Fundic region of stomach wall, showing glandular mucosa (GM) and surrounding muscle bilayer (M). (d) Salivary glands, showing parotid (P) in the lower left and sublingual or submandibular in the remaining area. (e) Lung, showing bronchioles (B) surrounded by alveolar tissue and blood vessels. Kidney is shown in Fig. 3. Samples were autoradiographed for 2 (a), 3 (b), or 4 (c–e) days, and dark-field images were photographed and printed under identical conditions. Negative controls showed negligible hybridization signal (see Fig. 1). Scale bar represents 0.2 mm.

male. This difference may have both sex-specific and strain-specific components, as described below, for the endogenous mouse gene. The possibility of a line-specific component was not investigated.

Apart from expression in the kidney, expression was similar in all respects in the line 16 female and the line 39 male studied.

Human α_1 AT expression in normal human tissues

To compare the regulation of human α_1 AT expression in transgenic mice with endogenous expression in humans, a number of human tissues were examined by *in situ* hybridization. In both fetal and adult human liver, strong hybridization was observed in the hepatocytes but not the portal tract (Fig. 4a). Hybridization was ob-

served in villi in fetal gut sections and in Paneth cells of adult gut sections (Fig. 4b). Some signal was seen in 15.5- and 18-week fetal pancreas sections despite histological deterioration of these samples (data not shown). Adult pancreas and fetal and adult stomach samples were unavailable for testing.

Sections of snap-frozen human fetal lung showed hybridization in epithelia lining the bronchi and bronchioles (Fig. 4c). A post-mortem adult lung sample showed hybridization in scattered single cells only; these may have been macrophages, which are known to be rich in α_1 AT (Isaacson et al. 1981).

Sections of snap-frozen fetal kidneys at 13- and 19-weeks gestation failed to show hybridization. A post-mortem adult kidney sample showed hybridization in ring-shaped groups of cells that may have been loops of

Figure 1. Human α_1 AT RNA in 14.5 dpc transgenic mouse embryos. Columns 2 and 3 show dark-field images of equivalent areas on adjacent sections hybridized with antisense RNA probe, and sense control RNA probe, respectively. Bright-field images of corresponding sections are shown in column 1, the appropriate area being indicated with a rectangle where applicable. All sections in the sagittal plane (a–g) are shown in the same orientation. Samples from both lines 16 and 39 are included: (a) yolk sac (YS), adjacent to the snout; (b) abdominal region showing liver (L), stomach (S), pancreas (P), gut (G), and metanephros (M); (c) nasal sinus (NS) and nasal cartilage (NC); (d) pharynx (Ph) and basisphenoid cartilage (BC); (e) liver (L), lung (Lu), and ribs (R); (f) transverse section showing hybridization in rib cartilage (R) confined to the ossification center at the dorsal end. Spinal cord (SC) and forelimbs (F) are marked for orientation. (g) Spinal ganglia (SG), ribs (R), and vertebrae (V). Samples were autoradiographed for 4 days (except a, 7 days, and f, 3 days), and dark-field views photographed and printed under identical conditions. Scale bar represents 0.67 mm (b, dark field); 1 mm (remaining dark field; a, bright field); 3 mm (remaining bright field).

Figure 3. Expression of human and endogenous α_1 AT in mouse kidney. Dark-field photomicrographs of hybridized kidney sections (b–f) are shown with their corresponding areas marked on whole kidney section (a): (C) cortex; (M) medulla; (P) renal papilla. (b) Corticomedullary region (b) and papillary region (c) of line 39 transgenic mouse kidney are shown hybridized with human α_1 AT probe. Corticomedullary regions of female and male CBA mouse kidneys (d,e), respectively, and papillary region of male CBA mouse kidney (f), are shown hybridized with mouse α_1 AT probe. All samples were autoradiographed for 4 days, and dark-field images were photographed and printed under identical conditions. Negative controls showed negligible hybridization signal (see Fig. 1). Scale bar represents 3 mm (a), 1 mm (b–f).

Henle (Fig. 4d), although the histology of the sample was too poor for firm conclusions to be drawn.

Samples of human yolk sac, salivary gland, spinal ganglia, and ossifying cartilage were unavailable for testing, although previous RNase protection studies have shown expression in yolk sac (Kelscy et al. 1987).

