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Disruption of the LF-A1 and LF-B1 binding sites in the human alpha-1-antitrypsin gene has a differential effect during development in transgenic mice

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Previous work in transfected cell lines and in nuclear extracts has led to the identification of two *cis*-acting elements important for transcription of the human alpha-1-antitrypsin (A1AT) gene, which bind to two liver specific *trans*-acting factors, LF-A1 and LF-B1. Mutations EM3 and PM1, which abolish the binding of LF-A1 and LF-B1 respectively, drastically reduce transcription activity of the A1AT gene *in vitro* and in cell culture. The same mutants have now been introduced in a larger DNA context and their effect has been tested in transgenic mice. A stretch of DNA was constructed which carries two transcriptional units: 18 kb of the human retinol binding protein (RBP) gene, driving the expression of the bacterial chloramphenicol acetyl transferase, linked to 17.5 kb containing the entire A1AT coding sequence with additional 5′ and 3′ flanking sequences. Transcription from the RBP promoter was shown to predominate in liver, and could be used as an internal marker of 'active copy number'. Mutations in the A1AT gene promoter were introduced by homologous recombination in bacterial cells. The results show that base pair substitutions in the binding site for LF-A1 and LF-B1 drastically reduce transcription in non-hepatic adult tissues, yolk sac, and fetal liver, whereas only LF-B1 binding site mutations have a marked, albeit variable, effect in adult liver.

Key words: alpha-1 antitrypsin/development/transcription factors/transgenic mice

Introduction

The identification of *cis*-acting elements within the promoters and enhancers of many genes, and the purification and cloning of the genes coding for the corresponding *trans*-acting factors, are essential steps for the understanding of the mechanism of regulation of gene expression in higher eukaryotes. However, apart from the few cases of naturally occurring mutants (e.g. Ronchi *et al.*, 1989, for the γ-globulin gene; Crossley *et al.*, 1990, for factor IX) that have been identified, most of the current information in this field has been obtained in extremely simplified experimental systems in which the 'gene' tested is reduced to minimal segments, containing only putative promoters and/or enhancers. Moreover, the expression systems themselves are often cultured cell lines or nuclear extracts and the importance of a particular sequence is usually established by the effect of specific mutations on transcription. The simplification of the experimental systems, while unavoidable for the fast and reproducible collection of results, might lead to a correspondingly simplified picture of the mechanism of regulation of transcription. It is therefore desirable to set up experimental systems in which the role of *cis*-acting elements, the identity of which has been established by transfection or by *in vitro* transcription, is reexamined both in the context of larger genomic segments, and during development and differentiation *in vivo*.

We have therefore initiated a study of the regulation of expression of the human alpha-1-antitrypsin (A1AT) gene in transgenic mice. The gene was chosen because of the large amount of available background information, both on the expression of the wild-type gene in transgenic mice (Kelsey *et al.*, 1987; Rüther *et al.*, 1987; Sifers *et al.*, 1987) and on the effect of small mutations in the 5′ region of the gene on the expression of a reporter gene in cultured cells and *in vitro* (Ciliberto *et al.*, 1985; De Simone *et al.*, 1987; Monaci *et al.*, 1988).

In previous work a minimal promoter element for the human A1AT gene, from N −137 to −37, was found to be sufficient for specific transcription *in vivo* in cultured hepatoma cell lines, and *in vitro* with rat liver nuclear extracts (Ciliberto *et al.*, 1985; De Simone *et al.*, 1987; Monaci *et al.*, 1988). Within this minimal promoter, two important *cis*-acting elements were identified: element A, defined by the mutation EM3, and element B, defined by the mutation PM1. The integrity of each of these two elements is essential for the binding of two transcription factors, LF-A1 and LF-B1 respectively (De Simone 1987; Hardon *et al.*, 1988; Monaci *et al.*, 1988; Frain *et al.*, 1989; Nicosia *et al.*, 1990). The aim of the present study is to test whether the A and B elements, which appeared to be important in the context of minimal promoter segments, are as important in the context of the entire human A1AT gene (including the whole coding region with 7.3 kb of flanking regions) and in a whole organism *in vivo*.

In the present paper we show the relevance of LF-A1 and LF-B1 binding sites in maintaining an efficient transcription of the A1AT gene throughout development and in the adult animal.

