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Assignment of the gene for cytosolic alanine aminotransferase (AAT1) to human chromosome 8

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SUMMARY

The segregation of human cytosolic alanine aminotransferase (AAT1) and the individual human chromosomes has been studied in 27 secondary and tertiary rat hepatoma-human (liver) fibroblast hybrids. The staining solution used to visualize AAT activity on starch gels was specific for AAT since it was visualized only when all components of the stain were present. The locus for human AAT1 has been assigned to chromosome 8.

INTRODUCTION

Alanine aminotransferase (AAT) (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2) catalyses the reversible reaction:

$$\text{alanine} + \text{2-oxoglutarate} \rightleftharpoons \text{glutamate} + \text{pyruvate}.$$

Cytosolic and mitochondrial AAT activities have been found in mammals (Swick, Barnstein & Stange, 1965). These enzymes play a key role in gluconeogenesis via the glucose-alanine cycle (Felig, 1975). In muscle, pyruvate is transaminated by the cytosolic enzyme (AAT1) to alanine (DeRosa & Swick, 1975), which is then released into the circulation for uptake by the liver. In the hepatocyte, alanine is converted to pyruvate primarily by mitochondrial AAT (Dieterle *et al.* 1978), and the hepatic pyruvate is then metabolized to glucose.

The cytosolic enzyme has been characterized in rat liver (Gatehouse *et al.* 1967; Matsuzawa & Segal, 1968) pig heart (Saier & Jenkins, 1967) and human liver (Hopper & Segal, 1964; Kamoda *et al.* 1980). In addition, the cytosolic enzyme is present in human kidney, muscle, pancreas and erythrocytes (Maclagan, 1970; Kamoda *et al.* 1980). The specific activity of the cytosolic enzyme varies markedly with the tissue source, the hepatic enzyme having the highest specific activity (Kamoda *et al.* 1980). Although human erythrocytes have only 10% of the hepatic specific activity (Maclagan, 1970), the erythrocyte enzyme has been the subject of extensive genetic studies. Chen & Giblett (1971) were the first to find a polymorphism for human cytosolic erythrocyte AAT. To date, at least 11 different alleles have been identified at the erythrocyte AAT locus (Chen *et al.* 1972; Kompf & Ritter, 1979). It should also be noted that the serum AAT activity has proven to be a useful indicator of hepatocellular injury (Combes & Schenker, 1969).

The previous demonstration that rat hepatoma cells expressed cytosolic AAT1 and that the activity was inducible by dexamethasone (Lee & Kenney, 1970; Sparkes & Weiss, 1973) suggested that somatic cell hybrids made from these hepatoma cells and human fibroblasts derived from liver might be useful for the chromosomal localization of the human cytosolic AAT1

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gene. We report here the assignment of the structural gene for cytosolic AAT1 to human chromosome 8 using rat hepatoma-human (liver) fibroblast hydrids. These hybrids expressed the human and rat AAT isozymes at high levels without glucocorticoid induction. Since a previous report (van Someren *et al.* 1974*b*) had suggested that the staining solution used to visualize AAT also detected other enzymes, the specificity of the stain was investigated in both hybrid cells and other tissues.

MATERIALS AND METHODS

Cell lines and hybrid cell formation

The rat hepatoma cell line, FU5AH (HGPRT$^-$), was initially established by Dr Mary Weiss (Schneider & Weiss, 1971; Weiss & Chaplain, 1971) and was generously provided by Dr Marcello Siniscalco. Fresh human foetal liver was minced, treated with 0·05 % collagenase, washed in serum free Dulbecco's Medium (DMEM, GIBCO, Grand Island, NY) and then the individual cells were established in culture using standard techniques. The cells were grown in culture for several weeks before fusion, at which time the majority of these cells appeared fibroblastic in morphology. Hybrids were produced by the fusion of rat hepatoma FU5AH cells and the foetal human (liver) fibroblasts. For fusion, the cells were mixed together in serum free DMEM containing 47 % polyethylene glycol (molecular weight 6000) and 15 % dimethylsulphoxide for 2 min (Shay, 1977). Additional serum free medium was added, and the cells were washed twice before plating in DMEM containing 10 % foetal calf serum (GIBCO), 10 mM hypoxanthine, 40 μM aminopterin, 16 μM thymidine and 5 μM ouabain. A single colony was observed after several weeks in the selective medium. This colony was isolated, expanded and subcloned by placing 100 cells in 25 cm^2 plastic tissue culture flasks. Individual clones, usually one from each flask, were isolated with cloning rings. Several of these secondary clones were further subcloned to give tertiary clones.

