INTERACTING GENES CONTROL GLYCEROL-3-PHOSPHATE DEHYDROGENASE EXPRESSION IN DEVELOPING CEREBELLUM OF THE MOUSE

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Manuscript received September 7, 1984
Revised copy accepted January 16, 1985

ABSTRACT

The cerebellum of BALB/cJ mice has approximately 2.5 times as much glycerol-3-phosphate dehydrogenase (GPDH) as that of C57BL/6J mice. This difference in enzyme levels, which positively correlates with similar differences in the levels of hybridizable GPDH mRNA, is controlled by at least two unlinked regulatory loci and the structural gene, *Gdc-1*, located on chromosome *15*. These regulatory loci, which act predominantly during the second and third weeks of postnatal cerebellar development and differentiation, have been separated from each other in the CXB recombinant inbred strains of mice. One regulatory locus, *Gdcr-1*, although unlinked to the structural gene, has an allele in BALB/c mice that preferentially enhances expression of the BALB/c structural allele at *Gdc-1*. The other locus, *Gdcr-2*, which may or may not be single, enhances GPDH expression at *Gdc-1* irrespective of the allele present, as is commonly observed for loci acting from a distance. Measurements of GPDH mRNA in the recombinant inbred mice suggest that these regulatory genes act by modulating mRNA levels. Accordingly, the regulation of GPDH expression in the cerebellum of mice depends on a complex interaction of unlinked regulatory elements with regulatory elements near the structural gene. Furthermore, since the *Gdc-1* locus is expressed in virtually every tissue of the mouse except blood and since the observed genetic variation is restricted to the cerebellum, it is likely that other tissues will have their own distinctive genetic mechanisms for modulating *Gdc-1* expression.

BY examining tissue-specific genetic variation in the quantitative expression of structural genes, one can begin to identify and analyze genes controlling the differentiation of individual cell types within an organism. This approach has been particularly successful for amylase expression in mice where it has been found that the parotid gland and liver genomes utilize one of two promoters of different strengths at the 5′ end of the *Amy-1* gene so that amylase mRNA and protein production in parotid gland is more than 100-fold greater than it is in liver (YOUNG, HAGENBUCHLE and SCHIBLER 1981; SCHIBLER *et al.* 1983). The pancreas, on the other hand, has its own transcriptional unit, *Amy-2*, closely linked to *Amy-1* and represented by multiple copies (HJORTH, LUSIS and NIELSEN 1980; NIELSEN 1982; SCHIBLER *et al.* 1982). A second gene-enzyme system that is well defined genetically (PAIGEN 1979), although less so

molecularly (PALMER *et al.* 1983), is the β-glucuronidase enzyme encoded by the *Gus* gene. Although the *Gus* structural gene functions in several tissues, elements, genetically inseparable from the structural gene, control genetic variations for testosterone induction in the proximal tubule cells of kidney (SWANK, PAIGEN and GANSCHOW 1973), for developmental variation in the liver (PAIGEN 1961) and for a generalized systemic variation (PAIGEN 1979). Although these systems provide strong evidence implicating sequences in and around the structural gene in tissue-specific gene regulation, they cannot, in themselves, explain the coordinated expression of genes in a developing complex organism. Other unlinked genes must be involved in the activation of structural genes in a specific tissue at a defined point in development; in multicellular eukaryotic organisms we know very little of the structure and function of these genes.

Between C57BL/6J and BALB/cBy mice a quantitative variation in *sn*-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8;GPDH) expression is determined by a complex interaction between genes, one of which is the structural gene, *Gdc-1*, on chromosome *15* (KOZAK 1972). Since the variation is restricted to the cerebellum and occurs during postnatal development, it is possible that the variation is controlled by regulatory elements. Additional experiments showing that the kinetic properties of the purified enzymes of the two strains are indistinguishable (KOZAK 1974) and that higher levels of immunoprecipitable protein and translatable mRNA are detected in the cerebellum of BALB/cJ(By) mice (KOZAK and RATNER 1980) suggest that these loci are involved in tissue-specific, developmentally dependent gene regulation.

This paper elucidates our attempt to unravel the complexity of the quantitative variation in cerebellar GPDH by using recombinant inbred strains of mice to separate major interacting genes. The results indicate that the structural gene by itself does not possess properties that can account for the variable quantitative expression but that two regulatory elements, *Gdcr-1* and *Gdcr-2*, not linked to one another nor to the structural gene, are interacting with the structural gene. One element interacts preferentially with BALB/cJ allele at the *Gdc-1* locus to increase the number of GPDH-C subunits while the other interacts with the *Gdc-1* gene to enhance expression of both structural alleles. Both regulatory elements modulate the number of GPDH mRNA molecules. We think that these unlinked genetic regulatory elements which interact with the GPDH structural gene, or elements modulating its expression, may represent tissue-specific regulatory genes.

