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Assignment of the gene for neutral α -glucosidase AB to chromosome 11

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Abstract. Human tissues contain two isozymes of neutral α -glucosidase, neutral α -glucosidase AB and neutral α -glucosidase C (α -D-glucoside glucohydrolase, EC 3.2.1.20). The two isozymes, initially defined on the basis of differences in electrophoretic mobility in starch gel, have also been shown to have other distinguishing biochemical characteristics including different substrate specificities. Rodent tissues contain apparently homologous isozymes of neutral α -glucosidase. The mouse and human α -glucosidase C isozymes, but not the AB isozyme(s), can be distinguished by the difference in their electrophoretic mobility. This difference has previously enabled us to use human-mouse somatic cell hybrids to assign the structural gene for human α -glucosidase C to chromosome 15. We now report the differentiation of mouse and human neutral α -glucosidase AB isozymes by rocket immunoelectrophoresis, using an antibody raised in mice against purified human placental neutral α -glucosidase AB. This antibody precipitated both the A and B bands of human neutral α -glucosidase AB and did not cross react with mouse enzyme as determined by Ouchterlony double immunodiffusion and by rocket immunoelectrophoresis. Using this antibody, the segregation of human neutral α -glucosidase AB was examined in 41 mouse $\times$ human hybrid clones. Thirty-eight hybrid clones, derived from fusions of RAG $\times$ seven different human cells, showed 100% concordant segregation of human neutral α -glucosidase AB and the 11. Three additional clones, derived from a fusion of tetraploid murine erythroleukemia cells (2S-MEL) $\times$ diploid human fibroblasts carrying a translocation chromosome(s) allowed the regional localization of the gene to the long arm of 11 (11q13 $\rightarrow$ 11qter).

Two major forms of neutral α -glucosidase activity are observed when extracts of human cells are electrophoresed in starch gel and α -glucosidase activity is detected at neutral pH using the fluorogenic substrate 4-methyl-umbelliferyl- α -D-glucopyranoside. In the human, these isozymes have been called neutral α -glucosidase AB and neutral α -glucosidase C. Neutral α -glucosidase AB appears to consist of two related

isozymes which differ in degree of sialation and which migrate rapidly toward the anode at pH 6.5. Neutral α -glucosidase C is unaffected by treatment with neuraminidase and migrates less rapidly towards the anode. The α -glucosidase AB and C isozymes also differ in their substrate specificities, molecular weights, solubility in ammonium sulfate, binding to Concanavalin A, isoelectric points, and inhibition by some cations (Swallow et al., 1975; Martiniuk and Hirschhorn, 1981; Martiniuk, 1982). Human neutral α -glucosidase C is genetically polymorphic with seven phenotypes detected, resulting from the expression of four different alleles, one of which is a silent or null allele. Individuals homozygous for the null allele of the α -glucosidase C gene still express α -glucosidase AB, and no variation in the mobility of α -glucosidase AB is detected in individuals with different α -glucosidase C phenotypes (Martiniuk and Hirschhorn, 1980). These

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results suggest that the two neutral α -glucosidase isozymes are products of different genetic loci.

Two major forms of neutral α -glucosidase activity are also seen upon electrophoresis of extracts of mouse tissues. The most anodally migrating mouse neutral α -glucosidase binds to Sepharose-Concanavalin A, indicating that this form is glycosylated, and therefore apparently homologous with the most anodally migrating human glycosylated neutral α -glucosidase AB. The more slowly migrating isozyme is presumably homologous with human neutral α -glucosidase C. The mouse and human neutral α -glucosidase C isozymes have different electrophoretic mobilities, so the human neutral α -glucosidase C could be mapped to chromosome 15 by studies of human $\times$ mouse cell hybrids (Martiniuk et al., 1980). However, under the conditions used, no differences in mobility between human and mouse neutral α -glucosidase AB could be detected. We have now been able to distinguish mouse from human neutral α -glucosidase AB by immunologic methods and report the assignment of neutral α -glucosidase AB to the human 11.