Expression of endogenous mouse α_1 AT

We used a probe specific for mouse α_1 AT to examine the

endogenous expression of α_1 AT in normal mouse 14.5-dpc embryos and adults. In the embryos, strong expression was observed in yolk sac endoderm and liver (Fig. 5). Some hybridization signal may have been present in fetal stomach, gut, and pancreas, which appeared to be above background (Fig. 5a). No hybridization was observed in the metanephros (Fig. 5a) or elsewhere in the fetus (data not shown). The pattern of hybridization in transgenic fetuses was identical to that in nontransgenic littermates, suggesting that expression of the human

Figure 4. α_1 AT expression in human tissues. Corresponding bright-field (upper) and dark-field (lower) images of hybridized tissue sections are labeled as in Fig. 2: (a) adult liver; (b) adult small intestine, longitudinal section; (c) fetal lung; and (d) adult kidney. Samples were autoradiographed for 3 (a and b) or 4 (c and d) days and dark-field images photographed and printed identically. Negative controls showed negligible hybridization (see Fig. 1). Scale bar represents 0.2 mm.

α_1 AT transgene did not alter the expression of endogenous α_1 AT.

Expression of α_1 AT in the adult mouse was confined to the hepatocyte population of the liver (not shown) and to the kidney (see below). No expression was detected in gut, stomach, pancreas, salivary gland, or lung.

In the kidney, α_1 AT expression was confined to a region surrounding the cortico-medullary boundary (Fig. 3e,f), in contrast to the expression of the human α_1 AT transgene (Fig. 3b,c). It is not clear whether the hybrid-

ization was associated with the distal convoluted tubules or loops of Henle. Interestingly, expression of endogenous α_1 AT was much weaker in a C57BL/6 kidney than in a CBA kidney of the same age, and weaker in females of both strains (Fig. 3d,e).

Discussion

Technical assessment

This study has demonstrated that the human α_1 AT gene is expressed in a far greater variety of tissues in transgenic mice than previously suspected. We take this as likely to reflect normal expression of the gene in humans because the pattern of expression observed did not differ from that seen in the limited number of human tissues available for study and was identical in two independent transgenic lines. The approach of using transgenic mice rather than human material has several advantages for studying the expression of a human gene. First, the quantity and variety of tissues of any developmental stage and in suitable condition for RNA analysis is virtually unlimited. Second, expression of a transgene is often stronger and easier to detect than endogenous expression, possibly due to multiple gene copy number. The transgenic mice used in the present studies had 2.5- to 5-fold higher liver α_1 AT mRNA levels and approximately 4-fold higher serum α_1 AT levels compared to normal human levels (Kelsey et al. 1987). Third, the only human genes present in the transgenic mice are the transgene. This precludes detection of transcripts of other partially homologous human genes as might occur when using human material. The cosmid insert borne by the transgenic mice used in the present study carries a 5 kb sequence with homology to, and 10 kb downstream from, the α_1 AT gene. It is unclear whether this α_1 AT-related gene, designated ATR, is expressed. ATR appears to lack promoter sequences (Hofker et al. 1988), and transcripts have not been detected in human tissues (Kelsey et al. 1988). However, the physical properties of this sequence are not consistent with those of a pseudogene (Bao et al. 1988), and ATR is capable of transient expression in L cells (Kelsey et al. 1988). The formal possibility that ATR expression could have contributed to our findings is being tested using a transgene devoid of ATR.

Thorough investigation of sites of gene expression relies on detection of either the RNA or protein products in situ. We have chosen to examine transcription because location of α_1 AT protein may reflect sites of adhesion or endocytosis rather than de novo synthesis, whereas efficient export of α_1 AT protein from some sites of synthesis, such as the liver, may result in false negatives. On the other hand, transcription does not necessarily result in protein synthesis, so results from both in situ RNA and immunohistochemical analyses should ideally be considered together. Recently, Carlson et al. (1988) have produced an immunohistochemical survey of human α_1 AT synthesis in transgenic mice. Although the transgenic mice studied by these authors bear an insert with less flanking sequence, the observations of

Figure 5. Endogenous α_1 AT RNA in 14.5-dpc mouse embryos. Each bright-field photomicrograph is followed by corresponding dark-field images of sections hybridized with antisense and sense control probes, respectively. (a) Abdominal region, comparable to and labeled as in Fig. 1b. (b) Head region showing forebrain (F), yolk sac endoderm (YSE), and yolk sac mesoderm (YSM). Scale bar represents 0.5 mm (a) and 0.2 mm (b).