MATERIALS AND METHODS

Animals: Mice were bred and maintained in the research colonies of The Jackson Laboratory. Breeding pairs of CXB recombinant inbred mice were obtained from DONALD BAILEY. The C57BL/6J.CAST-*Gdc-1*^{d} (N_{11} F_{9} generation) congenic line was obtained from THOMAS RODERICK.

Expression of the genetic variation which results in differences in GPDH enzyme levels among inbred strains is maximal between 10 and 20 days of age. Since this is also the period in the cerebellum of active growth and differentiation when the GPDH specific activity increases sharply, small differences in age (12–24 hr) can contribute substantially to the nongenetic variance. To minimize this nongenetic variance we carefully recorded the birth dates of litters and did not analyze litters of four pups or less.

All crosses were made reciprocally; however, since in every case the direction of a cross did not affect the distribution of the phenotypes, the data has been pooled and presented as coming from a cross made in one direction only. This practice has been followed for all of the intercrosses and backcrosses reported in this paper. We have not detected differences among the By, J and Wt sublines of BALB/c or the By and J sublines of C57BL/6 for any of the GPDH phenotypes described in this project. Because B6 and B6.CAST do not show significant differences in activity levels at 18 days of age, we initially concluded that these two strains were identical with respect to the quantitative aspects of GPDH expression and that B6.CAST provided a strain that could be conveniently used in further genetic analysis since cosegregation of structural alleles with activity phenotypes would be easier to score with an electrophoretic variant than a heat stability variant. In retrospect, however, the *Gdc-1*^{d} allele should not be considered as simply an electrophoretic variant of the *Gdc-1*^{b} allele, and the conclusions derived from the quantitative expression of the *Gdc-1*^{d} allele should not be considered as necessarily pertaining also to the *Gdc-1*^{b} allele.

Enzyme assays: The GPDH activity level was determined on the 100,000 × *g* supernatant fraction as described by KOZAK and JENSEN (1974). Heat denaturation of GPDH activity was carried out as described by KOZAK (1972).

RNA assay: Total RNA from the cerebellum and cerebral cortex was extracted by guanidinium-HCl (COX 1968) from a pool of at least 20 mice. Quantitation of the amount of GPDH mRNA in a preparation was made by Northern blot analysis as described by DERMAN *et al.* (1981) using Zetabind filters (AMF). Filters were hybridized with a T4 polymerase-labeled genomic DNA probe prepared essentially as described by O'FARRELL (1981). This genomic DNA fragment (isolated from a BALB/c embryo DNA library in Charon 28, obtained through the courtesy of PHILIP LEDER) is a 0.8-kilobase *Hin*dIII fragment carrying coding sequence for amino acids 14 to 73 of the GPDH protein. A manuscript describing the characteristics of the genomic sequence is in preparation. Autoradiographs of the filters carrying variable levels of RNA and standards were scanned at 600 nm with a Gilford Instruments linear transport device. Quantitation of RNA on Northern blots is subject to many variables. To obtain reliable estimates the following procedures were used. Precipitated RNA to be analyzed was centrifuged out of an ethanol solution shortly before electrophoresis and dissolved in a small volume of H_2O previously treated with diethyl pyrocarbonate. One aliquot was used to measure the concentration of RNA by UV absorption and a second aliquot containing the desired amount of RNA added to the sample buffer. Following hybridization with the probe for GPDH mRNA, filters were hybridized with additional unique sequence probes including a probe from the 5′ end of the *Gdc-1* gene and a probe to mouse malic enzyme (SUL *et al.* 1984). The mRNAs that hybridize to these probes do not vary between BALB/c and B6 mice. Finally, filters were stained with methylene blue to obtain a visual impression of the amount of RNA bound to the filter (ZINN, DI MAIO and MANIATIS 1983).

Allozyme assay: Allozymes of GPDH were separated by electrophoresis on cellulose acetate plates (Helena) as described by KOZAK, BURKART and HJORTH (1982). Stained plates were scanned with the Helena Quick Scan and areas under each peak determined using an Apple Graphics Tablet and the area function.

