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Genetics

Trans-acting temporal locus within the β-glucuronidase gene complex

(mice/gene regulation/systemic regulator/*cis*)

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ABSTRACT **Mice carrying the [*Gus*]^H haplotype of the β-glucuronidase gene complex have considerably decreased enzyme levels and a decreased rate of enzyme synthesis. This is now shown to result from the action of two regulatory loci within the gene complex. One is a systemic regulator, *Gus-u*, that acts *cis* to cause a uniform reduction in enzyme levels in all tissues. The other is a temporal locus, *Gus-t*, that acts *trans* to cause abrupt switches in the rate of enzyme synthesis in only certain tissues and at characteristic stages of development. The distinction between these two loci was made possible by the introduction of a method for quantitating the relative numbers of A and H allozyme subunits in β-glucuronidase tetramers. The procedure involves purification of the enzyme, cleavage at methionyl residues with CNBr, isoelectric focusing to separate the peptides, and quantitation of the peptide containing the A/H amino acid substitution. The presence of a *trans*-acting regulatory locus within a gene complex raises evolutionary and functional questions about why it is located there and how it acts.**

In differentiating organisms the rate of synthesis of each enzyme varies among cell types and changes during development. The set of loci encoding the information determining this program are termed temporal genes, and temporal loci are now known that control the developmental expression of a variety of enzymes in mouse, *Drosophila*, and maize, with each structural gene apparently controlled by its own unique set of regulators (reviewed in refs. 1, 2). Temporal gene systems appear to be organized in a bipartite manner. In several cases genetic variation in developmental programing has been mapped to sites in close proximity to the relevant structural gene, in other cases to distant sites, and in at least one case, murine β-galactosidase (3), both proximate and distant sites interact to determine the phenotype.

An important question relating to the proximate sites is whether they act *cis* or *trans*. *Trans*-acting sites are presumed to release diffusible regulatory signals in contrast to *cis*-acting sites that serve as intrinsic regulators of their own chromosome, perhaps by acting as receptors for diffusible signals. Among the proximate sites tested so far, those of *Drosophila* aldehyde oxidase (4, 5) and amylase (6), maize esterase (7) and alcohol dehydrogenase (8), and mouse alcohol dehydrogenase (9) and glucose phosphate isomerase (10) act *cis*. The report of a *cis*-acting temporal locus at mouse H2 (11) is now rendered uncertain by the finding that this developmental control may be post-translational in mechanism (12). In contrast to these cases, the proximate temporal locus of the β-glucuronidase gene complex has been reported to act *trans* by both Herrup and Mullen (13) and Meredith and Ganschow (14), who used terminal sensitivity of the enzyme to estimate the β-glucuronidase production from each chromosome in appropriate heterozygotes, although our own laboratory had reached a contradictory conclusion from the isoelectric focusing pattern of the enzyme seen in other heterozygotes (unpublished data).

Because the β-glucuronidase gene complex is one of the most extensively studied gene complexes in higher organisms (reviewed in ref. 15), the finding of a *trans*-acting regulatory element there would have appreciable significance. Therefore, we have reexamined this question using a quantitative test for *cis* vs. *trans* action of regulatory loci at the [*Gus*] complex (16) and succeeded in resolving the ostensible contradiction.* It now appears that two proximate regulatory elements differ among the [*Gus*] haplotypes that have been used to study the developmental regulation of β-glucuronidase. One is a previously unrecognized mutation in the *cis*-acting systemic regulator *Gus-u*; the presence of a mutant allele at this site decreases the rate of enzyme synthesis to one-third of the usual level at all stages of development. The other is the previously recognized temporal gene, *Gus-t*, where the *Gus-t*^{h} allele acts *trans* to superimpose a further developmentally specific reduction in enzyme synthesis in some tissues at fixed stages of development. The *trans* action of *Gus-t* is thus confirmed, and the reasons for the apparent contradiction are now understood.

