Cloning of the human α_1 antichymotrypsin gene and genetic analysis of the gene in relation to α_1 antitrypsin deficiency

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SUMMARY Deficiency of α_1 antitrypsin (Pi) is clinically heterogeneous and the unpredictability of the clinical manifestation in a person of phenotype PiZ, which may vary from severe childhood liver disease to normal health, is a problem in genetic counselling. This problem may increase as couples at risk who have not had an affected child are identified in screening programmes. One possibility is that genetic variation of other protease inhibitors may influence the prognosis.

With this in mind we report the isolation of the human gene for α_1 antichymotrypsin (AACT) on a series of cosmid clones, with restriction mapping of about 70 kb around the gene. A probe pACE3-4 derived from the 5' end of the gene defines sequences which have been assigned to chromosome 14 using somatic cell hybrids and has been used to show a common *TaqI* polymorphism with allele frequencies of $AACT^6=0.7$ and $AACT^3=0.3$ in Europeans. pACE3-4 is closely linked to α_1 antitrypsin (maximum lod score in males $+2.29$ at $\theta=0$; in females $\hat{Z}=+6.11$ at $\theta=0.032$). Analysis of Pi-AACT haplotypes in 31 families ascertained through PiZ or PiSZ subjects did not show any linkage disequilibrium. The distribution of $AACT^6$ and $AACT^3$ alleles in 16 unrelated PiZ patients presenting with childhood liver disease and five unrelated PiZ patients with adult chest disease did not differ significantly from each other. These results suggest that if genetic variation at the AACT locus does influence the outcome of α_1 antitrypsin deficiency, such variation is not in linkage disequilibrium with the AACT polymorphism reported here.

The human genes coding for the two serine protease inhibitors α_1 antitrypsin (AAT or Pi) and α_1 antichymotrypsin (AACT), which have approximately 42% homology at the protein level,¹ have both been mapped to 14q31→32.2.² AAT has been widely studied and is a major serum protein whose principal target is thought to be leucocyte elastase. Many genetic variants have been described, the most important clinically being the PiZ allele. Homozygotes of type PiZ occur at a frequency of about 1 in 4000 in North European populations; these subjects are at risk of developing emphysema and a proportion of them develop severe liver disease, the pathogenesis of which is not understood.^{3,4}

Much less is known about genetic variation of AACT, a protease inhibitor whose physiological substrates are thought to be cathepsin G and mast cell chymase.^{5,6} It has also been found to reduce the cytotoxic activity of T cell killer lymphocytes.⁷ AACT is one of the major acute phase reactants in plasma, rising up to four times its basal level within eight hours of trauma or endotoxic shock.⁸ This response is greater and more rapid than that of AAT. The exact role of AACT in man is not understood.

We report here the isolation of the human AACT gene and preliminary analysis to establish its genetic distance from AAT. We were also interested to investigate whether the striking linkage disequilibrium which has been reported between the Z allele of AAT and various restriction sites in the neighbourhood of AAT might extend as far as AACT.

Materials and methods

CONSTRUCTION OF GENOMIC LIBRARY

A cosmid library was constructed using a fibroblast cell line CAR, grown out from a female abortus of approximately 19 weeks' gestation, which had been typed as homozygous type PiZ for AAT. High molecular weight DNA from CAR was partially digested with *Sau3A* and cloned into the *Bam*HI site of the vector Homer IV.⁹ The library comprised 650 000 independent recombinants and was maintained in an unamplified form. The techniques used were essentially those of Grosveld *et al.*¹⁰

SCREENING THE LIBRARY

pHAC1, a cDNA probe for human antichymotrypsin, was kindly provided by Dr Robert Hill.¹¹ The 1.1 kb insert was gel purified before use. Duplicate pairs of filters on Genescreen plus were screened using pHAC1. Hybridisation was carried out at 42°C overnight in a mixture containing 50% deionised formamide, 1 mol/l NaCl, 5×Denhardt's, 1% SDS, 100 µg/ml herring sperm DNA, 10 µg/ml poly A, and 1.5 ng/ml of ³²P labelled probe. Filters were washed to a final stringency of 3×SSC, 0.1% SDS (1×SSC=0.15 mol/l NaCl+0.015 mol/l trisodium citrate) at 65°C and exposed to XOMAT AR film at -70°C with intensifying screens for between six and 18 hours.

