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A Locus Controlling the Activity of UDP-Galactose:G_{M2}(NeuGc) Galactosyltransferase in Mouse Liver is Linked to the H-2 Complex

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Genetic polymorphism in the expression of the G_{M1}(NeuGc) ganglioside has been shown in the liver of inbred strains of mice. Through analysis of the gangliosides of H-2 congenic and recombinant strains, this polymorphism was demonstrated to be controlled by a locus mapped left outside of the H-2 complex on chromosome 17, and the locus was assumed to control the level of the activity of G_{M1}(NeuGc) synthetase, UDP-galactose:G_{M2}(NeuGc) galactosyltransferase (E.C.2.4.1.62) [Hashimoto *et al.*, J Biochem (1983) 94:2049-54].

In the present study we analyzed the genetic linkage between the activity of the galactosyltransferase and the H-2 haplotype. For this purpose, we selected two inbred strains of mice, WHT/Ht and BALB/c, because they have different levels of the transferase activity and show different H-2 haplotypes; the specific activity of the transferase obtained with BALB/c was one-eighth of that with WHT/Ht, and BALB/c expressed the Ia.7 antigen as one of the products encoded in their H-2^d complex, whereas WHT/Ht did not. To analyze the linkage between these two phenotypes, WHT/Ht were mated with BALB/c to obtain the F_1 mice, and the female F_1 mice were then backcrossed to WHT/Ht. It was found that one half of the backcross generation expressed the Ia.7 antigen derived from BALB/c and had a significantly lower specific activity of the transferase than that of WHT/Ht, while the other half did not express the Ia.7 antigen but had the same specific activity of the transferase as that obtained with WHT/Ht.

These results suggest that the locus controlling the level of the transferase activity in mouse liver is linked to the H-2 complex on chromosome 17.

Abbreviations: The ganglioside nomenclature is based on the system of Svennerholm, J Neurochem (1963) 10:613-23. The sialic acid species present is shown in parentheses after the ganglioside abbreviation. NeuGc, *N*-glycolylneuraminic acid.

Glycolipids are ubiquitous on cellular membranes but their composition differs from organ to organ even in one species. Furthermore, polymorphic variations of the glycolipid composition have been recognized in several organs [1, 2]. Some of these variations are considered to be under genetic control, although the precise mechanism has not yet been elucidated.

In order to establish a model system for analyzing the mechanism, we have studied the polymorphic variations of the glycolipid composition in several mouse organs, and found that the ganglioside composition in the liver differs from strain to strain. On the basis of the ganglioside composition, we classified the inbred strains into three groups [3-5]; the first group possessed G_{M2}(NeuGc) as a major ganglioside, to which belonged strains such as BALB/c (H-2^d), DBA/2 (H-2^d) and C57BL/10 (H-2^b), the second group including WHT/Ht possessed no G_{M2}(NeuGc) but did possess G_{M3}(NeuGc), and the third group possessed both G_{M2}(NeuGc) and G_{M1}(NeuGc), to which belonged strains such as SJL/J (H-2^s), SWR/J (H-2^q), SL/QDJ (H-2^q), PL/J (H-2^u) and RFM/Ms (H-2^f).

In previous papers [3, 6], we reported that the expression of G_{M2}(NeuGc) was inherited as an autosomal dominant trait and was directly regulated by the activity of G_{M2}(NeuGc) synthetase, UDP-*N*-acetylgalactosamine:G_{M3}(NeuGc) *N*-acetylgalactosaminyltransferase. We also reported [5] that the expression of G_{M1}(NeuGc) was controlled by a gene located left outside the H-2 complex, and suggested that the gene directly controlled the level of the activity of G_{M1}(NeuGc) synthetase, UDP-galactose:G_{M2}(NeuGc) galactosyltransferase, in a similar way to the regulation of G_{M2}(NeuGc) expression.

In the present study we analyzed the genetic linkage between the activity of the galactosyltransferase and the H-2 haplotype in the backcross generation of (BALB/c × WHT/Ht)-F_1 to WHT/Ht, and demonstrated that the locus controlling the level of the galactosyltransferase activity is linked to the H-2 complex on chromosome 17.