Preparation of cell extracts

Confluent hybrid cells were removed from 75 cm^2 plastic tissue culture flasks using a rubber policeman. The cells were washed twice with Dulbecco's phosphate buffered saline (GIBCO) and after centrifugation at 200 g, the cells were either used immediately or frozen at −70 °C. For enzyme analysis, the cells were suspended in 25–100 μl of water, subjected to one cycle of freezing (−70 °C) and thawing (37 °C), centrifuged at 14000 g for 20 min, and the supernatant was removed for electrophoretic studies. Fresh rat or fresh frozen human liver was homogenized in an equal volume of water or 0·1 M Tris-HCl buffer, pH 7·5, centrifuged at 14000 g for 20 min and then the supernatant was removed for electrophoresis.

Analysis of AAT

AAT activity in tissues and hybrid cells was analysed by starch gel electrophoresis. The electrophoretic system was adapted from the method used for separation of phosphoglycolate phosphatase (Povey *et al.* 1980). The bridge buffer was TEMM (0·1 M Tris-HCl/0·01 M disodium EDTA/0·1 M maleate/0·01 M-$MgCl_2$) adjusted to pH 7·2 with NaOH. The gel buffer was a 1 to 10 dilution of the bridge buffer. In some gels 2-mercaptoethanol (0·1 ml of 0·1 M mercaptoethanol per 100 ml) was added to the gel buffer. No differences were observed in the separation of the

Table 1. *Enzyme markers examined in rat-human hybrids*

Chromosome	Enzyme*	Reference	Required modification†	
			Bridge buffer	Dilution for gel buffer
1	PGM1	Harris & Hopkinson, 1976	—	—
2	MDH1	—	TEMM, pH 7·4	1/10
3	ACY1	Voss *et al.* 1980	—	—
4	PGM2	Harris & Hopkinson, 1976	—	—
5	HEXB	—	0·2 M-Na_2HPO_4/ 0·03 M-H_3BO_3, pH 7·0	1/10
6	ME1	—	TEMM, pH 7·4	1/10
7	βGUS	Chern & Crose, 1976	—	—
8	GSR	Van Someren *et al.* 1974*a*	—	—
9	AK1, 3	Wilson *et al.* 1976	—	—
	ACO1	Meera Khan *et al.* 1978	—	—
10	GOT1	—	0·24 M-NaH_2PO_4/ 0·15 M-$Na_3C_6H_5O_7$, pH 7·0	1/80
11	ESA4	—	TEMM, pH 7·2	1/10+2 ME
12	PEPB	—	TEB, pH 8·6 (1/7 dil)	1/10
13	ESD	—	TEMM, pH 7·2	1/10+2 ME
14	NP	—	0·24 M-NaH_2PO_4/ 0·15 M-$Na_3C_6H_5O_7$, pH 7·0	1/10
15	MPI	—	TEMM, pH 7·2	1/10
16	PGP	Povey *et al.* 1980	—	—
17	—	—	—	—
18	PEPA	—	TEB, pH 8·6 (1/7 dil)	1/10
19	GPI	—	0·1 M-Tris-HCl/ 0·1 M-NaH_2PO_4, pH 7·4	1/10
20	ADA	Harris & Hopkinson, 1976	—	—
21	SOD1	—	0·1 M-phosphate, pH 7·5	1/10
22	ACO2	Meera Khan *et al.* 1978	—	—
X	G6PD	Meera Khan, 1971	—	—

* Shows & McAlpine, 1979.

† Buffer system modifications required for optimal separation of rat and human isozymes. TEMM = 0·1 M Tris-HCl/0·1 M maleate/0·01 M-Na_2EDTA/0·01 M-$MgCl_2$; TEB = 0·9 M Tris-HCl/0·5 M boric acid/0·02 M EDTA; ME = 2-mercaptoethanol.

isozymes in the presence or absence of 2-mercaptoethanol. The gel was run overnight at 5 V/cm at 4 °C.