MATERIALS AND METHODS

Mice. Mice of the C57BL/6J (B/6) and A/J (A) strains were obtained from The Jackson Laboratory; C3H/HeHa (C3H) and DBA/LiHa (DBA) mice were bred at Roswell Park Memorial Institute. Strain DBA/LiHa was originally known as DBA/2Ha, but it is now recognized as an independent strain. DBA/LiHa carries the [*Gus*]^A haplotype in contrast to DBA/1 and DBA/2 strains, which carry the [*Gus*]^B haplotype. The congeneic strains B6.A and B6.H were constructed by transferring the [*Gus*]^A and [*Gus*]^H haplotypes from A and C3H onto the B/6 genetic background by repeated backcrossing (17). These strains are summarized in Table 1.

Assays. β-Glucuronidase specific activity was determined in earlier experiments by using *p*-nitrophenyl β-D-glucuronide as substrate at 56°C (18) and in later experiments by using 4-methyllumbelliferone-β-D-glucuronide as substrate at 37°C (19). Specific activity is expressed as μmol of product per hr/g of tissue and 1 4-methyllumbelliferone unit is equivalent to ≈2

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* Genetic nomenclature: The β-glucuronidase gene complex is designated [*Gus*]; alternative forms or haplotypes are designated [*Gus*]^A, [*Gus*]^B, and [*Gus*]^H; the loci within the complex are *Gus-s* (structural gene), *Gus-r* (androgen response regulator), *Gus-t* (temporal gene), and *Gus-u* (systemic regulator).

Table 1. Glucuronidase genotypes and phenotypes of mouse strains

Strain	Haplotype	Alleles at t-s-u	Heat stability	Electrophoretic mobility	Adult liver activity*
A	A	a-a-b	Stable	Fast	26
B6.A	A	a-a-b	Stable	Fast	30
B6.H	H	h-h-h	Labile	Slow	2.5
C3H	H	h-h-h	Labile	Slow	2.8
B6	B	a-b-b	Stable	Slow	32
DBA	A	a-a-b	Stable	Fast	29

* Adult enzyme activity is expressed as 4-methyllumbelliferone units/g of tissue.

p-nitrophenol units. *Cis–trans* testing was carried out as described (16), except that in some of the experiments peptide bands were visualized by using a silver stain procedure (20). The validity of the silver stain for quantitation of peptides was confirmed by finding a linear staining intensity with concentrations of purified glucuronidase allozymes over the experimental range.

Rate of Enzyme Synthesis. For each measurement three adult mice were injected intraperitoneally with 0.5 mCi (1 Ci = 3.7×10^{10} Bq) of [^{3}H]leucine, and 1 hr later animals were sacrificed and the pooled tissues were homogenized in 10 vol of 0.02 M imidazole at pH 7.2. Homogenates were adjusted to pH 4.5 with acetic acid and heated at 56°C for 30 min. The precipitate was removed by centrifugation and ammonium sulfate was added to the supernatant solution to 55% of saturation. The ammonium sulfate pellet was resuspended in 0.02 M Tris/0.15 M NaCl, pH 7.4, and glucuronidase was purified from the solution by using antibody affinity chromatography as described (21). The purified glucuronidase was precipitated with goat antibody to mouse glucuronidase and the immunoprecipitates were subjected to polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. The radioactivity migrating at the position of glucuronidase (subunit M_r = 70,000) was adjusted for loss of glucuronidase activity during the purification (the yield averaged 60–70%) and this was used in calculating the rate of glucuronidase synthesis. The relative rate of enzyme synthesis is expressed as isotope incorporated into β-glucuronidase relative to total trichloroacetic acid precipitate.

RESULTS

Genetics. Law *et al.* (22) originally reported that a single locus with two alleles determines the inheritance of low β-glucuronidase activity in crosses between mice of the C3H and A strains. Subsequently, C3H was found to produce a structurally altered enzyme as well as having decreased activity, and the loci determining the two phenotypes cosegregated (23). These results are confirmed in Fig. 1, showing the segregation of the structural variant, which was scored by changes in the thermolability of the enzyme, and the determinant of enzyme activity, which was scored by assays of enzyme levels in adult liver. The cross was between the haplotypes $[Gus]^H$ in strain C3H and $[Gus]^A$ in strain DBA.