Results

Experimental design

The work presented in this paper is based on the observation that a DNA segment containing the entire coding sequence of the human A1AT gene, plus an additional 7 kb and 0.3 kb

at the 5′ and 3′ sides respectively, directs efficient and tissue-specific transcription in transgenic mice (Rüther et al., 1987). Similar results with different human A1AT genomic fragments were obtained by Kelsey *et al.* (1987) and Sifers *et al.* (1987). We wished to introduce small (4−5 bp) mutations into the context of this large DNA segment. For this purpose we set up an *in vivo* homologous recombination protocol in *E.coli* which is described in Materials and methods and in Tripodi *et al.* (1990). This technique was to be used to introduce mutations which were expected to decrease or abolish transcription of the transgene. In an attempt to take into account factors such as position effects, copy number, mosaicism and genomic imprinting, all of which could affect the expression of the transgene (Palmiter *et al.*, 1986), a cosmid was constructed containing, in addition to the wild type or mutated A1AT gene, another transcriptional unit that would serve as an internal marker of expression for the normalization of the results. For this purpose, we chose 18 kb of the human Retinol Binding Protein (RBP) gene, carrying the bacterial chloramphenicol acetyl transferase (CAT) coding region within the first exon.

Two of the mutations previously shown to abolish transcription from the A1AT gene promoter in transfected cells (De Simone *et al.*, 1987) and to reduce it in an *in vitro* transcription assay (Monaci *et al.*, 1988) were chosen for analysis in the transgenic system. EM3 is a 4 bp substitution (−118 to −115) which inhibits the binding of the transcription factor LF-A1 to the promoter; PM1 is a 5 bp substitution (−77 to −72) which similarly inhibits the binding of the transcription factor LF-B1.

We analysed three constructs in transgenic mice: pCos-RBP-CAT/A1AT-WT (referred to in the text as WT), which has an intact A1AT gene linked to the RBP-CAT internal control, pCos-RBP-CAT/A1AT-EM3 (referred to in the text as EM3), which differs from the wild type construct only in that it contains the previously described EM3 mutation, and pCos-RBP-CAT/A1AT-PM1 (referred to in text as PM1) which harbours the PM1 mutation (see Figure 1).

Transgenic lines obtained

Three independent founder transgenics were produced for each of the three constructs. Two wild type lines (WT13, WT16) and two EM3 lines (EM3-2, EM3-7) were analysed in detail. In the case of the PM1 transgenic mice, only one founder (PM1-6) was able to transmit the transgene to its offspring. The other two founders (PM1-67, PM1-71) were eventually killed and analysed. Copy number and CAT activity of these lines are summarized in Table I.

Activity of the RBP-CAT gene

Transcription from the RBP promoter was monitored by measuring CAT activity in tissue extracts. In each case expression was highest in liver (see Figure 2A) CAT activity was also detectable, albeit at a greatly reduced level, in other tissues such as kidney, brain, lung and spleen. The levels of CAT activity in liver varied in the different lines tested, and there was no correlation with the copy number of the human gene (see Table I and Figure 2B). The conclusion that RBP is predominantly expressed in liver confirms and extends the previous findings (D'Onofrio *et al.*, 1985; Colantuoni *et al.*, 1987).

In order to use RBP-CAT activity as an internal control for normalization of the A1AT gene expression, we made