ANALYSIS OF COSMID CLONES BY RESTRICTION MAPPING

Restriction maps of recombinant cosmids were constructed using the partial digestion strategy previously described,⁹ slightly modified. Cosmid miniprep DNA was linearised at the *Sal*I site within the vector. Approximately 1 µg amounts of linearised DNA were subjected to partial digestion with additional enzymes in the presence of 4 mmol/l spermidine. One tenth of each digest was loaded on to a 0.3% agarose gel. Duplicate nitrocellulose blots from these gels were hybridised separately to a 2.2 kb *Hind*III-*Sal*I and a 1.7 kb *Pst*I-*Sal*I fragment of Homer IV, labelled by nick translation. From the sizes of the partially digested cosmid fragments, indirectly end labelled with the vector probes, approximate restriction maps were deduced. These were verified by standard single and double digestion followed by electrophoresis on 0.7% agarose gels.

ANALYSIS OF HUMAN GENOMIC DNA

High molecular weight DNA was prepared as previously described.¹² In general, approximately 5 µg of DNA from each sample were digested according to manufacturer's instructions in the

presence of 4 mmol/l spermidine and electrophoresed in 1% agarose gel using tris/acetate/EDTA or tris/borate/EDTA buffers.¹³ Samples were blotted onto Genescreen Plus and hybridised at 65°C overnight without formamide, as described in the Genescreen instructions. DNA probes were labelled with α^{32} PdCTP (Amersham) either by nick translation or by random priming.¹⁴

SOMATIC CELL HYBRIDS

DNA from a previously characterised panel of human-rodent somatic cell hybrids¹⁵ was available. Two additional hybrids were used. One, FST7, has been previously described.¹⁶ SCAF 8 is the result of a fusion between a rat hepatoma and a human fetal fibroblast of phenotype PiZ, and is reported here for the first time. The method of analysis of this DNA was exactly as described above for human genomic DNA except that approximately 10 µg of DNA were loaded per track.

IDENTIFICATION OF PI PHENOTYPES

Plasma samples from blood collected in EDTA were treated with dithiothreitol and iodoacetic acid before polyacrylamide gel isoelectric focusing. The pH gradient was established with 'Pharmalyte' pH 4.2 to 4.9 and ACES. The focused gels were stained with Coomassie blue.

IDENTIFICATION OF PI GENOTYPES

Many of the samples were analysed for various restriction fragment length polymorphisms in the region of the *AAT* gene. These included the *Ava*II polymorphisms previously described by Cox *et al.*,¹⁷ where there are two sites with alternate alleles designated 5 or 7 and 1 or 4 respectively,^{17 18} and the *Mae*III polymorphism also previously described by Cox and Billingsley¹⁹ with alternate alleles of 2.7 and 2.4. This variation was detected using a 6.5 *Bam*HI fragment from the 3' end of the *AAT* gene. In addition, a clone derived from a related sequence *ATR*, situated 10 kb downstream of *AAT*, was used to detect an *Eco*RI polymorphism.^{20 21} This allowed the *M1* allele identified by isoelectric focusing to be subdivided into *M1A* (usually *Ava*II 74, *Mae*III 2.7 *ATR* 5.6) or *M1B* (usually *Ava*II 51, *Mae*III 2.4 *ATR* 7.0). It also allowed the confirmation of each *PiZ* allele as (*Ava*II 71, *Mae*III 2.7, *ATR* 7.0).