Materials and Methods

Chemicals and Reagents

The materials used in this work were as follows: Ficoll-Paque from Pharmacia Fine Chemicals (Uppsala, Sweden), fetal calf serum and RPMI 1640 medium from Gibco Labs. (New York, USA), monoclonal antibody against Ia.7 and Low-Tox-M rabbit complement from Cedarlane Labs. (Hornby, Canada), SEP-PAK C18 cartridges from Waters Associates (Milford, USA), *N-p*-tosyl-L-lysine chloromethylketone (TLCK), L-1-tosylamide-2-phenylethylchloromethylketone (TPCK), and phenylmethylsulfonylfluoride (PMSF) from Sigma Chemical Co. (St. Louis, MO, USA), pepstatin A from Peptide Institute (Osaka, Japan), Triton CF-54 from Supelco (Bellefonte, PN, USA), UDP-[6-^{3}H]galactose (15.7 Ci/mmol) and UDP-[U-^{14}C]galactose (330 mCi/mmol) from Amersham Japan (Tokyo, Japan), Direct Exposure Film from Kodak (Rochester, NY, USA), and Ultrofilm from LKB (Bromma, Sweden). Brij 96 was a generous gift from Mr. Hasegawa, Kao Corporation (Tokyo, Japan). G_{M3}(NeuGc), G_{M2}(NeuGc) and G_{M1}(NeuGc) were prepared from liver of ICR mice, and their structures were confirmed by several methods including TLC and GLC analyses, methylation study and sialidase treatment (unpublished results).

Mice

The WHT/Ht strain was originally established by Dr. Hewitt at Westminster Hospital London [7] and brought to Japan by Dr. Sakamoto, Tohoku University, Japan. The strain used in this work was a generous gift from Drs. Suzuki and Sudo, Institute of Medical Science, University of Tokyo, Japan, and maintained in our institute. BALB/c crS1c ($H\text{-}2^d$) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Male WHT/Ht were mated with female BALB/c to obtain the F_1 mice, and then the female F_1 were backcrossed to WHT/Ht. Mice were killed by decapitation at the age of 8-12 weeks, and both the spleen and liver were removed immediately. The spleen was subsequently processed to monitor the Ia.7 antigen as a marker of the $H\text{-}2^d$ haplotype. One half of the liver (about 0.5 g) was quickly frozen by immersing it in liquid nitrogen and stored at -70°C until the galactosyltransferase activity was determined. The activity was stable at least for six weeks. The other half of the liver was processed for ganglioside analysis.

Detection of the Ia.7 Antigen

Spleen cells were monitored for the Ia.7 antigen by the method of Gorer and O'Gorman [8] with slight modifications as follows. Splenocytes were isolated with Ficoll-Paque and then suspended in RPMI 1640 medium containing 5% fetal calf serum at a final concentration of 6×10^6 cells per ml. Serial dilutions of the anti-Ia.7 antibody solution were made and 50 μl of each solution were dispensed in a microtiter plate. To each well were added 50 μl of the cell suspension, and then the plate was incubated at room temperature for 30 min. Then, 50 μl of rabbit-complement solution were added to each well and the plate was incubated for another 30 min. After the incubation, 100 μl of trypan blue solution were added to each well, and the proportion of stained cells was determined under a microscope. The percentage of stained cells with saline instead of the antibody solution was used as a control, and the control value was below 5%.

Analysis of Gangliosides in the Liver of Mice

The ganglioside pattern was analyzed in a similar way as described previously [5]. Briefly, the procedures included extraction with a chloroform/methanol mixture, 2/1 by vol, separation by partition according to Folch *et al.* [9], reversed phase column chromatography with SEP-PAK C18 cartridges [10, 11], and TLC on HPTLC plates (Merck, Darmstadt, W. Germany). The solvent systems used for TLC analysis were chloroform/methanol/5 N ammonium hydroxide/0.4% $CaCl_2$ in water, 55/50/4/6 by vol, and chloroform/methanol/0.2% $CaCl_2$ in water, 55/45/10 by vol. Spots of gangliosides were detected with resorcinol reagent [12]. The ganglioside mixture prepared from the liver of ICR mice was used as a reference for TLC analysis, which consisted of G_{M3}(NeuGc), G_{M2}(NeuGc) and G_{M1}-(NeuGc).