Materials and methods

Cell lines and hybridization procedures. A total of 41 clones (four primary and 37 secondary clones) derived from fusions between eight different human cell lines and murine RAG or tetraploid murine erythroleukemic (2S-MEL) cells were isolated and cloned according to the cloning procedures of Ham and Puck (1962) and were characterized. Table I lists the 38 clones which were isolated from seven fusions of RAG with different human parental lines.

Three additional clones were analyzed from a fusion of Lesch-Nyhan (HLN) fibroblasts and 2S-MEL (tetraploid Friend murine erythroleukemia cells) (Willing et al., 1979). These clones have been described previously (Wang et al., 1981) and were a primary (XX-8) and one tertiary clone and one quaternary clone from the same primary. The primary clone (XX-8) contained human chromosomes 4, 6, 8, 9, 11-15, 18, 21, 22, and two human-human translocations between 4 and 11. The tertiary clone XX-8-A31-23 contained human chromosomes 4, 9q, 15, 21, and the human-human translocation chromosome t(11;4) (11pter $\rightarrow$ 11q12::4q21 $\rightarrow$ 4qter). The quaternary clone XX-8-H21-H7-10 contained only the human-human translocation chromosome t(4;11)(4pter $\rightarrow$ 4q21::11q13 $\rightarrow$ 11qter).

Chromosome analysis. Metaphase spreads for chromosome analysis were prepared as previously described (Smith et al., 1975). Chromosomes were banded using quinacrine hydrochloride fluorescence and trypsin-Giemsa treatment. The Giemsa-11 staining method was used to distinguish mouse and human chromosomes (Friend et al., 1976). Determination of chromosome content of the clones was as previously described by both karyotype and/or iso-

zyme analysis (Smith et al., 1975). The chromosomes of the secondary hybrid clones were determined chiefly by isozyme analysis, however, chromosomes 3, 6, and 17 were only identified cytogenetically.

Marker enzymes. Known and well characterized human enzyme markers for 20 of the total 23 chromosomes were determined as indicated in table I. The enzymes were analyzed by horizontal starch gel or cellulose acetate electrophoresis using methods described by Harris and Hopkinson (1976). In general, LDHA was used as the isozyme marker for 11, but both LDHA and ESA4 were scored in the XX-8 clones.

Sample preparation. For analysis of neutral α -glucosidase AB, cell extracts were prepared from the cells grown in one 75 cm² flask, approximately 10×10^6 cells, and frozen at -60°C . The cells were suspended in H₂O at a total volume of 70 μl , and sonicated three times (5 sec/burst) (Heat Systems-Ultrasonics, Inc.) at 4°C in 400 μl micro-centrifuge tubes (Eppendorf), the extract was centrifuged for 5 min at 4°C in a microcentrifuge (Beckman), and the supernatant was used for electrophoresis. Cell extracts for other enzyme analyses were prepared in distilled water (the cells of one 75 cm² flask/0.1–0.3 ml water) either by five cycles of freezing and thawing or by sonication and centrifugation in a micro centrifuge. Analyses of all the enzyme marks required the cells from three 75 cm² flasks. Almost all enzymes were stable when the extracts were stored at -60°C .