RESULTS

Strategy: The original data suggesting a two-locus model for inheritance of cerebellar GPDH variation were based on backcross analysis of the BALB/cJ and C57BL/6J mice (KOZAK 1972). In addition to showing the quantitative difference, these strains also expressed alternate alleles at the structural gene which changed the thermal stability of the enzyme. The allele in BALB/c, *Gdc-1*^{c}, determines the heat-sensitive enzyme GPDH-C; the allele in C57BL/6, *Gdc-1*^{b}, determines the heat-stable enzyme GPDH-B. Since the modal distribution of 1:2:1 for the activity level in the backcross generation was consistent with two loci and since there was a strong association between the heat-sensitive *Gdc-1*^{c} allele and the high activity phenotype (as well as recombinant phenotypes, which were homozygous *Gdc-1*^{c} and had intermediate enzyme levels),

we concluded that the activity phenotype was controlled by two regulatory elements unlinked to each other; one, however, seemed to be linked to the structural locus, *Gdc-1*.

The availability of recombinant inbred mice derived from the BALB/cBy and C57BL/6By progenitor strains (BAILEY 1971) allowed us to test further the adequacy of the regulatory gene model and to determine whether these genes, when together or recombined, acted by controlling mRNA levels. We first analyzed C57BL/6By × BALB/cBy (CXB) recombinant inbred (RI) lines of mice at the protein and mRNA level in order to identify RI lines in which the two regulatory loci had been separated from each other and possibly other minor genetic influences. Second, we analyzed offspring from backcross and intercross matings made between the RI lines with recombinant genotypes to determine the mode of inheritance of these genotypes. Third, we measured the relative production of protein subunits in F_1 offspring made between CXB recombinant inbred mice and a congenic strain, C57BL/6J.CAST-*Gdc-1*^{d} (B6.CAST), which carries an electrophoretically variant allele at *Gdc-1* but which is similar to the C57BL/6J parental strain in the level of GPDH expression.

Heat inactivation and enzyme activity levels in progenitor and RI strains: The RI mice and the parental strains were characterized by heat inactivation of GPDH to determine whether they carried the heat-labile *Gdc-1*^{c} allele or the heat-stable *Gdc-1*^{b} allele (Table 1). The resulting strain distribution pattern (SDP) of the heat sensitivity phenotype is characteristic for the segment of chromosome *15* which carries the *Gdc-1* gene. This is the only chromosome *15* marker known for the RI mice (TAYLOR 1981). In addition, Table 1 lists the cerebellar GPDH activity levels found in mice at 18 days of age. The RI strains are grouped into one of two classes, the high activity class comprising CXBG and CXBH and an intermediate class including CXBK, CXBI, CXBJ, CXBD and CXBE. A low activity class, characteristic of the C57BL/6 progenitor strain, was not represented among the RI strains, not an unexpected finding in view of the small number of RI strains available for analysis.

F_1 hybrids constructed by crossing B6.CAST to each of the RI lines or their progenitor strains are intermediate in their level of enzyme expression (Table 2) with the rank order paralleling that observed with the progenitor strains (Table 1). This indicates that the effects of these genes are generally additive, although a dominance effect seems to be present in the (BALB/c × B6.CAST)F_1 and the (CXBG × B6.CAST)F_1 hybrid. The significance of this effect is not understood and has not been pursued further in this study.

GPDH mRNA levels: With a DNA hybridization probe derived from the coding region of the *Gdc-1* gene, we measured the level of GPDH mRNA transcripts. Total RNA isolated from the cerebellum of each RI strain was electrophoresed through agarose gels, blotted onto a nylon-based filter paper (Zetabind, AMF) and hybridized with a T4 polymerase-labeled fragment of DNA containing GPDH protein-coding sequence. Autoradiographs revealed the presence of a single hybridizing species of mRNA approximately 3500 nucleotides long (Figure 1). Densiometric scanning and integration of the peak

TABLE 1

Properties of GPDH expression in cerebellum of RI mice

Strain	Heat inactivation[a]	Enzyme activity at 18 days of age[b]
BALB/cBy	5.9	0.273 ± 0.043 (18)
CXBG	6.3	0.250 ± 0.046 (41)
CXBH	4.3	0.242 ± 0.016 (41)
CXBK	4.8	0.192 ± 0.023 (26)
CXBI	47.3	0.167 ± 0.015 (22)
CXBJ	36.1	0.166 ± 0.019 (26)
CXBD	8.0	0.156 ± 0.017 (18)
CXBE	37.6	0.153 ± 0.022 (44)
C57BL/6J.CAST-*Gdc-1*$^{d/d}$	45.5	0.125 ± 0.023 (50)
C57BL/6J	37.7	0.117 ± 0.022 (18)

Statistical analysis by the Student-Newman-Keuls multiple range test at $P = 0.01$; strains grouped by braces are not significantly different.