Preparation of the Microsomal Fraction

All procedures were carried out at 4°C. Frozen liver was cut into small pieces and put into 2 ml of ice-cold buffer consisting of 30 mM Tris-HCl (pH 7.0), 250 mM sucrose, 1 mM

EDTA, 3 μM pepstatin, 5 μM TLCK, 3 μM TPCK and 300 μM PMSF, and then homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10 000 $\times$ g for 12 min and the supernatant obtained was further centrifuged at 105 000 $\times$ g, for 2 h. The precipitate obtained was suspended in the same buffer as above and then used as the microsomal fraction. The protein concentrations were determined by the method of Lowry *et al.* [13].

Assay of G_{M1}(NeuGc) Synthesis

The activity of UDP-galactose:G_{M2}(NeuGc) galactosyltransferase was determined in a similar way as described previously [6]. Unless otherwise stated, the incubation mixture contained, in a total volume of 50 μl, 2.5 μg G_{M2}(NeuGc), 6 nmol UDP-[6-^{3}H]galactose (50 mCi/mmol), 50 μg enzyme protein, 150 μg Triton X-100, 5 μmol sodium cacodylate (pH 6.5), 500 nmol $MnCl_2$, 0.15 nmol pepstatin, 0.25 nmol TLCK, 0.15 nmol TPCK, and 15 nmol PMSF. After incubating the mixture at 37°C for 30 min, the reaction was terminated by adding 100 μl of 250 mM EDTA solution containing 150 μg of egg lecithin. The mixture was sonicated for 5 min, and then applied to a SEP-PAK C18 cartridge after dilution with 10 ml of 100 mM KCl solution [10, 11]. The cartridge was washed with 35 ml of distilled water, and then lipids were eluted with 6 ml of methanol. Radioactivity incorporated into the lipid fraction was determined with a liquid scintillation counter (Aloka LSC-700).

The experiment was performed in duplicate, and the value obtained without the added lipid acceptor was taken as a control, which was subtracted from all data. The control values were between 50 and 150 dpm or between 200 and 300 dpm per tube, when the microsomal fractions prepared from WHT/Ht or BALB/c liver were used, respectively.

One unit of activity is defined as the amount of enzyme that transfers 1 μmol of galactose per min under the standard assay conditions.

Product Identification

The products synthesized during the incubation were identified by TLC followed by fluorography or autoradiography. Fluorography was carried out according to the method of Kundu [14] with the pooled products from five incubation mixtures. Autoradiography was performed as follows; after incubating the reaction mixture containing UDP-[1-^{14}C]galactose (330 mCi/mmol) instead of UDP-[6-^{3}H]galactose, the lipid fraction was recovered from the SEP-PAK C18 cartridge as described above. To this fraction were added 2 μg of G_{M1}(NeuGc) as a carrier, and then aliquots of the mixture were spotted on an HPTLC plate. The plate was developed with a solvent system of chloroform/methanol/5 N ammonium hydroxide/0.4% $CaCl_2$ in water, 55/50/4/6 by vol, or chloroform/methanol/0.2% $CaCl_2$ in water, 55/45/10 by vol, and then exposed to a Kodak Direct Exposure Film for a week. Gangliosides were detected with resorcinol reagent.

Results

Characterization of the Galactosyltransferase

The microsomal fraction prepared from WHT/Ht liver was used as an enzyme preparation. After the reaction mixture containing UDP-[^{14}C]-galactose had been incubated as described in the Materials and Methods section, the reaction product was analyzed by TLC followed by autoradiography. As shown in Fig. 1, the reaction product showed a single band on the autoradiogram (lane 1), which corresponded to that of G_{M1}(NeuGc) (lane 2). A similar result was obtained on thin-layer fluorography when UDP-[^{3}H]-galactose was used as the sugar nucleotide.