Preparation of human placental neutral α -glucosidase AB for immunization. Placental neutral α -glucosidase AB was partially purified by precipitation with 40% ammonium sulfate followed by sequential chromatography on hydroxyl apatite, DEAE-Sephacel, and Sepharose 6B (Martiniuk and Hirschhorn, 1981; Martiniuk, 1982). An approximately 1,000-fold purification of the enzyme was obtained with yields of 2–4%. All procedures were done at 4°C . The ammonium sulfate precipitate, obtained from a homogenate of a placenta as previously described (Martiniuk and Hirschhorn, 1980), was resuspended in a minimum volume of 0.01 M sodium phosphate, pH 7.0, and dialyzed overnight against three changes of 4 l each of 0.1 M sodium phosphate, pH 7.0. The dialyzed sample was centrifuged at $15,000 \times g$ for 10 min, and the supernatant was applied to a 2.5×50 cm hydroxyl apatite column with a flow rate of 50–70 ml/hr. After washing the column with 0.13 M sodium phosphate, pH 7.0, a linear gradient was generated using 550 ml of 0.13 M sodium phosphate, pH 7.0, and 550 ml of 0.35 M sodium phosphate, pH 7.0. Fractions containing enzyme activity were pooled, concentrated using a YM 30 membrane, and dialyzed against 0.1 M Tris-HCl, 0.2 M NaCl, pH 7.0. The sample was applied to a 7.0 ml DEAE-Sephacel column previously equilibrated with dialysis buffer, and the column was washed at a flow rate of 25 ml/hr until the A_{280} of the effluent had decreased to that of the buffer. The enzyme was eluted with a linear gradient generated using 50 ml of starting buffer and 50 ml of 0.01 M Tris-HCl, 0.45 M NaCl, pH 7.0. Under these conditions, two peaks of enzyme activity were detected, one peak in the void volume and a second peak which was bound and eluted with the NaCl gradient. Each peak was concentrated on YM 30 membranes, and a sample of each was electrophoresed in starch gel. The bound peak contained only neutral α -glucosidase A, while the peak in the void volume contained both neutral α -glucosidase B and a band with a mobility between A and B. The peak bound to DEAE-Sephacel, containing neutral α -glucosidase A, was applied to a 1.5×100 cm Sepharose 6B column in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at 10 ml/hr and 1.0 ml fractions were collected into tubes containing 0.5

ml of 20% glycerol-buffer. The fractions containing enzyme activity were pooled and concentrated on a YM 30 membrane. Enzyme activity in column fractions was assayed by incubating a sample of the fraction with 50–100 μ l of 4-methylumbelliferyl- α -D-glucopyranoside (4-MUF) in 0.1 M sodium phosphate, pH 7.5, for 30–60 min, the reaction was stopped with 0.2 M glycine, 0.2 M NaCl, pH 10.4, and the fluorescence was measured in a Turner fluorometer as described previously (Swallow et al., 1977; Martiniuk and Hirschhorn, 1981).

Further purification and immunization. The purified human placental neutral α -glucosidase AB was electrophoresed in a vertical polyacrylamide gel (4.5% stacking and 6% running gel; 1.5 mm thick $\times$ 130 mm $\times$ 100 mm) using a gel buffer of 0.05 M sodium phosphate, pH 6.5, and a chamber buffer of 0.05 M sodium phosphate, 1.0 M glycine, pH 6.5. Ten to 15 μ g of protein were placed in each slot, and the gel was electrophoresed for 18–20 h at 15 mAmps constant current. Enzyme activity was visualized by staining one channel with 4-MUF. The equivalent location was cut out of the other five channels, emulsified with complete Freund's adjuvant, and injected subcutaneously in the lower back of five female BALB/c mice weighing between 14–16 grams. Identical material was injected 4, 9, 13, and 15 wk after the initial immunization. Mice were bled prior to each boost from the tail, and the serum was pooled at each bleeding and kept at -60°C until used. Antibody was detected by Ouchterlony double immunodiffusion performed in 0.05 M sodium phosphate, pH 7.5, with 0.02% sodium azide. Immunoprecipitin lines were detected visually and then by enzymatic stain for neutral α -glucosidase activity as described previously (Swallow et al., 1977; Martiniuk and Hirschhorn, 1980). Antibody was detected in the serum from week 13 on.

Immunoprecipitation. Whole cell homogenates and partially purified neutral α -glucosidase AB preparations (obtained from DEAE-Sephacel chromatography as described above) were diluted 1:1 with the antisera or buffer and incubated at 4°C for 3 h. One half volume of a 50% slurry of protein A-Sepharose was added, incubated for 60 min, and centrifuged in a microcentrifuge. The supernatant extracts were then electrophoresed in starch gel (Martiniuk and Hirschhorn, 1981) and stained for neutral α -glucosidase activity.