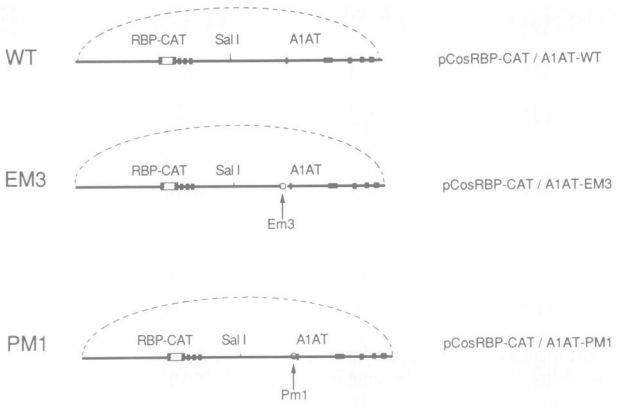

Fig. 1. Constructs used in this study

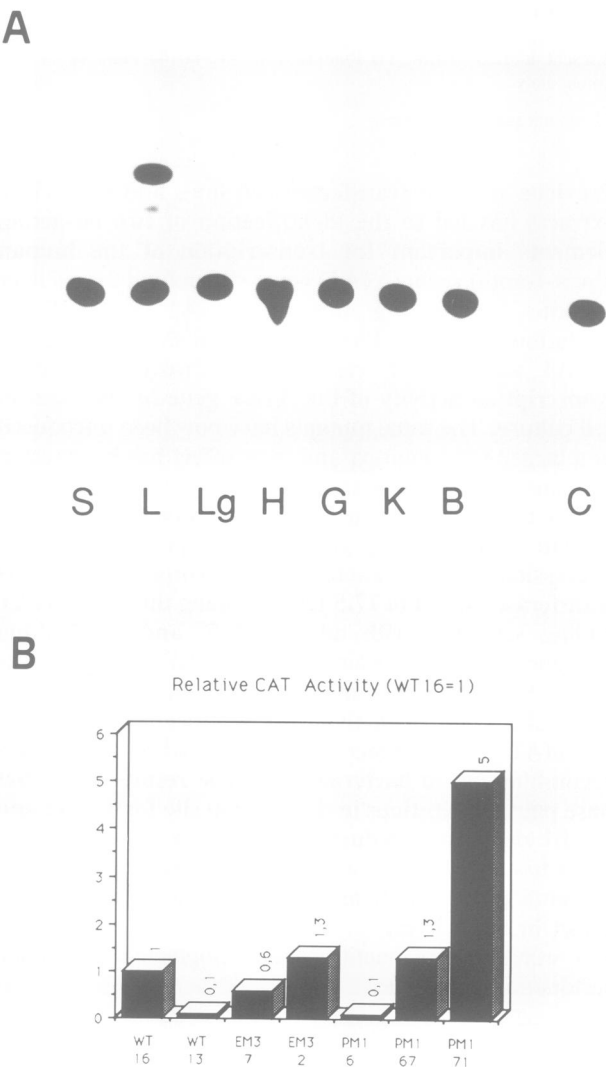

Fig. 2. A. Tissue-specific expression of human RBP-CAT. CAT activity assayed in extracts of various tissues from a mouse of the WT16 line. S, spleen; L, liver; H, heart; G, gut; K, kidney; B, brain; C, control mouse liver. Overexposure of this TLC plate shows a signal in other tissues (see text). **B.** Histogram of CAT activity in liver extracts of transgenic lines examined expressed relative to that of WT16.

the admittedly simplified assumption that positional effects due to the surrounding mouse genome are exerted on the entire foreign insert and in an equivalent manner on both

Table I. Human A1AT expression in WT, EM3 and PM1 trangenic lines

	WT16	WT13	EM3-2	EM3-7	PM1-6	PM1-67	PM1-71
Copy number	1	2	10	4	1	1	5
Normalization to the endogenous mouse A1AT							
Human A1AT adult liver	100%	16%	152%	67%	2%	1%	13%
Human A1AT fetal liver	100%	–	31%	14%	<1%	–	–
Human A1AT yolk sac	100%	–	<1%	<1%	<1%	–	–
Normalization to the endogenous mouse A1AT and to the CAT activity							
Human A1AT adult liver	100%	160%	116%	111%	20%	<1%	2%
Human A1AT fetal liver	100%	–	24%	23%	<1%	–	–

The results were obtained by analysing Southern and Northern autoradiographs in an A300 Molecular Dynamics Computing Densitometer. The appropriate exposure of gels was chosen. Different experiments were analysed and normalized separately with respect to endogenous mouse A1AT expression. 100% value for human A1AT expression and value 1 for CAT activity was arbitrarily attributed to family WT16

the transcriptional units RBP-CAT and A1AT. As the RBP-CAT activity should serve as an estimate of 'active copy number', its values in liver extracts of each of the different transgenic lines were quantified by densitometry (see Figure 2B) and the A1AT expression was normalized with respect to it.