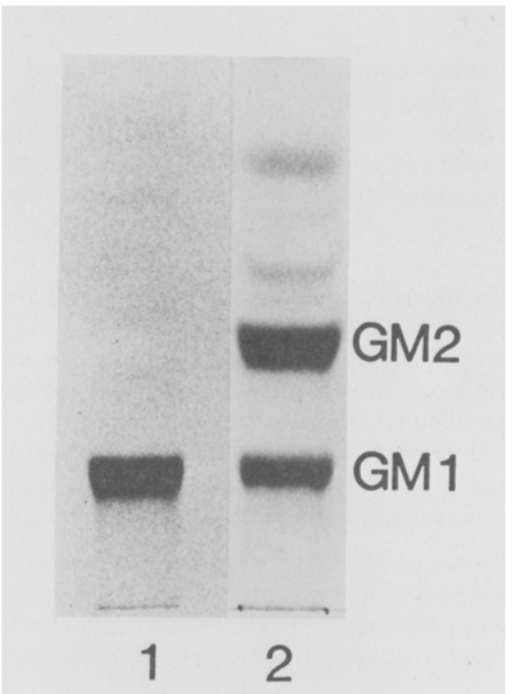

Figure 1. Product identification by autoradiography. The microsomal fraction prepared from WHT/Ht liver was used as the enzyme source. Lane 1 is an autoradiogram of lane 2. Note that the spot corresponding to G_{M1}(NeuGc) carries most of the radioactivity. Lane 2 is a thin-layer chromatogram of the lipid fraction, which was recovered from the incubation mixture by reversed phase column chromatography with a SEP-PAK C18 cartridge and then applied to a HPTLC plate together with 2 μg of G_{M1}(NeuGc) as a carrier. The plate was developed with a solvent system of chloroform/methanol/5 N ammonium hydroxide/0.4% $CaCl_2$ in water, 55/50/4/6 by vol. Since the incubation mixture contained 2.5 μg of G_{M2}(NeuGc) as a lipid acceptor, G_{M2}(NeuGc) was also detected with the resorcinol reagent.

The incorporation of [^{3}H]-galactose into the lipid fraction increased linearly with increasing amounts of the enzyme protein up to 100 μg per tube, and the reaction with 51 μg of the enzyme protein continued linearly for 75 min. The enzyme lost its activity on boiling for 10 min. The optimum pH of the enzyme was 6.5. Mn was essential for the

Table 1. Activity of UDP-galactose: G_{M2}(NeuGc) galactosyltransferase in the liver of WHT/Ht and BALB/c mice.

Strain	Enzyme activity[a]	
	male	female
WHT/Ht	5.9 ± 0.7	7.1 ± 1.1
BALB/c	0.8 ± 0.3	0.9 ± 0.1

[a] Mean ± S.D. × 10^{-6} units/mg protein.

transferase activity, the optimum concentration being 10 mM, and Co^{++} could partially (about 30%) replace Mn^{++}. The presence of 0.3% (w/v) of Triton X-100 in the reaction mixture was required to obtain the maximum activity. The apparent K_M values for UDP-galactose and G_{M2}(NeuGc) were 240 μM and 2 μM, respectively. From these results the standard assay conditions were determined as described under Materials and Methods.

The Activity of the Galactosyltransferase in the Liver of WHT/Ht and BALB/c Mice

A significant difference in the level of the galactosyltransferase activity was observed between male and female mice. As shown in Table 1, the specific activities of the galactosyltransferase of male and female WHT/Ht were 5.9 × 10^{-6} and 7.1 × 10^{-6} units/mg protein, respectively. A similar tendency was also observed for male and female BALB/c, although the values were relatively low. Thus, it was decided to calculate the mean values separately for the respective sexes in the following experiments.

The ratio of the specific activity of WHT/Ht liver to that of BALB/c liver was seven in the case of male mice and eight in the case of female mice. These results indicate that the phenotype of a high level of galactosyltransferase activity can easily be distinguished from that of a low level under the assay conditions established in the present study.