AAT was visualized using the method described by Harris & Hopkinson (1976), except the concentrations of the components were modified. 700 mg of DL-alanine, 200 mg of 2-oxoglutaric acid monosodium salt, 10 mg NADH, 75 μl of lactate dehydrogenase (LDH, 10000 units/ml; Sigma Chemical Co., St. Louis, MO) in 25 ml of 0·5 M Tris-HCl buffer, pH 7·5, and 25 ml of 2% agar noble (Difco Laboratories, Detroit, MI) were present in the complete staining solution. In the stain containing only 2-oxoglutaric acid and NADH, ammonium chloride (10 mg) was added. In some experiments the initial staining was followed by a second staining step. Methyl thiazolyl tetrazolium (MTT; 5 mg) and meldola blue (MLB; 100 μg) in 25 ml of 0·5 M Tris-HCl buffer, pH 7·5, and 25 ml of 2% agar were applied to the surface of the gel after incubation with the above stain. All chemicals were from Sigma Chemical Co. (St. Louis, MO) except for MLB which was obtained from Boehringer-Mannheim (Indianapolis, IN).

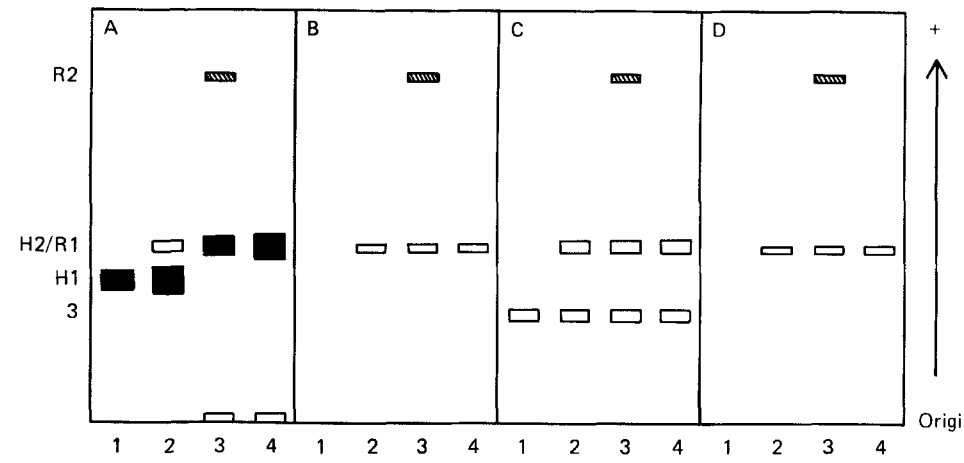

Fig. 1. Diagram showing the activity bands visualized following starch gel electrophoresis and staining for AAT isozymes. Gel A, complete stain containing alanine, 2-oxoglutarate, NADH and LDH; gel B, complete stain without alanine; gel C, complete stain without LDH; and gel D, stain without alanine and LDH. Lane 1, human erythrocytes; lane 2, human liver; lane 3, rat hepatoma FU5AH cells; lane 4, rat liver.

Identification of human chromosomes in hybrids

The human chromosomes in the hybrid cells were identified by karyotype and/or enzyme marker analyses. Metaphase spreads were stained first with Giemsa-11 (Friend *et al.* 1976), destained and then restained with quinacrine hydrochloride for Q banding (Breg, 1972).

The enzyme markers examined are listed in Table 1. In many cases, the published electrophoretic conditions were modified as indicated (Table 1) for optimal separation of the rat and human isozymes. All the enzymes were separated by starch electrophoresis, except for glutathione reductase, aconitase, and glucose-6-phosphate dehydrogenase which were separated by cellulose acetate electrophoresis. The staining systems used were from the published methods referenced in Table 1 or from Harris & Hopkinson (1976).

RESULTS

Specificity of AAT staining system

The complete staining system used to visualize AAT on gels contained alanine, 2-oxoglutarate, LDH and NADH. In this system, pyruvate generated by AAT was converted to lactate by LDH, and fluorescent NADH was converted concurrently to non-fluorescent NAD. Under ultraviolet light, AAT activity (i.e. the newly generated NAD) appeared as dark bands against a light background. The specificity of this stain was examined with samples of human liver, human erythrocytes, rat liver and rat hepatoma FU5AH cells. With the complete stain (Figs 1 and 2, gel A), human erythrocytes had only one activity band, indicating that the donor of the erythrocytes was homozygous at the AAT locus. Human liver had two bands; one (labelled H1) was a strong band which migrated to the same position as the erythrocyte isozyme, and the second was a much weaker band (H2) which migrated more anodally. FU5AH hepatoma cells had one activity band (R1) which migrated to the same position or slightly more anodally than