A1AT gene expression in WT cosmid constructs

The human A1AT expression in the context of the cosmid constructs was consistent with that reported previously (Kelsey *et al.*, 1987; Rüther *et al.*, 1987; Sifers *et al.*, 1987), i.e. transcription of the human A1AT gene occurred predominantly in liver and lower levels of expression were also seen in kidney and gut. The results for WT16 are shown in Figure 3. The level of transcription, normalized with respect to the transcription of the endogenous mouse A1AT gene, appears to be consistent within individuals of a single family and varies by a factor of six between WT13 and WT16 families. The CAT activity driven by the RBP-CAT gene follows the same trend of expression as that of A1AT in the two families. After normalization to CAT activity, the values of human A1AT transcription were comparable in WT13 and WT16 families. This may be taken as an indication that the internal control could be used for the normalization of the transcriptional activity of the test A1AT gene in WT and mutant constructs.

Mutation of the LF-B1 binding site of the A1AT gene strongly reduces transcription

Previous work has shown that the interaction between the transcription factor LF-B1 and the A1AT promoter is important for transcription of the A1AT gene (De Simone *et al.*, 1987; Monaci *et al.*, 1988). Mutations in the LF-B1 binding site, particularly the 5 bp substitution PM1, inactivate transcription in transfected cell lines (De Simone *et al.*, 1987) and reduce it *in vitro* (Monaci *et al.*, 1988).

In mice of the three PM1 lines produced we found different levels of A1AT expression. In the case of PM1-6 and PM1-67, expression in adult liver, after normalization with respect to the endogenous mouse A1AT gene, was found to be much lower than WT13 and WT16, while in the case of PM1-71 the levels of A1AT transcription were comparable to the WT13 family (see Figure 3 and Figure 4).

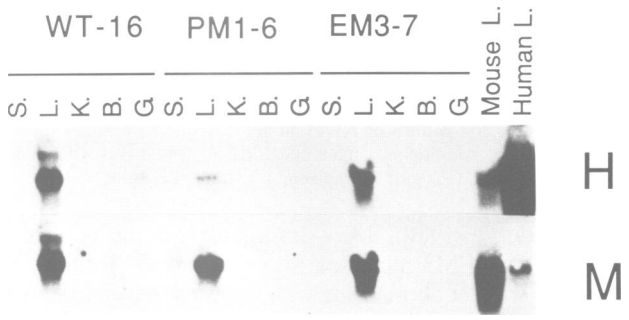

Fig. 3. Expression pattern of WT, PM1 and EM3 mutants A1AT genes in adult mice. RNA was extracted from transgenic mice of the lines WT16, PM1-6 and EM3-7. Two autoradiographs of a Northern blot are shown: H is hybridized with the human cDNA coding for A1AT; M is the same filter rehybridized with a mouse cDNA coding for A1AT. S, spleen; L, liver; K, kidney: B, brain; G, gut; mouse L, mouse control liver; human L, human liver.

After normalization with respect to the CAT activity, the three PM1 mutants showed a significant reduction of expression, varying from 20% to <1% of that seen in WT transgenic mice (see Table I). The PM1 mutation clearly has a marked but variable effect on expression of the human A1AT transgene in adult liver.

The most extreme effect of the PM1 mutation was seen in embryonic tissues. In mice of the PM1-6 line, no expression of the transgene was detected in either liver or yolk sac of 17.5 days *post coitum* (d.p.c.) embryos (data not shown). Unfortunately, PM1-67 and PM1-71 mice did not transmit the transgene to their offspring and therefore in these lines the expression of the transgene during development could not be analysed.

Mutation of the LF-A1 binding site markedly reduces transcription in embryonic tissues but not in adult liver

In previous studies the *cis*-acting element A was described as playing an important role in the transcription from the A1AT promoter, both in transfected cells and *in vitro*. Mutations in this element, most notably the 4 bp substitution EM3, prevent the binding of the *trans*-acting factor LF-A1 that is known to be present in liver (De Simone *et al.*, 1987;