Rocket immunoelectrophoresis for detection of human neutral α -glucosidase AB in hybrids. Forty microliters of serum (obtained 15 and 17 wk after the initial immunization) was incorporated into 4 ml agarose (1% in 0.05 M sodium phosphate, pH 7.5, with 0.02% sodium azide) and a $70 \times 80 \times 0.5$ mm gel was poured. Five microliter samples were added rapidly to 4 mm diameter wells with a chamber buffer of 0.05 M sodium phosphate, pH 7.5, and a constant 25 V across the gel at $8\text{--}12^{\circ}\text{C}$. After sample application, the voltage was increased stepwise to 80 volts in three hours and then maintained for 18–20 hours. Gels were washed in multiple changes of saline at 25°C for 3–6 h and then stained for enzymatic activity with 4-MUF as previously described (Martiniuk and Hirschhorn, 1981). Enzymatic activity was seen in rockets after 10–20 min.

Results

A series of preliminary experiments were performed to determine if the antibody against human neutral

α -glucosidase AB could detect human neutral α -glucosidase AB and not rodent α -glucosidase AB (data not shown). The mouse anti-human neutral α -glucosidase AB antisera reacted with human placental extracts to yield a single precipitin line on double immunodiffusion and a single, symmetric rocket on immunoelectrophoresis, both of which stained for neutral α -glucosidase activity and protein. The antisera did not cross react with extracts of the parental mouse RAG or 2S-MEL cells, which were used to form hybrid cell clones, in either double immunodiffusion or rocket immunoelectrophoresis. Additional experiments were performed to confirm that the antibody was directed against human neutral α -glucosidase AB and to determine if the antibody might be directed preferentially to one of the various sialated or desialated forms of the enzyme. Crude placental extracts and partially purified extracts containing either α -glucosidase A, α -glucosidase AB, or α -glucosidase B were incubated with varying concentrations of the anti-sera, and the antigen-antibody complexes were precipitated with protein A-Sepharose. The supernatants from antibody treated extracts, as well as from extracts that were incubated without antibody, were then electrophoresed in starch gel and stained for enzymatic activity. There were no bands of neutral α -glucosidase AB in their respective samples, indicating that the antibody was able to precipitate the sialated, the intermediate bands, and the desialated forms of human neutral α -glucosidase AB.

Varying amounts of the antisera in agarose were then tested to determine the optimum concentration for detection by rocket immunoelectrophoresis of neutral α -glucosidase AB in cultured human cells and in various mixtures of human and RAG cells. Under optimal conditions, the height of the rocket, as detected by staining for enzymatic activity, was proportional to the amount of human cell extract, and no rocket was seen with extracts of the parental mouse cells. Only a single rocket was seen after staining for enzymatic activity. This rocket coincided with a rocket seen after staining for protein when human placental extracts were used. When other cell extracts containing less enzymatic activity, such as those from hybrid cell clones, were used, a single rocket was also seen by staining for enzymatic activity, but a rocket could not always be easily distinguished when staining for protein (data not shown). Therefore, all hybrid clones were tested using at least two different dilutions of the

Fig. 1. Rocket immunoelectrophoresis of human neutral α -glucosidase AB from human placenta and human $\times$ RAG hybrid clones. Lane 1: human placenta; lanes 2 and 3: hybrid clones positive for human neutral α -glucosidase AB; lane 4: mouse RAG cells.

extract and staining for enzymatic activity, a procedure which was more sensitive than staining for protein and which gave unequivocal results.

Rocket immunoelectrophoresis of 38 human $\times$ RAG hybrid clones revealed clones which were positive and negative for human neutral α -glucosidase AB (fig. 1). Thirty-eight hybrid clones (three primary and 35 secondary clones derived from 16 different primary clones in seven separate fusions) of RAG with human cells were examined for the segregation of human neutral α -glucosidase AB, and human chromosomes, or enzyme markers, or both. All three primary clones (table II) showed concordant segregation of human neutral α -glucosidase AB and the 11 (as detected by the presence of human lactate dehydrogenase A). Analysis of the 35 secondary clones (table II) showed 35 clones concordant for human neutral α -glucosidase AB and the 11. The combined three primary and 35 secondary clones showed 100% concordant segregation of human neutral α -glucosidase with the 11 (table II).