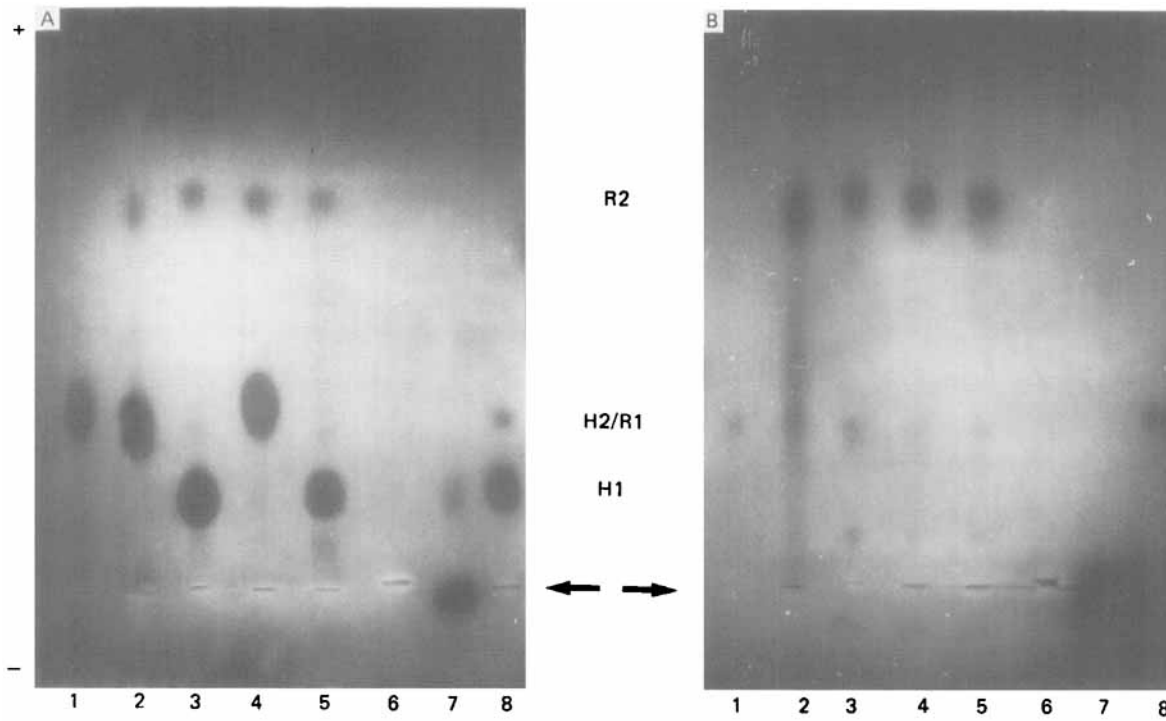

Fig. 2. Photograph showing AAT activity following starch gel electrophoresis of extracts from rat-human hybrids, parental cells and control tissues. Gel A was stained with the complete AAT staining solution and gel B was stained with the solution lacking alanine. Lane 1, rat liver; lane 2, rat hepatoma FU5AH cells; lanes 3 and 5, rat-human hybrids positive for human AAT; lane 4, hybrid negative for human AAT; lane 6, human fibroblasts; lane 7, human erythrocytes; lane 8, human liver.

the minor human liver band (H2), and a second band (R2) which migrated close to the anode. Rat liver had only a single activity band (R1). In some gels, an additional weak band was visible in rat liver and hepatoma cells at the origin after prolonged incubation (Fig. 1, gel A).

No bands were seen when 2-oxoglutarate was omitted from the stain. Exclusion of alanine or both alanine and LDH from the staining solution (Fig. 1, gels B and D), resulted in the detection of a weak band in human liver which migrated to the same position as band H2 when visualized with the complete stain; no such band was detected in human erythrocytes with the modified stains. When only LDH was omitted (Fig. 1, gel C), a single activity band (3) which migrated more cathodally than band H1 was seen in erythrocytes; human liver had the more cathodal band 3 as well as the H2 band. Both rat liver and FU5AH cells had a band in the same position as band R1 when alanine, LDH or both were absent (Fig. 1 and Fig. 2). They also had an additional band which migrated to the same position as 3 in the absence of LDH (Fig. 1, gel C). Band R2 was present with equal intensity under all staining conditions, while all other rat and human activity bands were much weaker and took longer to visualize than the activity bands seen with the complete stain.