Carlson et al. are essentially similar to the *in situ* data presented here, as discussed below. This implies that protein synthesis usually accompanies α_1 AT transcription and that the transcripts detected in the present study are mRNA.

Control of α_1 AT gene expression

The results presented here have important implications for the understanding of the tissue and species specificity of gene regulation. Although it is formally possible that differential mRNA stability could contribute to our observations, the following discussion is based on the assumption that transcriptional regulation is of primary importance.

The sites of expression of human and mouse α_1 AT RNA described in the present study are summarized in Table 1. From these data we draw two conclusions. First, it appears that the human gene is regulated similarly whether in mouse or human cells, implying the involvement of human DNA sequences adjacent to or within the α_1 AT gene ('*cis*-sequences') in the developmental regulation of the gene. It has been shown previously that the 5'-flanking region of the human α_1 AT gene contains sufficient information for tissue-specific transcription, at least in hepatocytes (Ciliberto et al. 1985). Currently, we are investigating the involvement of *cis*-sequences in the overall regulation of the gene using transgenic mice bearing various 5' deletions and mutations. Second, the endogenous mouse α_1 AT transcripts accumulate in a limited but overlapping range of tissues, compared with their human counterparts. Therefore, regulatory molecules present in mouse cells must interact differently with the human and mouse α_1 AT genes. We can envisage two possible mechanisms for this differential interaction: (1) In some tissues, the human gene binds positive regulatory molecules that the mouse gene does not and vice versa. These *trans*-acting factors must be different from those that confer

expression in sites common to mouse and man. (2) Some tissues contain negative regulatory molecules that are recognized by mouse *cis*-sequences but not by human *cis*-sequences and vice versa.

Recently, five different proteins have been found to bind to the human α_1 AT promoter region (De Simone et al. 1987; Hardon et al. 1988; Monaci et al. 1988). Of these, two act as positive regulators in the liver and are absent from spleen and one appears to be a repressor. In view of the present observations, it would be informative to study the distribution of these proteins in a wider variety of tissues. If these are indeed transcription factors for the α_1 AT gene, the question becomes one of how these factors are developmentally regulated.

Physiological implications

Human α_1 AT RNA was found in several organs of the gastrointestinal tract of transgenic mice, notably stomach, gut, and pancreas. The human samples that were available for analysis accorded with these observations. Presence of the protein in gastric and intestinal mucosae is well documented in humans (Ray et al. 1982; Geboes et al. 1982; Kittas et al. 1982) and has been reported in transgenic mice (Carlson et al. 1988). Although both transgenic and human fetal pancreas were rich in human α_1 AT transcripts and both the mRNA and the protein have been reported in human adult pancreas (Ray et al. 1977; Ray and Desmet 1978; Carlson et al. 1988), we were unable to detect transcripts in sections of adult transgenic pancreas. Mature pancreas is rich in ribonucleases, and it is not clear whether our observations reflect lack of transcription of α_1 AT or simply degradation of RNA in the adult transgenic pancreas sections. Various α_1 AT deficiency phenotypes have been associated with celiac disease (Greenwald et al. 1975; Geboes et al. 1982; Nielsen 1984), gastric and duodenal ulcers (Andre et al. 1974), pancreatitis (Novis et al. 1975), and islet cell hyperplasia (Ray and Zumwalt

Table 1. Comparison of endogenous and transgenic α_1 AT RNA distribution

	Mouse α_1 AT (endogenous)		Human α_1 AT (transgene)		Human α_1 AT (endogenous)	
	fetal	adult	fetal	adult	fetal	adult
Liver	++	+++	+++	+++	+++	+++
Kidney	-	+++({ δ) +({ $\varnothing$)	-	+++	-	+
Gut	+/-	-	+++	++	++	+
Stomach	+/-	-	+	+++	a	a
Pancreas	+/-	-	+++	-	++	a
Salivary gland	a	-	a	+	a	a
Nasal sinus	-	a	+	a	a	a
Pharynx	-	a	+	a	a	a
Lung	-	-	++	++	++	b
Ossifying cartilage	-	c	+	c	a	c
Yolk sac	+++	c	+++	c	+++d	c
Spinal ganglia	-	a	+	a	a	a