Developmental Phenotypes. The original description (23) of developmental phenotypes of mice carrying different [*Gus*] haplotypes compared the $[Gus]^H$ haplotype in strain C3H/HeHa with the $[Gus]^A$ haplotype in strain DBA/LiHa. It was found that enzyme levels in adult C3H tissues, relative to their DBA counterparts, varied from one tissue to another and that this arose because of differences between tissues in the timing and extent of switches in enzyme activity. Subsequently, these switches were shown to involve changes in the rate of enzyme synthesis (24). The results for liver are confirmed in Fig. 2 and Table 2 which compare several inbred and congeneic strains and F_1 progeny of a cross.

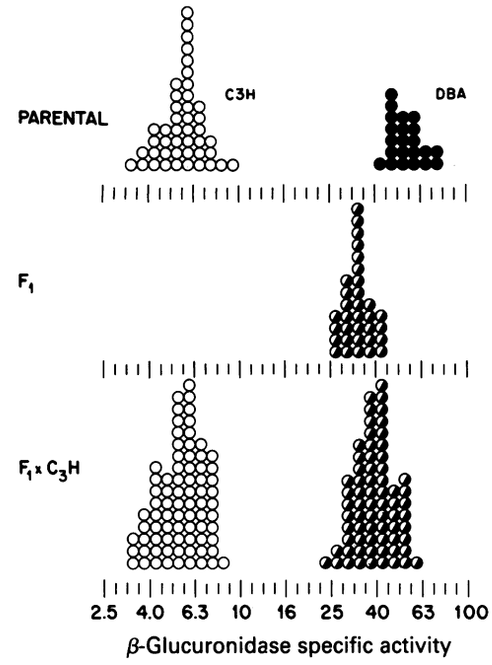

FIG. 1. Cross of C3H × DBA scored for β-glucuronidase specific activity and heat lability of the enzyme in liver. The abscissa is a log scale of enzyme specific activity in *p*-nitrophenol units (see *Materials and Methods*). Each point represents one mouse. The survival of the enzyme after a standard heat inactivation at 71°C is indicated as 0–4% (○), 11–18% (◕), and 24–33% (●); there was no overlap between groups.

These data (*i*) confirm the original observations on the altered developmental profile determined by $[Gus]^H$; (*ii*) show that the $[Gus]^A$ and $[Gus]^B$ haplotypes have the same developmental profile when tested on the same genetic background;

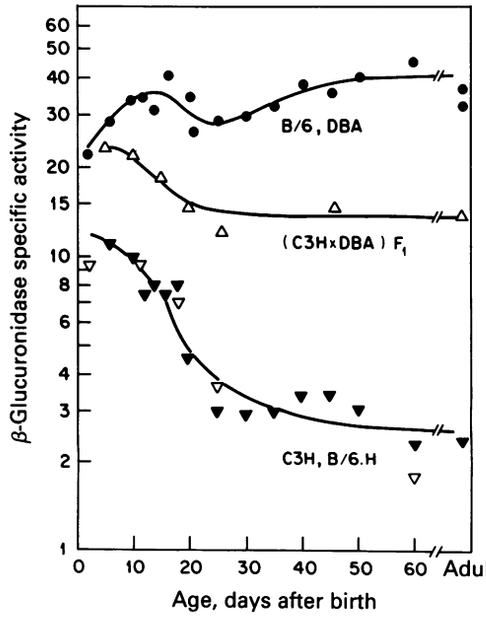

FIG. 2. Developmental profile for liver β-glucuronidase in various mouse strains. B6 (●); C3H (▽); B6.H (▼); and (C3H × DBA) F_1 (△). Each point is the mean of three to six animals measured at that age. Activity is in 4-methyllumbelliferone units.

Table 2. Segregation of hepatic β-glucuronidase activity in DBA × C3H cross

Strain or F_1 progeny	Mean	SD	*n*
DBA	56.1	9.5	22
C3H	5.7	1.2	44
(DBA × C3H) F_1	33.4	4.3	33
C3H × F_1			
High	39.5	7.6	69
Low	5.6	1.2	78

Mean activity is expressed as *p*-nitrophenol units/g of liver measured on adult mice.

(*iii*) show that the $[Gus]^{A/H}$ heterozygote exhibits intermediate inheritance; and (*iv*) establish that the *A*, *B*, and *H* haplotypes express the same phenotype in their strains of origin and when tested in a constant genetic background as C57BL/6 congeneic lines.