[a] Percent activity remaining after 4 min at 50°.

[b] Enzyme activity, given as the mean ± standard deviation, equals μmoles of NADH oxidized/min/mg protein; numbers in parentheses refer to number of mice analyzed.

TABLE 2

Specific activities of cerebellar GPDH in F_1 hybrids at 18 days of age

F_1 cross	Observed enzyme activity[a] (μmoles/min/mg protein)	Predicted value[b]
BALB/cBy × B6.CAST	0.238 ± 0.044 (31)	0.199
CXBG × B6.CAST	0.200 ± 0.020 (32)	0.188
CXBH × B6.CAST	0.187 ± 0.022 (28)	0.183
CXBK × B6.CAST	0.161 ± 0.012 (29)	0.159
CXBJ × B6.CAST	0.140 ± 0.013 (18)	0.146
CXBD × B6.CAST	0.136 ± 0.023 (17)	0.141
B6 × B6.CAST	0.122 ± 0.026 (23)	0.121

[a] Data are given as the means ± standard deviation. Numbers in parentheses refer to the number of animals analyzed.

[b] Mean of the two parental values is calculated from data in Table 1.

areas of the bands provided an estimate of the relative amount of RNA among the various RI strains. The correlation of the level of enzyme activity to hybridizable mRNA illustrated in Figure 2 strongly indicates that the variation in GPDH enzyme levels in the cerebellum is controlled by the number of GPDH mRNA transcripts. In addition, the data suggest that the SDP for enzyme activity is also the SDP for the number of mRNA molecules; other factors acting only at the translational level do not have a major role in determining the GPDH phenotype among RI strains.

Two important patterns are evident in this data:

1. Only strains with the *Gdc-1*^{c} structural allele at the structural locus have the high level of GPDH activity characteristic of BALB/c. This is consistent with data from earlier BALB/cJ × (BALB/cJ × C57BL/6J)F_1 backcross analysis