An additional three clones which were derived from a fusion of HLN $\times$ 2S-MEL cells were examined (Wang et al., 1981). The primary clone, XX-8, contained two human-human translocations between 4

Table I. Fusions of mouse RAG cells and human cells

Fusion	Parental cells		Primary clone	Number of secondary clones
	Mouse	Human		
1	RAG	ODY (lymphoid)	LB11	4
2	RAG	NB103 (lymphoid)	A (NA) ¹	3
			B (NA)	1
3	RAG	6323 (lymphoid)	A (NA)	7
			B (NA)	1
4	RAG	Angela (fibroblasts)	C	—
			B (NA)	3
			K (NA)	1
5	RAG	Fetal liver	A (NA)	1
			B (NA)	1
			C (NA)	1
6	RAG	Fetal kidney 2	T	—
			A (NA)	2
			Q (NA)	2
7	RAG	Fetal lung 2	A (NA)	6
			B (NA)	2

¹ NA: Not analyzed for neutral α -glucosidase AB but analyzed for chromosome content. Data not shown.

and 11 and was positive for neutral α -glucosidase AB. The tertiary hybrid clone, XX-8-A31-23, derived from this primary clone had the human-human rearrangement, t(11;4)(11pter→11q12::4q21→4qter) and was positive for LDHA, negative for ESA4, and negative for human neutral α -glucosidase AB. The other quaternary hybrid clone, XX-8-H21-H7-10, contained only human t(4;11), (4pter→4q21::11q13→11qter) and was negative for LDHA, positive for ESA4, and positive for human neutral α -glucosidase AB.

Because of the large size of the neutral α -glucosidase AB molecule (>250,000 D), we considered the possibility that a chromosome in addition to 11 might be required for the expression of neutral α -glucosidase. In the RAG $\times$ human clones, each of the other chromosomes, except for 21, could be shown to be absent in at least one clone which expressed neutral α -glucosidase AB (table II). However, the HLN $\times$ 2S-MEL clone XX-8-H21-H7-10, which contained the human-human t(4;11) rearrangement as the only human chromosome, expressed neutral α -glucosidase AB, eliminating 21 and indicating that only 11 was necessary for expression of neutral α -glucosidase AB.

Table II. Segregation of human chromosomes and human neutral α -glucosidase AB in human-mouse somatic cell hybrids: number of primary and secondary clones concordant and discordant for GANAB and chromosomes¹

Chromo- some	Enzyme marker	Phenotype ²								Frequency of discordancy
		Concordant				Discordant				
		+/+		-/-		+/-		-/+		
P	S	P	S	P	S	P	S			
1	PGM1	1	5	0	2	1	7	0	5	0.62
2	MDH1	1	12	0	0	2	1	0	3	0.32
3	—	1	5	0	0	1	1	0	3	0.46
4	PGM2	0	5	0	1	1	3	0	3	0.54
5	HEXB	2	3	0	2	0	2	0	1	0.30
6	SOF2/ME1	1	6	0	2	1	2	0	8	0.55
7	GUSB	1	4	0	2	1	1	0	5	0.50
8	GSR	0	8	0	7	2	3	0	4	0.38
9	AK1/AK3/ACON1	2	2	0	4	0	6	0	3	0.53
10	GOT1	1	9	0	1	1	1	0	3	0.31
11	LDHA/ESA4	3	15	0	20	0	0	0	0	0.00
12	LDHB/PEPB	2	10	0	1	1	1	0	8	0.44
13	ESD	0	2	0	2	1	0	0	5	0.60
14	NP	2	8	0	1	0	1	0	8	0.45
15	HEXA/MPI	3	12	0	1	0	1	0	8	0.36
16	—	1	0	0	2	1	1	0	2	0.57
17	—	0	0	0	2	2	1	0	1	0.67
18	PEPA	1	6	0	1	1	4	0	1	0.43
19	GPI	1	2	0	3	1	6	0	1	0.57
20	ADA	2	5	0	4	1	3	0	4	0.42
21	SOD1	3	14	0	8	0	0	0	10	0.40
22	ACON2	0	1	0	0	1	2	0	3	0.86
X	G6PD	2	5	0	0	0	0	0	4	0.36

¹ The table does not include the three clones derived from HLN $\times$ 2S-MEL primary fusions used for regional localization.