Additionally, the congeneic data serve as a further confirmation that *Gus-t*, the temporal locus determining the developmental phenotype, is closely linked to *Gus-s*, the structural gene for β-glucuronidase, because the linkage between these two loci was maintained after 20 generations of backcrossing during construction of the B6.H congeneic line. It is also apparent from the results with congeneic lines that *Gus-t* is the primary genetic determinant of the developmental phenotype and that any genetic variation at unlinked modifiers has only small effects (see below).

Additive Inheritance. Examination of the mean levels of enzyme in the various genetic classes shows intermediate enzyme levels in $[Gus]^{A/H}$ heterozygotes, indicating additive inheritance (Figs. 1 and 2 and Table 2). However, a rather small modifier effect can be detected when sufficient numbers of animals are compared. In the C3H × DBA cross, for example, H/A heterozygotes in the F_1 generation are 1.08 times the mid-parent value, whereas H/A heterozygotes arising in the backcross to C3H are 1.28 times the mid-parent value. Although this difference is statistically significant ($P < 0.01$), the magnitude of the difference is small compared to the primary observation that heterozygotes show intermediate enzyme levels.

***Cis* vs. *Trans* Action of *Gus-t*.** Herrup and Mullen (13) and Meredith and Ganschow (14) utilized the difference in thermolability of the GUS-H and GUS-B allozymes of β-glucuronidase to estimate the relative amounts of enzyme derived from each chromosome in heterozygotes. β-Glucuronidase is a tetramer of four identical subunits, and, if *Gus-t* acts *cis*, a majority of the enzyme present in $Gus\text{-}^{t^h,s^h/t^a,s^b}$ heterozygotes (see Table 1) should be in the form of B_4 homotetramers and hence thermostable. They did not find this result. Meredith and Ganschow also showed that the heat inactivation curves of the mixture of β-glucuronidase tetramers present in various tissues was the same despite the fact that *Gus-t* regulation is tissue specific and should lead to varying ratios of GUS-H and GUS-B subunits among tissues if there is *cis* regulation. Both groups therefore concluded that *Gus-t* acts *trans*. We had been reluctant to accept this conclusion because in our own experience isoelectric focusing of enzyme from $Gus\text{-}^{t^h,s^h/t^a,s^a}$ heterozygotes showed a clear predominance of GUS-A over GUS-H enzyme subunits, suggesting a *cis* control of enzyme synthesis (see below). The advent of a method for accurately quantitating the relative numbers of GUS-A and either GUS-H or GUS-B subunits has allowed us to reexamine this question and resolve the apparent discrepancy.

The method assays the amounts of the alternate forms of the peptide fragment containing the amino acid substitution that confers an extra negative charge to the GUS-A allozyme. Purified β-glucuronidase is isolated from each sample by a combination of physical and immunological methods and is treated with CNBr to cleave at methionyl residues, and the resulting peptides are separated by isoelectric focusing (16). These are then stained and quantitated by scanning densitometry (Fig. 3). As a control, equal amounts of GUS-H glucuronidase activity (using a testosterone-induced C3H kidney homogenate) and GUS-A activity (using a DBA liver homogenate) were mixed; the ratios of A to H peptides in these samples in three experiments were 1.1, 1.0, and 1.0, well within experimental error of the expected value of 1.0.

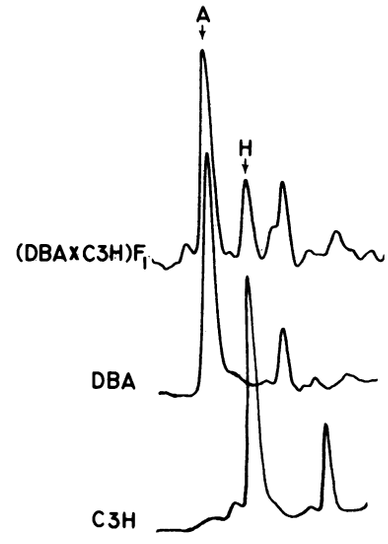

FIG. 3. Densitometric tracings of glucuronidase CNBr peptides from F_1 mice. A and H mark the positions of the major variant peptides. A minor derivative of the variant peptide, focusing more cathodically (at the right) was also resolved. Shown for comparison are the tracings of A and H peptides from DBA and C3H mice, respectively.