Some gels were also stained with MTT and MLB after staining with the AAT stain. Besides the bands observed under ultraviolet light, additional bands which migrated closer to the anode were observed (not shown). Similar bands also were seen on gels stained only with MTT and MLB, suggesting that these were superoxide dismutase.

Table 2. *Segregation of AAT and human chromosomes in rat-human hybrids*

Chromosome	Enzyme markers	AAT/Chromosome: Secondary clones C +/+	C −/+	D +/−	D −/+	Tertiary clones C +/+	C −/+	D +/−	D −/+	Total C	Total D	D (%)
1	PGM1	0	2	12	0	—	—	—	—	2	12	0·86
2	MDH1	2	2	10	0	—	—	—	—	4	10	0·71
3	ACY1	5	1	7	1	—	—	—	—	6	8	0·57
4	PGM2	11	0	2	1	—	—	—	—	11	3	0·21
5	HEXB	4	1	4	0	—	—	—	—	5	4	0·44
6	ME1/SOD2	12	0	0	2	9	0	0	0	21	2	0·09
7	βGUS	4	2	8	0	—	—	—	—	6	8	0·57
8	GSR	12	1	0	1	11	2	0	0	26	1	0·04
9	AK/ACO1	0	2	12	0	—	—	—	—	2	12	0·86
10	GOT1	12	0	0	2	6	0	0	2	18	4	0·18
11	ESA4	10	1	2	1	3	0	8	2	14	13	0·48
12	PEPB	11	0	1	2	—	—	—	—	11	3	0·21
13	ESD	7	0	5	2	—	—	—	—	7	7	0·50
14	NP	8	0	4	2	—	—	—	—	8	6	0·43
15	MPI	12	0	0	2	11	0	0	2	23	4	0·15
16	PGP	5	1	7	1	—	—	—	—	6	8	0·57
17	—	5	1	7	1	—	—	—	—	6	8	0·57
18	PEPA	0	2	12	0	—	—	—	—	2	12	0·86
19	GPI	3	0	7	2	—	—	—	—	3	9	0·75
20	ADA	9	0	3	2	—	—	—	—	9	5	0·36
21	SOD1	10	1	2	1	—	—	—	—	11	3	0·21
22	ACO2	1	0	4	0	—	—	—	—	1	4	0·80
X	G6PD	12	0	0	2	—	—	—	—	12	2	0·17

Human chromosomes were determined by enzyme marker and/or karyotype analysis.
C = concordant; D = discordant.

AAT activity in cultured human fibroblasts was examined and no AAT activity was observed even when extracts from $2{\cdot}5 \times 10^6$ cells were applied to the gel.

Analysis of AAT in rat–human hybrids

When visualized with the complete stain, 23 out of 27 secondary and tertiary subclones had an activity band which co-migrated with human liver AAT (H1) (Figure 2, gel A). Of these 23 hybrids, 4 also expressed a band which migrated to the same position as the H2 and R1 activity bands seen in human and rat liver, respectively. In one hybrid, the H2/R1 band consistently was equal in intensity to the human H1 band while in the other hybrids, the H2/R1 band was weaker than the human activity. No intermediate bands were detected which would correspond to human-rat AAT hybrid proteins. Nineteen of the 23 hybrid clones had either an extremely faint band or no activity at the H2/R1 position. The four hybrids which were negative for the human isozyme had a strong band which migrated to the same position as the H2/R1 band.

When the hybrids were stained without alanine (Fig. 2, gel B), no band corresponding to the human AAT isozyme H1 was observed in any of the hybrids. However, a faint activity which migrated in the H2/R1 position was seen in all hybrids examined, whether the H2/R1 band was present or absent when visualized by the complete stain.

The segregation of human cytosolic AAT and the individual human chromosomes in 14

secondary and 13 tertiary subclones is shown in Table 2. The human chromosome with the highest degree of concordant segregation with the expression of human AAT1 is chromosome 8. Only one out of 14 secondary and none of the 13 tertiary hybrids were discordant. A high degree of concordant segregation also was observed with chromosome 6.