FAMILY MATERIAL

Members of 30 families in which at least one subject had been typed as phenotype PiZ were collected from England and Scotland. Eight of these were ascertained through AAT deficiency related emphysema, mostly through a survey carried out by the British Thoracic Association,²² one through adult

liver disease (patient of Dr Gunn, Dundee), and 21 through liver disease in childhood, mostly children attending King's College Hospital. Two families were ascertained through a PiSZ proband, one because of chest disease, one by chance. Although in some of the PiZ families the proband was dead, with one exception at least one PiZ subject was available for testing in each family. The total numbers of PiZ subjects tested were seven with chest disease (including three sisters), 19 with liver disease (including a brother and sister), and seven apparently healthy (one father of a patient, four sibs of patients with chest disease, and two sibs of patients with liver disease). In one family the only PiZ subject available was a terminated fetus. All were of European origin.

One additional healthy (CEPH number 12) family was studied, kindly provided by Professor J M Dausset.

Results

ISOLATION OF COSMIDS FOR THE AACT GENE
Screening of 280 000 cosmids with the cDNA insert of pHAC1 resulted in the isolation of six inde-

pendent positive clones (fig 1). Each cosmid contained at least one *Eco*RI fragment of 4.6 kb and five also had a 1.85 kb band. The cDNA probe hybridised strongly to both the 4.6 kb band and the 1.85 kb band, and to a lesser extent to the 3.4 kb band seen in three of the cosmids. The sizes of these three bands agreed precisely with the size of fragments seen in genomic DNA probed with the cDNA probe pHAC1 (fig 1), and also with restriction maps obtained from two clones isolated independently from a bacteriophage library (data not shown). The *Eco*RI site map for each cosmid was determined and they were ordered into an overlapping series (fig 2a). The region hybridising to the cDNA comprised two *Eco*RI fragments of 4.6 kb, one of 1.85 kb, and one of 3.4 kb. Two recombinants were mapped with additional enzymes (fig 2b). All the AACT gene fragments seen on genomic Southern blots were identified within the cosmids as fragments hybridising to the cDNA probe. The transcriptional orientation of the gene was inferred from the hybridisation of two non-overlapping subfragments of the cDNA to restriction fragments of the cloned gene. A 3.4 kb *Eco*RI fragment, located towards the 5' end of the gene and contain-

FIG 1 ... (a) *Eco*RI digests run on a 0.7% gel of DNA of recombinants from the CAR library identified by hybridisation to the AACT cDNA pHAC1. (b) Autoradiograph of a blot of the gel in (a) hybridised against pHAC1. (c) Autoradiograph of a blot of human genomic DNA digested with *Eco*RI, run on 0.7% gel, and hybridised to pHAC1.

ing no repeat sequences, was subcloned into pUC9 and designated pACE 3-4 (fig 2b). The insert from this plasmid was used as a probe for AACT.

CHROMOSOME ASSIGNMENT OF pACE3-4

DNA from a panel of 11 independent somatic cell hybrids was digested with *TaqI* and probed with pACE3-4. All bands hybridising to this probe (see

below for description) could be unequivocally assigned to chromosome 14. There were at least three examples of discordance with every other chromosome (table 1).

GENETIC ANALYSIS OF AACT

Screening of genomic DNA from 10 unrelated subjects with pACE3-4 showed a common poly-

FIG 2 (a) The six HAC cosmids mapped in terms of *EcoRI* restriction sites. The approximate position of the AACT coding sequences are indicated by solid bars. The shaded bar indicates the position of a genomic region hybridising weakly to the cDNA clone, so the region of overlap may be small. (b) Detailed restriction map of two of the HAC cosmids, showing derivation of the probe pACE3-4. The asterisk indicates the polymorphic *TaqI* site.

TABLE 1 Segregation of pACE3-4 in 11 independent human rodent hybrids.

Hybrids	pACE3-4	Chromosomes																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
F4SC13cl12	+	·	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+
SCAF8	+	-	-	+	-	+	+	+	+	-	-	+	-	+	+	+	+	-	-	-	-	-	+	+
FST7	+	-	-	+	-	-	-	+	-	+	·	·	·	+	-	+	+	-	+	+	+	-	+	+
FST9/10	+	-	-	+	+	-	+	-	+	·	+	-	+	+	+	+	-	-	+	-	+	-	+	+
SIF4A31	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+
SIF5P5	+	-	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+
HORP9-5	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	·	+
HORL411B6	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-	+	-	-	+	-	-	-	-	+
Clone 21	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
SIF4A24E1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+
FG10	-	-	+	-	-	+	·	-	+	·	+	-	-	-	-	+	-	-	+	-	-	+	-	+
Concordant		3	4	7	5	5	7	6	5	4	7	4	8	4	11	4	7	3	4	5	6	4	7	8
Discordant		7	7	4	6	6	3	5	6	5	4	6	3	6	0	7	4	6	7	6	5	7	3	3