By using this procedure the H/A subunit ratio was ≈0.3 in the livers of adult $[Gus]^{H/A}$ heterozygotes arising from a cross between C3H and DBA or from a cross between the B6.H and B6.A congeneic lines (Table 3). This value was entirely unexpected because the *cis* hypothesis predicts a ratio of about 0.1 and the *trans* hypothesis a ratio of 1.0. The experimental results do not fit either hypothesis. Heterozygotes of $[Gus]^{B/A}$, where neither parent expresses the unusual developmental phenotype of $[Gus]^{H}$, gave a ratio of subunits close to 1.0, as expected. The subunit ratio was then examined in $[Gus]^{H/A}$ heterozygotes in liver at 10 days of age before the develop-

Table 3. Ratio of GUS-H (or GUS-B) to GUS-A enzyme in F_1 progeny

	Ratio	
Genetic cross	Ten days of age	Adult
C3H × DBA (H/A)	0.31, 0.34, 0.28, 0.29, 0.32	0.29, 0.40, 0.33
B6.H × B6.A (H/A)		
Liver		0.31, 0.29, 0.41
Spleen		0.43
Kidney		0.43, 0.37
DBA × B6 (B/A)		0.9, 1.3, 1.0

Glucuronidase was isolated from tissues by using antibody affinity chromatography and the ratio of GUS-H (or GUS-B) to GUS-A enzyme was determined by analysis of CNBr peptides. For determination of enzyme ratios in kidney, animals were treated with testosterone to induce β-glucuronidase (21). Each value represents a separate experiment.

mental switch that characterized $[Gus]^H$ occurs, and in other tissues. These included spleen, where the developmental difference between $[Gus]^H$ and $[Gus]^A$ is less pronounced, and kidney, where the difference is more pronounced. In each case the ratio of GUS-H/GUS-A subunits remained close to 0.3—that is, not only is the ratio not unity, but it is also not affected by the tissue-specific changes in β-glucuronidase activity determined by *Gus-t*.

We conclude that the $[Gus]^H$ haplotype, in fact, carries two regulatory changes from $[Gus]^A$ and $[Gus]^B$. One is in a *cis*-acting systemic regulator that decreases enzyme levels in all tissues and at all stages of development to 0.3 of the $[Gus]^A$ or $[Gus]^B$ level. The presence of a *cis*-acting systemic regulator, *Gus-u*, within the [*Gus*] complex, has also been detected in crosses between $[Gus]^B$ and two previously undescribed β-glucuronidase haplotypes, $[Gus]^{CS}$ and $[Gus]^{CL}$ (unpublished data). We designate the allele present in C3H as $Gus\text{-}u^h$ and the allele present in A and B/6 as $Gus\text{-}u^b$. The other regulatory element is the *Gus-t* temporal locus that acts *trans* on both chromosomes to impose a further reduction of enzyme activity selectively in some tissues. The detailed genotype of the $[Gus]^H$ haplotype is then $Gus\text{-}^{s^h,t^h,u^h}$; that of the $[Gus]^A$ haplotype is $Gus\text{-}^{s^a,t^a,u^b}$; and that of the $[Gus]^B$ haplotype is $Gus\text{-}^{s^b,t^a,u^b}$. In heterozygotes the *trans*-acting regulator causes a drop in enzyme activity during the developmental switch period (Fig. 2), with no change in the ratio of enzyme subunits caused by the presence of the *cis*-acting regulator (Table 3).

We have qualitatively confirmed these results by isoelectric focusing of intact enzyme molecules from fresh homogenates (Fig. 4). Heterozygotes showed a relative deficiency of GUS-H subunits, seen as a shift in the pattern away from the H_4 tetramer. Importantly, the magnitude of this effect did not change appreciably over the time period of the developmental switch.