DISCUSSION

Our studies on the specificity of the AAT staining methods were consistent with the strong human activity band (designated H1) in both human liver and erythrocytes being cytosolic AAT. In support of this was the fact that the H1 activity was present only with the complete stain and the fact that erythrocytes lack the mitochondrial enzyme. The weaker, more anodal band (H2) in human liver may be the mitochondrial activity; however, the presence of this band in human liver in the absence of alanine and/or LDH suggests that this band is not an AAT isozyme. The bands seen with the stain lacking LDH did not co-migrate with AAT and were much weaker in intensity, indicating that they also were not AAT isozymes.

Rat liver and hepatoma cells both had an activity band (R1) which was visible with both the complete stain and staining solutions lacking alanine and/or LDH. The R1 band was, however, markedly more intense with the complete stain. It appears that both rat AAT and an alanine and/or LDH independent enzyme(s) migrated to the same position on the starch gels. It is reasonable to assume that the R1 band seen with the complete stain is AAT since it is the only band present in rat liver, and it is much stronger in intensity than the other bands.

The bands seen in both rat and human sources in the absence of alanine alone or both alanine and LDH, could be glutamate dehydrogenase (GLUD, EC 1.4.1.3) since this enzyme can be visualized with a staining solution containing 2-oxoglutarate, NADH and an ammonium source (Nelson *et al.* 1977). The LDH used in our stain was in ammonium sulphate solution and ammonium chloride was added to the stain lacking LDH and alanine. Van Someren *et al.* (1974*b*) also found that LDH activity was visualized when gels were stained with 2-oxoglutarate and NADH in the presence or absence of exogenous LDH. The bands detected in our gels in the absence of exogenous LDH may also be LDH since similar bands were seen on a starch gel stained with only pyruvate and NADH (Harris & Hopkinson, 1976). The most anodal band, R2, seen in the rat hepatoma was not identified.

Intensely staining bands were present in the rat-human hybrids with the complete stain. The most cathodal band, H1, present in some hybrids was designated AAT1 since it co-migrated with human liver and erythrocyte AAT, and it appeared only with the complete stain. Identification of the more anodal band in the hybrids was more difficult since rat AAT (band R1) and human liver activity H2 migrated to the same position. In addition, the hybrids also expressed a weak band at the H2/R1 position when the stain lacked alanine. Clearly, further studies are necessary to identify electrophoretic and biochemical property differences between H2 and R1.

In the majority of hybrids positive for H1(AAT), little or no activity was seen in the H2/R1 position. This finding suggested that when human AAT1 is expressed, rat AAT and the rat alanine independent activity (R1) are not expressed, or the human activity H2 is not expressed, or that human AAT and H2 activity segregate independently.

It is of interest to note that in hybrids where both the H1 and H2/R1 bands were present,

no intermediate band occurred. Such a band would have suggested the presence of a rat-human AAT hybrid protein. Purified rat liver AAT has two subunits (Gatehouse *et al.* 1967; Matsuzawa & Segal, 1968) and the human erythrocyte enzyme also is dimeric since three isozymes have been seen on gels in erythrocytes from individuals heterozygous at the AAT locus (Chen & Giblett, 1971). Therefore, AAT heterodimers, similar to those seen with other dimeric enzymes in hybrid cells, were expected but were not observed. This finding suggested that either the clones are heterogeneous with rat AAT expressed in some cells and human AAT in the others, or that the H2/R1 band observed in certain hybrids positive for human AAT was not rat AAT.

Based on these studies, we have assigned the locus for cytosolic AAT1 to human chromosome 8. Giblett *et al.* (1978) had suggested, on the basis of linkage analysis, that *AAT1* and soluble glutamate oxaloacetate transaminase (*GOT*) were weakly linked and thus on chromosome 10. Four out of 22 of our hybrids were discordant for AAT and GOT. Kielty & Povey (1981) have also reported the assignment of *AAT1* to human chromosome 8 using rat-human hybrids. However, Wijnen & Meera Khan (1981) have assigned the locus to human chromosome 16. In our hybrids, 8 of 14 secondary clones were discordant for AAT1 and chromosome 16. If the locus coding erythrocyte AAT1 is also on chromosome 8, it can be deduced by exclusion mapping to be located in the region 8q13 → 8qter (Cook, Jeremiah & Buckton, 1981).

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