+ indicates human chromosome present. - human chromosome absent.
· means equivocal results or part of chromosome.
For sources of hybrids see text.

morphism detectable after *TaqI* digestion (fig 3). The two alternative alleles are referred to as *AACT*⁶ and *AACT*³ for convenience, although more accurate sizing suggests they are actually closer to 5.8 kb and 2.8 kb, with a constant band of 0.8 kb. Family studies showed Mendelian inheritance in a co-dominant manner. Double digestions of cloned and genomic DNA identified the site of this polymorphism (fig 2b).
Seven of the PiZ or PiSZ families were fully informative for linkage between *AACT* and the Pi protein polymorphism. CEPH family number 12

FIG 3 Autoradiograph of blot from three samples of human genomic DNA, digested with *TaqI* and probed with pACE3-4, to show the different phenotypes. Left to right, *AACT*³, *AACT*⁶⁻³, *AACT*⁶.

was also informative for *AACT* and *ATR*. The lod scores computed by LIPED using the whole data set are shown in table 2. Only one recombinant was found in at least 35 informative meioses, the maximum lod score in males being +2.29 at $\theta=0$, and in females +6.11 at $\theta=0.032$.
Of 62 unrelated subjects of European origin, 29 were *AACT*⁶, 28 *AACT*⁶⁻³, and five *AACT*³, giving a good fit to a Hardy-Weinberg distribution and gene frequencies of *AACT*⁶=0.7 and *AACT*³=0.3. Because many of these subjects were selected as members of PiZ families, and in view of the linkage disequilibrium already reported in the region of the AAT gene, it was recognised that this sample might represent a biased estimate of the *AACT* gene frequencies. Assuming no recombination it was possible to calculate the frequency of different Pi-*AACT* haplotypes in the families and these data are shown in table 3. The classification of Pi is mainly on the protein data but some of the M1 alleles have been subdivided by restriction fragment polymorphisms, as described in Materials and methods. There is no evidence for linkage disequilibrium. In particular, the distribution of *AACT*³ and *AACT*⁶ alleles in Z and non-Z haplotypes is identical ($\chi^2=0.07$, $p>0.1$).
The *AACT* genotypes in all PiZ subjects tested (some of whom are related to one another) are

TABLE 2 Lod scores for various values of the recombination fraction between *AACT* and Pi.

	Recombination fraction						
	0.00	0.01	0.05	0.1	0.2	0.3	0.4
Male	2.288	2.229	1.992	1.697	1.222	0.603	0.204
Female	-∞	5.907	6.047	5.629	4.420	2.999	1.475

TABLE 3 Distribution of AACT⁶ and AACT³ alleles in chromosomes carrying different Pi alleles.

Pi	AACT	
	6	3
M1 Total	32	14
M1A	5	4
M1B	14	6
M2	6	6
M3	4	1
S	1	1
Total non-Z	43	22
Z	41	19

TABLE 4 Distribution of AACT genotypes in PiZ subjects according to clinical presentation. Numbers in parentheses refer to unrelated persons.

AACT	Childhood liver disease	Lung disease	Healthy	Fetus
6-6	7 (7)	2 (2)	4 (3)	0
6-3	6 (6)	5 (3)	2 (1)	0
3-3	4 (3)	0 (0)	0	1 (1)

One patient of phenotype AACT 6-6 presented with adult liver disease and is not included. The genotypes of three small children were deduced from their parents. One family in whom the PiZ protein was unusual has been excluded and is the subject of further investigations.

shown in table 4, which has been divided up according to the clinical presentation of the subject. The numbers in parentheses are numbers of unrelated subjects. In this case the first PiZ subject in the family from whom DNA was obtained is listed. Although the numbers are very small there is no suggestion of a different distribution of genotypes in different clinical presentations.