The Expression of the Ia.7 Antigen in Splenocytes of WHT/Ht and BALB/c Mice

Anti-Ia.7 antibody killed 50-60% of the splenocytes obtained from BALB/c mice in the presence of complement but none of those obtained from WHT/Ht mice, which indicated that BALB/c expressed the Ia.7 antigen as one of the products encoded in their $H\text{-}2^d$ complex, whereas WHT/Ht did not. Thus, we used the presence of the Ia.7 antigen as a marker of the $H\text{-}2^d$ haplotype in the following experiment.

Linkage Analysis Between the Activity of the Galactosyltransferase and the Ia.7 Antigen

As described above, there was a clear difference between WHT/Ht and BALB/c mice in the level of the galactosyltransferase activity as well as in the expression of the Ia.7 antigen, and we analyzed the linkage between the galactosyltransferase activity and H-2 haplotype by performing mating experiments.

Table 2. Number of mice expressing G_{M2}(NeuGc) and the presence of the Ia.7 antigen in the backcross generation of (BALB/c × WHT/Ht)F_1 to WHT/Ht mice.

	G_{M2}(NeuGc)		
Ia.7 antigen	+	—	total
+	15	14	29
—	18	17	35
total	33	31	64

For linkage analysis, it is desirable to determine the galactosyltransferase activity under identical conditions. In previous papers [3, 6], however, we demonstrated that the expression of G_{M2}(NeuGc), an acceptor of galactose in the galactosyltransferase assay, was found only in half the backcross generation obtained on (BALB/c × WHT/Ht)F_1 to WHT/Ht mating. This meant that the assay conditions could not be identical if we used mice of the backcross generation at random, and so we decided to select the mice which did not express G_{M2}(NeuGc) and which showed little incorporation of galactose in the absence of exogenous G_{M2}(NeuGc). Through analysis of the liver ganglioside, we confirmed that the expression of G_{M2}(NeuGc) was segregated according to the Mendelian ratio for a single gene in the backcross generation, i.e. the G_{M2}(NeuGc) positive to negative ratio was 33 to 31, as shown in Table 2. We also found that the Ia.7 antigen was expressed in 29 out of 64 mice in the backcross generation, but its segregation was independent of that of G_{M2}(NeuGc). This result suggests that the locus controlling the expression of G_{M2}(NeuGc) is not linked to the H-2 complex, and that the selection of mice not expressing G_{M2}(NeuGc) has no effect on the following linkage analysis between the level of the galactosyltransferase and the H-2 haplotype.

The strategy for the linkage analysis is shown in Fig. 2. Assuming that the level of the galactosyltransferase activity is controlled by a pair of allelic genes which are linked to the H-2 complex, chromosome 17 of WHT/Ht should carry a gene expressing a high level of the galactosyltransferase activity, whereas that of BALB/c should carry a gene expressing a low level of the galactosyltransferase activity. Since chromosome 17 of BALB/c should be inherited by (BALB/c × WHT/Ht)F_1 mice and then by half the backcross generation, this half that expresses the Ia.7 antigen should show a significantly lower activity of the galactosyltransferase than either WHT/Ht or the other half that does not express the Ia.7 antigen.

This assumption proved to be the case in this work as shown in Fig. 3. Nine out of 17 females in the backcross generation expressed the Ia.7 antigen, and at the same time showed a specific activity of the galactosyltransferase which was about half of that of WHT/Ht or that of the rest of the females, i.e. 4.0×10^{-6} as opposed to 7.1×10^{-6} or 7.0×10^{-6} units/mg protein, respectively. As for the males of the backcross generation, five out of 14 expressed the Ia.7 antigen and had significantly lower activity than that of WHT/Ht or the rest of the males, i.e. 3.2×10^{-6} as opposed to 5.9×10^{-6} or 5.2×10^{-6} units/mg pro-