The present studies used $[Gus]^{H/A}$ heterozygotes, whereas $[Gus]^{H/B}$ heterozygotes were used in the heat inactivation studies of Herrup and Mullen (13) and Meredith and Ganschow (14). It is not possible to confirm the *trans* action of *Gus-t* in H/B heterozygotes by using the peptide method because there are no charge differences between GUS-B and GUS-H enzyme subunits. Therefore, we have compared the heat inactivation rates of enzyme from *H/A* and *H/B* heterozygotes to test whether the same developmental phenotype is expressed in the two heterozygotes. Our results (Fig. 5) confirm that the heat inactivation profiles of β-glucuronidase from heterozygotes is independent of the tissue examined or the stage of development chosen, and the results further indicate that there is no significant difference between $[Gus]^{H/A}$ and $[Gus]^{H/B}$ heterozygotes.

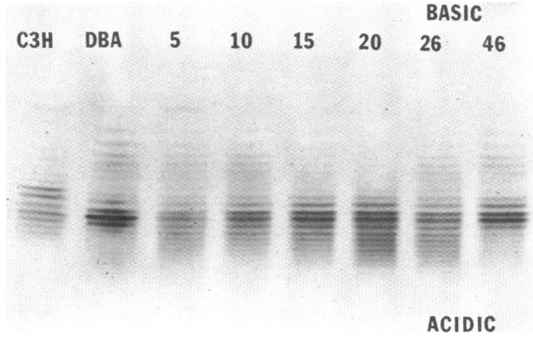

FIG. 4. Isoelectric focusing of whole glucuronidase from livers of C3H mice, DBA mice, and (C3H × DBA) F_1 mice of ages 5, 10, 15, 20, 26, and 46 days. After isoelectric focusing glucuronidase activity was visualized by using naphthol-AS-BI-glucuronide (25).

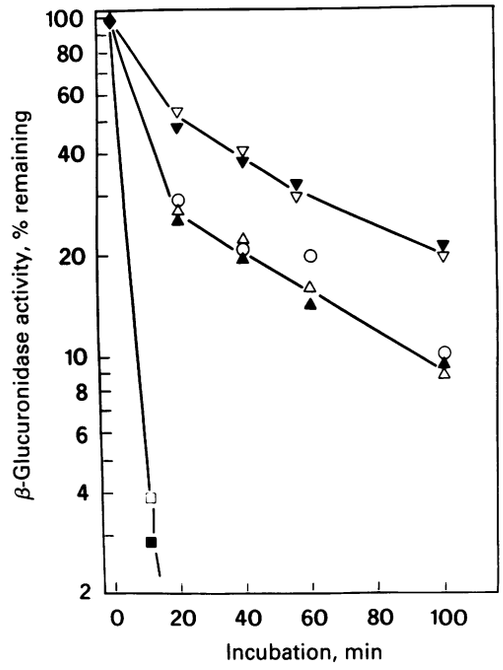

FIG. 5. Heat inactivation of liver glucuronidase. Liver homogenates were diluted in 0.1 M sodium citrate at pH 5.0 and heated at 71°C for various times, and the glucuronidase activity remaining was determined. B6 (▽); B6.A (▼); (C3H × B6) F_1 (○); (B6.A × B6.H) F_1 (△); (C3H × DBA) F_1 (▲); B6.H (■); and C3H (□).

Control of Enzyme Synthesis in Homozygotes and Heterozygotes. Ganschow (24) has shown that the decreased enzyme level in mice carrying $[Gus]^H$ results from decreased enzyme synthesis and is not the result of altered post-translational processing. We now confirm this (Table 4) and also show that the intermediate level of enzyme in heterozygotes reflects an intermediate rate of enzyme synthesis in these animals. This is true of $[Gus]^{H/A}$ heterozygotes arising from crosses between C3H and DBA and from crosses between the congeneic lines B6.A and B6.H.

DISCUSSION

Combining our present results with the previous data of Herrup and Mullen (13) and Meredith and Ganschow (14), it appears that the *Gus-t* temporal locus in the [*Gus*] complex acts *trans* and that alleles of *Gus-t* exhibit additive inheritance. This result raises the question of why a *trans*-acting regulatory locus is located within the [*Gus*] complex, when, in principle, such a locus could be located anywhere in the genome. It also presents a potential access to molecular studies of a *trans*-acting DNA regulatory sequence through cloning the adjacent β-glucuronidase structural gene. Our results also confirm several major features of *Gus-t* action—namely, that *Gus-t* is located in the [*Gus*] complex, that alleles of *Gus-t* show additive expression, and that *Gus-t* is the predominant genetic locus determining differences in β-glucuronidase developmental programing among inbred mouse strains.