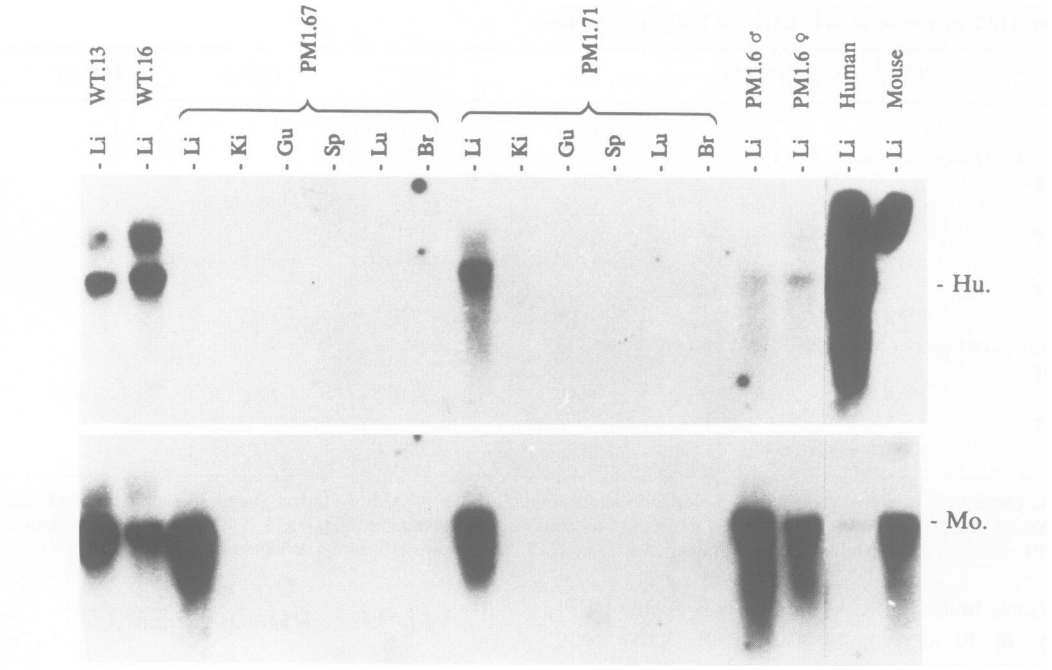

Fig. 4. Expression pattern of A1AT in adult tissues of founder mice PM1-67 and PM1-71. RNA was extracted from tissues of the PM1-67 mouse, which was a male and PM1-71 which was a female. Both male and female mice of the line PM1-6 are shown as controls. Li, liver; Ki, kidney; Gu, gut; Sp, spleen; Lu, lung; Br, brain.

Hardon *et al.*, 1988). Mice transgenic for the construct bearing the EM3 mutation show a novel pattern of expression. After normalizing with respect to the endogenous mouse A1AT gene, the families carrying the EM3 mutation showed the following pattern of expression: EM3-2 was higher than WT16 and EM3-7 was intermediate between WT16 and WT13. The CAT activity measured in EM3-2 and EM3-7 followed the same trend (see Table I).

When the A1AT expression was normalized with respect to the corresponding CAT levels, the same values, comparable to WT, were obtained for both the mutant families. Taken together, our results suggest that the EM3 mutation has no effect on A1AT expression in adult liver. However, it appears to affect transcription during development. In liver RNA from 17.5 d.p.c. transgenic mouse embryos, EM3-2 and EM3-7 expression, when normalized to the endogenous mouse A1AT gene, was reduced from 3 to 5-fold with respect to the WT construct (see Figure 5). When normalized to the CAT activity the level of transcription in the two EM3 families was 13% and 31% of the human wild type. Moreover, no expression of human A1AT could be detected in EM3 transgenic mouse yolk sac, suggesting that the mutation has a stronger effect on transcription in embryonic tissues than in adult tissues (see Figure 5). It is also of note that no expression of human A1AT could be detected in adult tissues such as kidney and gut, even though expression was seen in these tissues in WT transgenic mice.

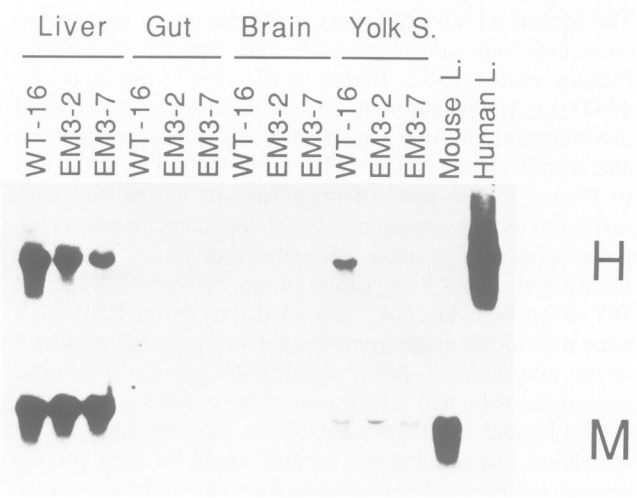

Fig. 5. Expression pattern of WT and EM3 mutants A1AT genes in embryos. RNA was extracted from transgenic embryos (17.5 d.p.c.) of the lines WT16, EM3-2 and EM3-7. Two autoradiographs of a Northern blot are shown. H is hybridized with the human cDNA coding for A1AT. M is the same filter rehybridized with a mouse cDNA coding for A1AT.