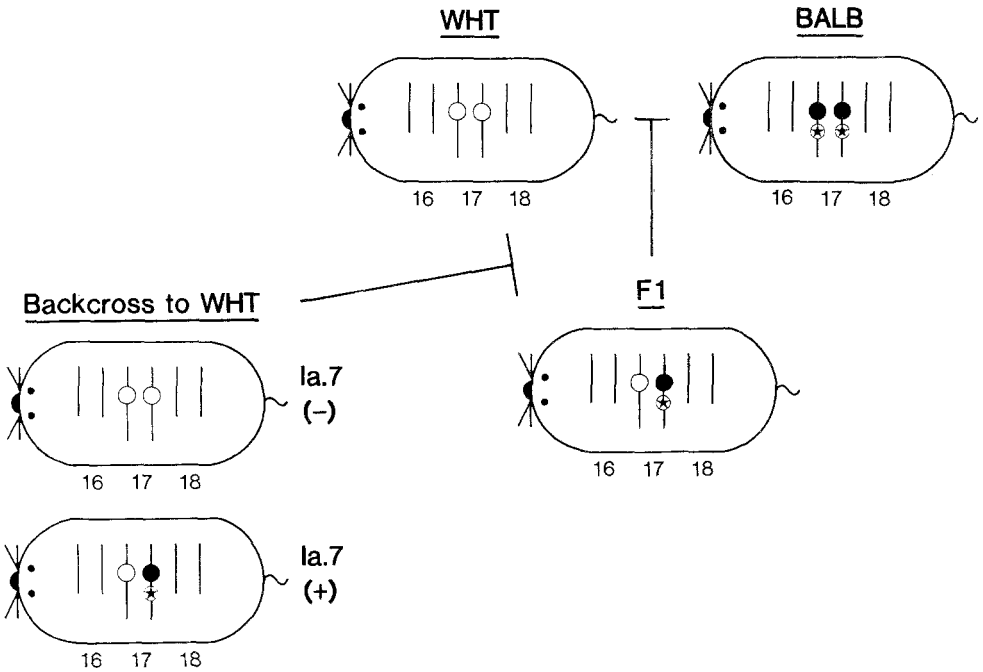

Figure 2. The strategy for the linkage analysis between the two loci controlling the level of the activity of UDP-galactose:G_{M2}(NeuGc) galactosyltransferase and the expression of the Ia.7 antigen. If the locus for the enzyme activity is linked to the H-2 complex, chromosome 17 of WHT/Ht carries a gene coding high enzyme activity (○), whereas that of BALB/c carries a gene controlling low enzyme activity (●), and the Ia.7 antigen ⊛. Chromosome 17 of BALB/c should be transferred to half the backcross generation, and these mice showing expression of the Ia.7 antigen sould have significantly lower activity than WHT/Ht or the rest of the generation.

tein, respectively. These results clearly demonstrate that genetic linkage is present between the level of the transferase activity and the H-2 haplotype in the backcross generation, and thus we concluded that the locus controlling the level of the galactosyltransferase activity is linked to the H-2 complex on chromosome 17.

Discussion

In the present study, we analyzed the characteristics of UDP-galactose:G_{M2}(NeuGc) galactosyltransferase in the microsomal fraction of WHT/Ht liver and noticed that the enzyme had a low K_M value for G_{M2}(NeuGc). This result indicates that when the enzyme preparation contains a significant amount of endogenous G_{M2}(NeuGc), the blank value without added G_{M2}(NeuGc) will be rather high, which will prevent accurate determination of the activity of galactosyltransferase. As described in a previous paper [3], however, WHT/Ht possess G_{M3}(NeuGc) but not G_{M2}(NeuGc) in the liver, and this enables the precise determination of the transferase activity without the effect of endogenous G_{M2}(NeuGc). Furthermore, the segregation of G_{M2}(NeuGc) was demonstrated to be independent of that of the Ia.7 antigen in the backcross generation of (BALB/c × WHT/Ht)F_1 to WHT/Ht (Table 2). Therefore, it was possible to select mice not expressing G_{M2}(NeuGc) from the backcross generation to determine the galactosyltransferase activity in the linkage analysis.