(+++) Strong expression; (++) moderate expression; (+) weak expression; (+/-) possibly trace amounts; (-) absent; (a) not tested; (b) present in macrophage population only, tissue may have deteriorated before analysis; (c) not applicable; (d) see Kelsey et al. (1987).

1986]. These observations suggest a local role of α_1 AT in the gastrointestinal tract, possibly to afford local protection against attack by proteases involved in digestion.

The most unequivocal evidence for an important role of α_1 AT is provided by the rare homozygotes or compound heterozygotes for the so-called Pi-null allele, characterized by a lack of production of α_1 AT. These individuals present with severe emphysema in early adult life (Garver et al. 1986; Cox and Levison 1988). Although this has been thought to be due to reduced plasma α_1 AT level, some of the problem could be due to lack of α_1 AT produced locally, either by alveolar macrophages, or, as suggested here, by bronchiolar epithelium. Expression of α_1 AT in the human lung warrants further investigation because fresh tissue samples were unavailable for the present study.

Deficiency of α_1 AT has been associated with glomerulonephritis (Miller and Kuschner 1969; Moroz et al. 1976; Fagerhol and Cox 1981), again suggesting a local role for the protein in the kidney. Carlson et al. (1988) have detected α_1 AT in the kidney, and have proposed that it could serve to protect the urinary tract from bacterial proteases during infection, or from proteases normally present in urinary tissue, such as kallikrein and urokinase. We found a striking difference in gross distribution of human and mouse α_1 AT transcripts, which seemed to occupy mutually exclusive sites in the mouse kidney. The significance of this observation and of the sex dependence of the expression levels of both human and endogenous α_1 AT genes in the mouse kidney remains obscure. Renal α_1 AT expression has been reported to be regulated by testosterone in *Mus caroli* (Latimer et al. 1987). Sex or strain dependence could explain why several groups have found only weak expression of human α_1 AT in transgenic mouse kidney (Kelsey et al. 1987; Ruther et al. 1987; Sifers et al. 1987).

The observation of human α_1 AT transcripts in ossifying cartilage of transgenic fetuses presents the intriguing possibility that α_1 AT may play a role in maintaining the protease-antiprotease balance in the breakdown of cartilage during bone development and in elastin turnover during adult life. This possibility is supported by the association between various α_1 AT deficiency phenotypes and rheumatoid arthritis (Cox and Huber 1976, 1980; Buisseret et al. 1977; Geddes et al. 1977; Arnaud et al. 1979; Mitchell and Fabb 1984; Teh et al. 1985; Cox and Levison 1988), although some workers have disputed this association (Backert and Keupers 1977; Sjoblom and Wollheim 1977; Sanders et al. 1986). No endogenous α_1 AT RNA was detected in ossifying cartilage of normal mice. This may reflect differences between human and mouse metabolism because arthritis is not prevalent in normal mice and osteogenesis is not impaired noticeably in the transgenic mice. It remains to be established whether α_1 AT RNA and protein are produced during ossification in humans. If so, normal development in Pi-null homozygotes suggests that any role of α_1 AT in osteogenesis can be circumvented by other means.

α_1 AT protein has been found in the brain in man

(Dziegielewska et al. 1986) and transgenic mice (Carlson et al. 1988). Although we did not observe human α_1 AT transcripts in the developing transgenic mouse brain, spinal ganglia clearly expressed the gene. Transgenic adult brain remains to be tested. The possibility of α_1 AT production in the brain is of interest since some PiZZ cases are diagnosed following catastrophic cerebral hemorrhage, not always explicable by vitamin K deficiency resulting from liver disease (G. Mieli-Vergani, pers. comm.).