Discussion

In this paper we have attempted to evaluate in transgenic mice the individual contribution of single *cis*-acting elements and therefore of the corresponding transcriptional factors on the expression of the human A1AT gene during development and differentiation. The importance of extending the studies, originally carried out in simplified cell systems, to transgenic mice is twofold. Firstly, there is the obvious advantage of being able to study transgene expression in multiple tissues throughout development. Secondly, previous work has shown that data from experiments involving transfected cells are not always consistent with that from transgenic mice. For some genes, additional *cis*-acting elements are required for expression in transgenic mice. For example, albumin expression in transgenic mice requires an element some distance away from the promoter, which is not necessary for expression in transfected cells (Pinkert *et al.*, 1987). Also, studies of the MHC gene Eα have shown that the

removal of a 680 bp fragment from the promoter of the gene has much less influence on Eα expression in cultured B cell lymphoma lines that it does in transgenic mice (Dorn *et al.*, 1988).

We wanted to establish whether elements judged to be important in transfection experiments and in *in vitro* transcription are of comparable significance in the context of large genomic fragments including the relevant coding sequence, and in different tissues throughout development. In this study we have analysed the behaviour of mutations in the binding sites of the two transcriptional factors LF-A1 and LF-B1.

Through the homologous recombination protocol, we introduced the desired mutation within the context of a much larger DNA segment, containing all the introns and exons of the A1AT gene and additional 5′ and 3′ flanking sequences; we also normalized our results either to the expression of the mouse endogenous gene or to an internal marker, the RBP-CAT transcriptional unit. We believe that this type of internal control is an advantage in studies that attempt to abolish the expression of a transgene. To try and quantify the level of expression of the test gene in the absence of such an internal control could yield misleading results, as a consequence of variable copy numbers of the transgene or of different random insertion sites in the genome. We cannot of course exclude that the presence of the RBP gene in front of the A1AT gene affects the expression of the latter. However, the difference in expression observed in WT and mutants suggests a neutral effect of the RBP gene.

The role of LF-B1 in the transcription of the human A1AT gene

The *trans*-acting factor LF-B1 is involved in the regulation of several liver-specific genes (De Simone and Cortese, 1988). We have shown in the present study that the integrity of the binding site for LF-B1 is essential for the expression of the A1AT gene in mouse embryos and in non-hepatic tissues and is necessary for maximal A1AT expression in adult liver. The results suggest that the binding of LF-B1 to the unique site in the proximal promoter element of the A1AT gene is essential in yolk sac and in fetal liver and cannot be compensated by the presence of several thousand bases upstream or downstream of the transcription start site. This result confirms and extends the previous evidence that LF-B1 plays an important role in regulating the transcription of A1AT. In particular the data show lack of functional redundancy of the B element within the context of what might legitimately be considered the entire gene.

Differential effect of disruption of the LF-A1 binding site during development

LF-A1 is a transcription factor involved in the regulation of a subset of genes expressed in liver, partially overlapping with the subset of genes dependent on LF-B1 (De Simone and Cortese, 1988). Mutations in the LF-A1 binding site abolish transcription from the A1AT promoter, as established in transfection experiments (De Simone *et al.*, 1987) and reduce it *in vitro* in nuclear extracts (Monaci *et al.*, 1988). In the present study we show that destruction of the LF-A1 binding site causes no change in transcription in the adult liver. It seems likely therefore that the result of the *in vitro* experiment with constructs carrying only the promoter sequence, from base −137, have over-emphasized the importance of the A element, which appears to be dispensable in adult liver. This result can be compared with those obtained by Swift *et al.* (1989) in a study of the pancreas specific elastase promoter. Mutation at three different sites led to transcriptional inactivation of this promoter in transfected pancreatic acinar cell lines but had no effect on the expression of the same construct in transgenic mice. In the latter system, multiple mutations were needed to reduce transcription, leading to the interesting conclusion that cultured cell lines might have lost the capacity to synthesize a transcription factor normally present in adult pancreas. Our experiment on the A1AT gene differs in an important aspect from the experiment carried out on the elastase promoter (Swift *et al.*, 1989). These authors have constructed a test gene carrying only the promoter of the elastase gene, from N −205 to N +8, fused to either CAT or the human growth hormone gene, and therefore could not analyse the potential enhancement or restriction to the expression which might be exerted by sequences outside the short DNA fragment used. More interestingly, mutation in the A site causes reduction in transcriptional activity in fetal liver and abolishes transcription in the yolk sac. This supports the conclusion that the A element of the A1AT promoter has a 'specialized function' particularly in 'extra-hepatic' tissues.