Discussion

In contrast to the many studies on AAT, the genetics of AACT at the protein level has received little attention, and normal electrophoretic variation of this protein in man has not been described. There are, however, several reports of partial deficiency, some of which are genetically determined.^{23 24} Although several of these families have been ascertained through a patient with liver or lung disease it is not clear whether the frequency of partial AACT deficiency is greater in such patients than in normal healthy controls.²³ The isolation of genomic clones for AACT, reported here and also previously,² should allow an understanding of the deficiency at a molecular level. It should also be possible to devise experimental systems to analyse the regulation of

expression of the human AACT gene. The assignment of pACE 3.4 to chromosome 14 using somatic cell hybrids provides an independent confirmation of the previous result,² which was obtained using in situ hybridisation.

One of the problems which prompted this study was the clinical heterogeneity of α_1 antitrypsin deficiency. It now seems well established that the molecular basis of the Z mutation is the same in all subjects.¹⁷ Only a small proportion of PiZ homozygotes (about 7%) develop severe liver disease in childhood. There is some evidence that severe liver disease is familial. In one study in England, in a family with a child already affected with severe liver disease, the chance of severe liver disease in a second PiZ child was estimated as 80%.²⁵ However, other authors have found a lower figure and a combination of all data would suggest about 40%.¹⁸ It is not known if the familial factor(s) is environmental or genetic. The occurrence of severe liver disease in only one of a pair of monozygotic twins²⁵ indicates that at least some non-genetic factor is involved. There have been two reports suggesting that breast feeding may protect against the liver disease.^{26 27} We have preliminary evidence for an increase in frequency of DR3 antigens in those PiZ patients with liver disease.²⁸ Another possibility is that genetic factors linked to AAT might influence the outcome. Two possible candidate genes would be the related sequence gene *ATR* or *AACT*. The likelihood that genetic variation at the *ATR* locus itself might influence the prognosis seems remote, especially since we have some evidence that this gene is not expressed.²⁹ It is, however, interesting that the linkage disequilibrium which has been reported in the Z gene appears to extend to the *EcoRI* site of the *ATR* gene.²¹ The *AACT* gene is a more serious candidate for some interactive effect. Our family studies show *AAT* and *AACT* to be tightly linked and preliminary mapping data from pulse field electrophoresis suggest they are within 120 kb of each other.³⁰ The *TaqI* polymorphism detected by pACE 3.4 may indeed have some use in prenatal diagnosis of PiZZ, since it gives rapid results on small quantities (about 1 μ g) of genomic DNA. We have so far no evidence for linkage disequilibrium between *Pi* and *AACT*; in particular the Z allele appears to be in equilibrium with the *TaqI* polymorphism of *AACT* reported here.

Although the data are small, there is at present no suggestion that the clinical status of a subject homozygous for PiZ can be predicted from the genotype for AACT. The question of whether genetic variation at the AACT locus may influence the clinical outcome of α_1 antitrypsin deficiency is not yet completely answered. Two arguments suggest

that it is probably not a major factor. One is that on our limited data the distribution of *AACT*³ and *AACT*⁶ alleles appears to be similar in patients with different clinical presentation. Hence if genetic variation at the *AACT* locus is involved, this variation is not in linkage disequilibrium with the polymorphism reported here. The other argument comes from the close genetic linkage found here between *Pi* and *AACT*. The existence of several sibships with one child severely affected with liver disease and one healthy would predict that a gene with a major effect would not be very tightly linked to *Pi*. However an interaction of a closely linked gene such as *AACT* and an environmental factor cannot be ruled out. It would be of interest to examine sibships discordant for clinical manifestations to see if any of them represent the rare cross over events between *Pi* and *AACT*.

After this manuscript was prepared it came to our notice that another map of the human antichymotrypsin gene has been published.³¹ This map differs significantly from our own, especially in the 3' region of the gene. Our own data are incompatible with the previously published map. The reason for this is not clear at present.

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