In summary, our studies with transgenic mice and with the limited human material available demonstrate an intriguing tissue distribution of α_1 AT. While no direct molecular connection can be proved, the sites of expression revealed in the present study show a striking correlation with the various pathological conditions associated with α_1 AT deficiency. Establishing the local roles of the protein implied by this correlation is the challenge of future research.

Methods

Transgenic mice

Transgenic mice bearing a cosmid insert comprising the human α_1 AT gene together with 8 kb of 5'- and 20 kb of 3'-flanking sequence were generated as described previously (Kelsey et al. 1987). Lines 16 and 39, used in this study, contain 10–15 and 15–20 copies of the cosmid insert, respectively. Founder mice were derived from (C57BL/6 $\times$ CBA) $F_1 \times F_1$ matings, and lines of mixed CBA/C57 BL/6 background were maintained by successive breeding with F_1 mice. A double immunodiffusion assay of amniotic fluid, as described by Kelsey et al. (1987), was used to identify transgenic embryos.

Probe preparation

A 1.2 kb *Pst*I fragment of the human α_1 AT cDNA clone p α AT10 (Kelsey 1987) and a 0.5 kb *Pst*I fragment of the mouse α_1 AT cDNA clone plv1796 (Hill et al. 1984) were subcloned into the plasmid vector pGem3Z (Promega Biotec). The mouse clone differs from the mouse α_1 AT cDNA clone plv3B described by Krauter et al. (1986) by only 3 nucleotides in the *Pst*I fragment, so the mouse probe used in the present study would be expected to hybridize to transcripts of all of the postulated mouse α_1 AT genes.

Sense and antisense probes, labeled with [35 S]UTP (>1000 Ci/mmol, Amersham International) to a specific activity of 1.6×10^8 dpm/ μ g, were generated from each cDNA insert using bacteriophage SP6 or T7 RNA polymerase (Promega Biotec) as appropriate. Probes were hydrolyzed at pH 10.2 to an average fragment length of 100 bp prior to hybridization (Cox et al. 1984).

In situ hybridization

Mouse tissues and embryos and human fetal tissues were frozen in liquid nitrogen immediately after dissection. Liver and gut samples were obtained from cadaver organ donors and were frozen in liquid nitrogen immediately. Kidney and lung samples were obtained from autopsies done within a few hours of death and also frozen in liquid nitrogen. All samples were stored at -70°C until sectioning. Cryostat sections of 7- μ m thickness were collected on L-lysine-coated glass slides, dried

briefly, fixed in 1% paraformaldehyde in PBS for 20 min at room temperature, rinsed thoroughly in PBS, dehydrated through graded alcohols, dried, and sorted desiccated at -70°C until hybridization. Before hybridization, slides were thawed, rehydrated through graded alcohols, treated with 1 $\mu\text{g}/\text{ml}$ proteinase K (Bethesda Research Laboratories) for 30 min at 37°C , rinsed thoroughly in PBS, fixed and rinsed again as above, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, rinsed thoroughly in PBS, dehydrated through graded alcohols, and dried.

RNA probe was applied to the sections at equimolar concentrations of 0.24 ng/ μl (human) or 0.1 ng/ μl (mouse) in a hybridization mixture containing hybridization mixture containing 10 mM dithiothreitol, 50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA fraction V), 1 M Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM EDTA, and 0.5 mg/ml yeast tRNA (Sigma). Hybridization proceeded for 16 hr at 42°C . After hybridization, slides were washed thoroughly in $4\times$ SSC ($1\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0), treated with 20 $\mu\text{g}/\text{ml}$ RNase A (Sigma) for 30 min at 37°C , rinsed thoroughly, washed in $0.1\times$ SSC for 30 min at 60°C , dehydrated in graded alcohols containing 300 mM ammonium acetate, and dried.

Slides were dipped in 50% Ilford G5 nuclear track emulsion, dried, and autoradiographed for 3–7 days at 4°C . Hybridization was visualized by developing in 20% Phenisol (Ilford) for 5 min at 18°C and counterstaining with hematoxylin and eosin.

Controls

Antisense and sense control RNA probes were applied to adjacent sections of each sample on the same slide, to ensure identical treatment of test and control sections. Nontransgenic littermate embryos did not bind the human probe, indicating that it does not cross-react with mouse $\alpha_1\text{AT}$.

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