In the light of results that revealed an unexpected A1AT expression during embryogenesis in several other sub-populations of cells in different organs (Koopman *et al.*, 1989) it will be interesting to examine in detail the pattern of expression of the mutant transgene thus clarifying in more detail the role of the A and B elements.

Materials and methods

Construction of plasmids and cosmids

pCos-RBP/A1AT cosmid was obtained by ligation of the 18 kb *SalI* fragment from the RBP gene (D'Onofrio *et al.*, 1985) and the 17.5 kb *SalI* fragment from the α1-antitrypsin gene λAT73 (Ciliberto *et al.*, 1985) into the *SalI* site of pCos2 (Poustka *et al.*, 1984). This cosmid was used as an acceptor in a homologous recombination reaction in *E.coli*, described in detail in Tripodi *et al.*. (1990). This led to the insertion of the CAT coding region downstream from the RBP promoter. The donor was a pEMBL-CAT derivative containing the *Hin*dIII−*Cla*I segment from pSV2-CAT (Gorman *et al.*, 1982) flanked at the 5′ end by a segment of the RBP gene, from N −1650 to N +36 and fused to the CAT coding region and at the 3′ end by another segment of the RBP gene, from N +59 to N +448. The resulting cosmid recombinant, pCos-RBP-CAT/A1AT (referred to as WT in the text) was used as an acceptor for the recombination with DNA segments carrying mutations in the A1AT promoter. The plasmid donors were pEMBL derivatives containing the A1AT DNA segment from N −261 to N −37, carrying the EM3 or the PM1 mutations (De Simone *et al.*, 1987). The resulting cosmids pCos-RBP-CAT/A1AT-EM3 (referred to as EM3 in the text) and pCos-RBP-CAT/A1AT-PM1 (referred to as PM1 in the text) were used for the construction of transgenic mice.

Transgenic mice

WT, EM3 and PM1 cosmids containing RBP-CAT-α1-antitrypsin genes were linearized in the *Cla*I site of the vector and were injected into fertilized (C56Bl/6J×CBA) F_1 mouse eggs according to Hogan *et al.* (1986). Founders were analysed by Southern blots in order to establish the overall structure of the inserted foreign fragment and the copy number. In order to confirm that the EM3 or the PM1 mutations were present, DNA from transgenic mice was amplified by PCR using primers specific for the 5′ region of the transgene. Since both the mutations created an artificial *Eco*RV site in the construct, digestion of the PCR product with *Eco*RV gave a pattern diagnostic for each mutation, and so confirmed the presence of the relevant mutation in all transgenic lines. Subsequent generations of mice were screened by PCR (Abbott *et al.*, 1988).

Transcriptional analysis

RNA extraction from various tissues was performed as described in Lovell-Badge (1988). Northern blots were performed using Hybond N membranes (Amersham); hybridization, washing and rehybridization were carried out according to the protocol recommended by the manufacturer. Autoradiographs (Underexposure of gels shown and of others not shown) were analysed using an A300 Molecular Dynamics Computing Densitometer. Protein extracts for CAT assays were made by freeze–thawing tissues in 0.25 M Tris–HCl pH 7.8. Protein concentrations of extracts were measured using the Bio-Rad protein assay kit. After heat inactivation for 10 min at 65°C, CAT enzymatic activity was assayed according to Gorman *et al.* (1982). The CAT assays were carried out in duplicate at three different protein concentrations in each case. Quantification was performed according to De Simone *et al.* (1987).

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