Table 4. Rates of β-glucuronidase synthesis in adult liver

Strain	Relative rate of synthesis × 10^5	% synthesis*	% activity*
DBA	1.4	100	100
(C3H × DBA) F_1	0.50	35	50
C3H	0.16	7	9

*DBA = 100%.

The *trans* action of *Gus-t* contrasts with what is known for other temporal gene systems, where only distant temporal loci have been found to act *trans* and all of the proximate loci tested to date act *cis* (4–9). *Trans* action also distinguishes *Gus-t* from the other regulatory loci within the [*Gus*] complex because both the androgen response regulator, *Gus-r*, and the systemic regulator, *Gus-u*, act *cis*.

The current findings also provide a better understanding of the developmental pattern of β-glucuronidase expression in mice carrying the $[Gus]^H$ haplotype. We now appreciate that this phenotype results from the combined action of two regulatory elements that are both located within the [*Gus*] complex. One is the *cis*-acting systemic regulator *Gus-u*, and the other is the *trans*-acting temporal gene *Gus-t*. The combined action of the two loci results in the presence of about 30% β-glucuronidase H subunits in A/H or B/H heterozygotes at all ages. This explains the apparent discrepancy between the conclusions originally drawn from heat inactivation studies of B/H heterozygotes (13, 14) and our own earlier results on the electrophoretic behavior of β-glucuronidase in A/H heterozygotes. The heat inactivation studies led to the conclusion of *trans* action because there was a deficiency of B_4 homotetramers compared to what was expected for complete *cis* action, whereas the electrophoretic studies led to the conclusion of *cis* action because there was a deficiency of H_4 homotetramers compared to what was expected for complete *trans* action.

It is useful to try to estimate the maximal distance between *Gus-t* and the β-glucuronidase structural gene within the [*Gus*] complex. In the crosses reported here no recombinants were observed in 147 tested chromosomes; no recombination occurred among the 13 C3H × C57BL/6 recombinant inbred lines (with an average of four opportunities for recombination during the establishment of each line), and no recombination occurred in 20 generations of backcrossing during construction of the B6.H congeneic. Together, this gives 0/219 recombination events. We estimate the maximal distance between *Gus-s* and *Gus-t* as the distance that would have a 0.50 probability of not giving a recombinant among 219 tested chromosomes. This calculates to be 0.3 centimorgan, which is equivalent to about 600 kilobases. The two loci could be much closer than this maximum.

The finding that *Gus-t* acts *trans* suggests that it determines a diffusible molecule that is capable of regulating the expression of the β-glucuronidase structural gene. As pointed out by Meredith and Ganschow (14), this molecule could be β-glucuronidase itself (or its mRNA). However, there is an accumulating body of evidence from other systems that temporal genes can act through diffusible regulatory substances that are not products of the enzyme structural gene that is being programed. The best characterized examples concern mouse α-galactosidase (26, 27), mouse β-galactosidase (3), *Drosophila* amylase (6), maize alcohol dehydrogenase (28), and maize catalase (29); the developmental programs of these proteins are determined by temporal genes located at some distance from the structural genes for these enzymes. Given this evidence for separate regulatory substances, one possible explanation of *Gus-t* action in particular suggests itself. *Gus-t* could code for a nonhistone chromatin protein specific to the [*Gus*] complex. This would explain the specificity of *Gus-t* action, *trans* action by the locus, and the additive inheritance of its alleles, because in heterozygotes such chromosomes could contain a mixture of the chromatin proteins coded by both chromosomes.

There is, presumably, some evolutionary reason why *Gus-t* is located within the [*Gus*] complex. If *Gus-t* does code for a nonhistone chromatin protein of the [*Gus*] complex, this would suggest that the protein components of the complex are coded by the complex itself. This idea is appealingly frugal and would allow for coselection and coevolution of the DNA sequences and protein components that collectively make up a gene complex.

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