² Hybrids were scored for the presence (+) or absence (-) of the human chromosome and human neutral α -glucosidase AB. P: primary clone, S: secondary clone.

Discussion

We have found concordant segregation of human neutral α -glucosidase AB, as detected by rocket immunoelectrophoresis, and the 11 by examination of human $\times$ mouse hybrid clones. The antibody used in rocket electrophoresis was produced by BALB/c mice injected with a partially purified human placental neutral α -glucosidase AB (purified 1000-fold), further purified by electrophoresis in polyacrylamide gels. The antibody precipitated both sialated and desialated forms of the human neutral α -glucosidase AB and reacted with extracts of human tissues in Ouchterlony double immunodiffusion and rocket immunoelectrophoresis. The antibody did not cross react with the mouse isozyme in Ouchterlony double immunodiffusion and rocket immunoelectrophoresis.

Rocket immunoelectrophoresis of the 38 human $\times$ RAG hybrid clones (three primary and 35 secondary clones) showed a total concordant segregation of human neutral α -glucosidase AB and 11. Analysis of an additional primary clone derived from 2S-MEL $\times$ HLN and two daughter clones containing translocations between the human 4 and 11 regionally localized the gene to 11q13 $\rightarrow$ 11qter.

Genes for several other enzymes and non-enzymatic proteins have been assigned to 11 (Gerald and Miller, 1982). Acid phosphatase-2, catalase, LDHA, hemoglobin epsilon, gamma, delta, and beta chains, and the structural gene for insulin have been assigned by somatic cell hybridization and/or recombinant DNA techniques to the short arm of 11. In addition, a deletion of 11p13 occurs in some patients with Wilms' tumor (Francke et al., 1979). Several

restriction fragment length polymorphisms occur in the same DNA region of hemoglobin chains as well as in the region of 11pter→11q13. Several surface antigens, as well as glutathione S-transferase and fibronectin, have also been mapped to 11 but have not been regionally assigned. Fewer proteins have been assigned to the long arm of 11. Cathepsin D appears to map to 11pter→q12, and ESA4 and porphobilinogen deaminase have both been assigned to the terminal portion of 11q. The regional localization of neutral α -glucosidase AB could only be defined to 11q13→qter.

The present studies also demonstrate that three different isozymes of α -glucosidase are controlled by different loci on three chromosomes: acid α -glucosidase on 17 (Solomon et al., 1979; Weil et al., 1979), neutral α -glucosidase C on 15 (Martiniuk et al., 1980), and neutral α -glucosidase AB on 11. These results are not surprising since previous genetic and biochemical evidence suggested that the three isozymes are encoded by different structural genes. The similarity of the acid α -glucosidase and neutral α -glucosidase C isozymes in molecular weight and ability to degrade glycogen raises the possibility that their genes have evolved from a common ancestral gene. The assignment of the two isozymes to separate chromosomes is not inconsistent with this possibility, since such genes for proteins that diverged from a common ancestor can be found on different chromosomes, for example, the alpha and beta globin chains (Deisseroth et al., 1977; Gusella et al., 1979), the alpha and beta hexosaminidase subunits (Lalley et al., 1974; Gilbert et al., 1975), and the LDHA and B subunits (Boone et al., 1971; Chen et al., 1973).

Since the loci for acid α -glucosidase and neutral α -glucosidase have been designated *GAA* and *GANC*, respectively (Shows and McAlpine, 1982), we suggest that the locus for neutral α -glucosidase AB be designated *GANAB*.

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