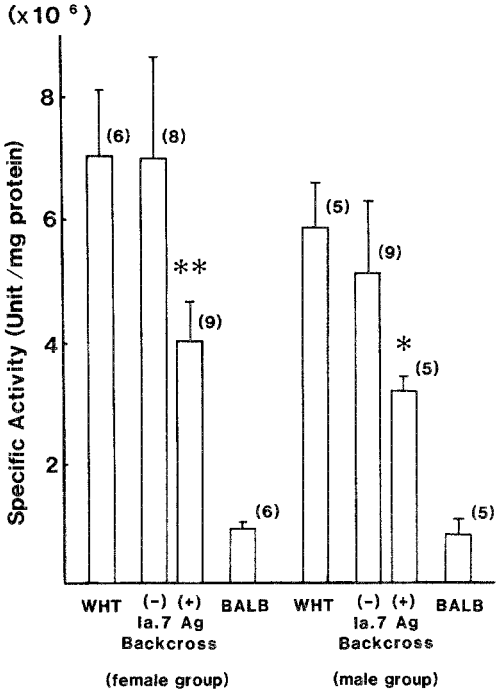

Figure 3. Activity of UDP-galactose:G_{M2}(NeuGc) galactosyltransferase in the liver of WHT/Ht and BALB/c strains, and the backcross generation of (BALB/c × WHT/Ht)F_1 to WHT/Ht. The activities in the two sexes are separately indicated. Figures in parenthesis are numbers of mice analyzed in each group. Note that mice with the Ia.7 antigen, (+), have a significantly lower activity than WHT/Ht or those withot the Ia.7 antigen, (-), in the backcross generation (P 0.005 (*) and P 0.001 (**) by Student's t test).

In a previous paper [5], we described that a locus designated as *Ggm-1* controlled the expression of G_{M1}(NeuGc) in mouse liver, which was mapped left outside of the H-2 complex. The present results indicate that the gene controlling the level of the galactosyltransferase activity is linked to the H-2 complex. It is thus conceivable that *Ggm-1* controls the expression of G_{M1}(NeuGc) by regulating the level of the galactosyltransferase activity. Furthermore, the gene dosage effect of *Ggm-1* was observed in the backcross generation, as shown in Fig. 3, in which mice with the Ia.7 antigen had half as much specific activity as that of mice without the Ia.7 antigen, or the specific activity of the heterozygote of *Ggm-1* was half as much as that of the homozygote. This observation suggests that *Ggm-1* encodes the galactosyltransferase itself. It is also possible, however, that *Ggm-1* controls other factors such as activators or inhibitors of the transferase, or regulators of gene expression of the enzyme protein. To clarify the function of *Ggm-1* as well as its gene product, it is essential to purify the galactosyltransferase and to demonstrate its characteristics.

We found that the expression of G_{M1}(NeuGc) also exhibited polymorphism in mouse erythrocytes and coincided exactly with that in liver, suggesting that the expression in erythrocytes was controlled by *Ggm-1* in the same way as in liver. In other organs, however, such as the thymus, spleen, heart, lung, kidney and testis, the expression was not necessarily correlated with that in the liver, which suggested that the regulation of the expression of G_{M1}(NeuGc) through *Ggm-1* was limited to the liver and erythrocytes (Nakamura *et al.* unpublished results).

As previously described [5], *Ggm-1* is mapped at the centromeric side of the H-2 complex, or within the T/t complex [15]. Several genes included in the T/t complex are considered to code antigens on the cellular surface which are involved in embryogenesis or organ differentiation, and their mutation causes abortion of mouse embryos or failure in organogenesis. For example, Shur [16] reported that such a mutation caused elevation of the activity of UDP-galactose:*N*-acetylglucosamine galactosyltransferase on the cellular surface of mouse embryos, and suggested that the increase in the enzyme activity resulted in abnormal cell-cell interactions during embryogenesis. Thus, it is interesting to note that *Ggm-1* also regulates the activity of the galactosyltransferase in mouse liver, which seems to be different from the enzyme in mouse embryos as described above. The galactosyltransferase for G_{M1}(NeuGc) synthesis may be also involved in the cell-cell interactions during ambryogenesis, and it is an interesting problem to analyze the expression of G_{M1}(NeuGc) in mouse embryos in relation to the expression of the galactosyltransferase.

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