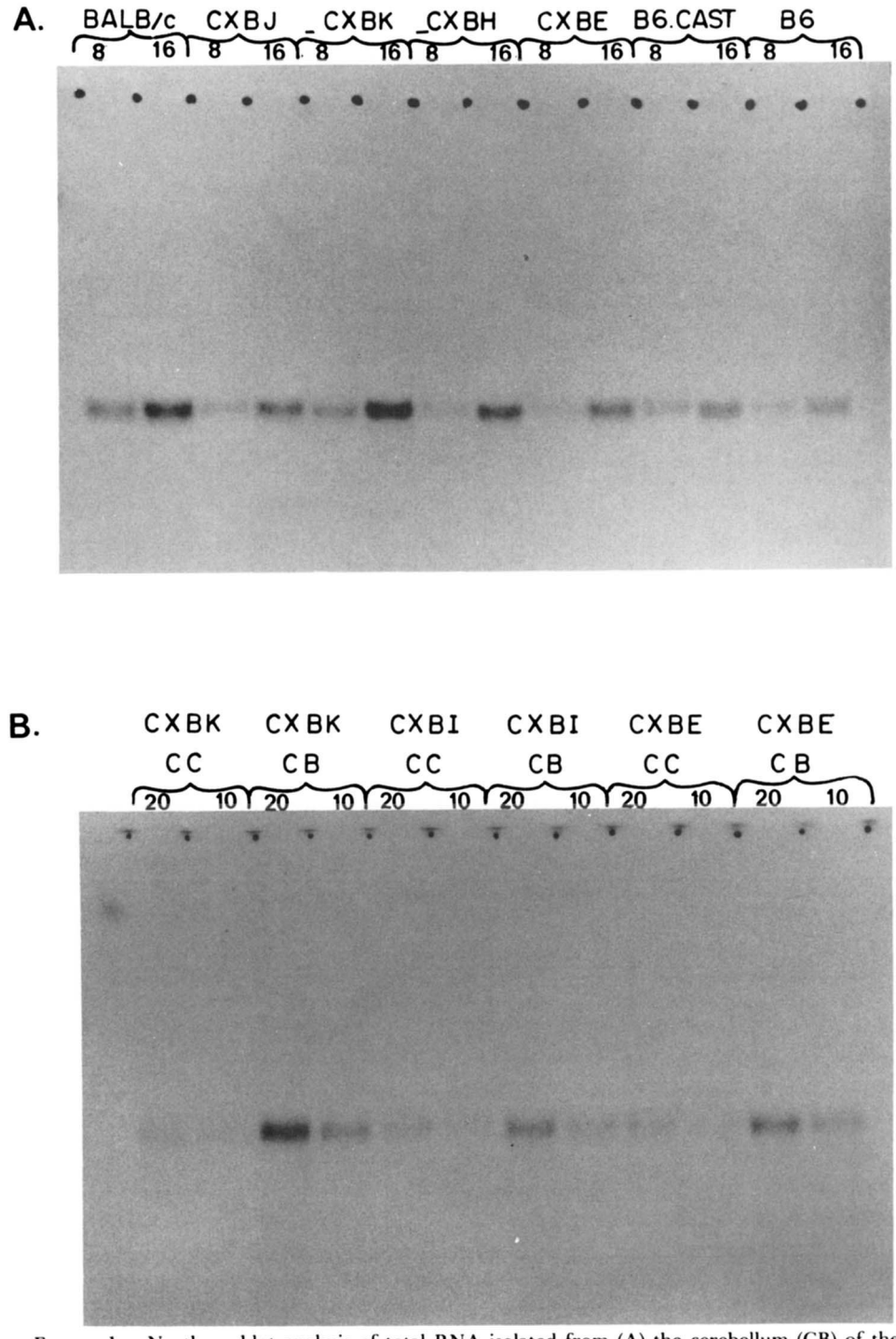

FIGURE 1.—Northern blot analysis of total RNA isolated from (A) the cerebellum (CB) of the indicated strains of mice at 18 days of age and (B) the cerebral cortex (CC) of the indicated strains of mice also at 18 days of age. Numbers at the top of each lane refer to the number of micrograms of RNA electrophoresed in the gel. Blotting and hybridization procedures are described in MATERIALS AND METHODS.

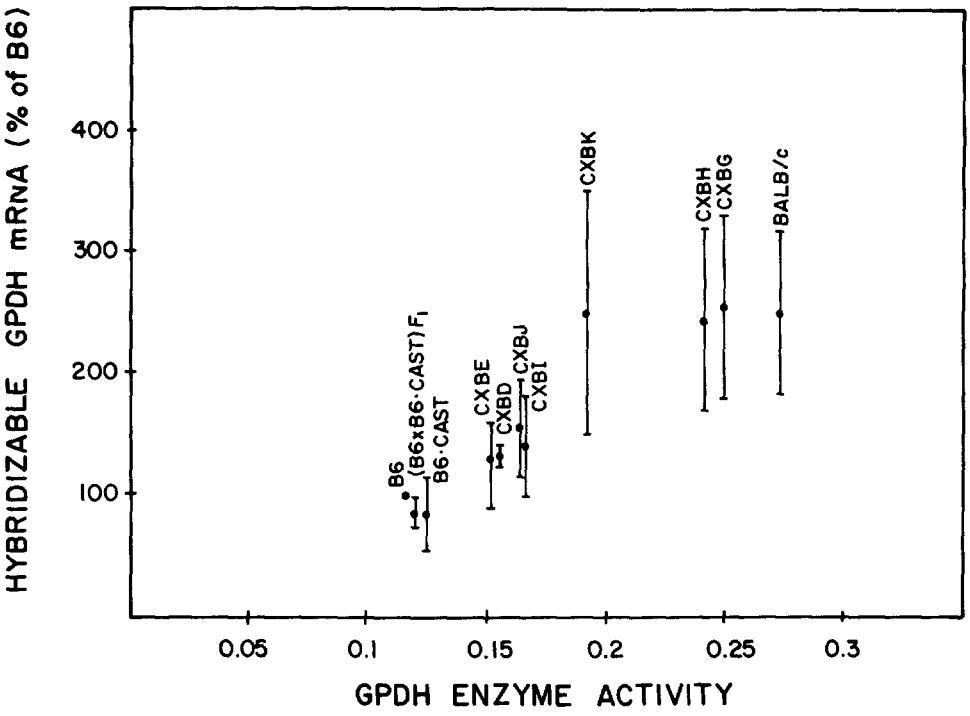

FIGURE 2.—Correlation between hybridizable GPDH mRNA and enzyme activity in the cerebellum of recombinant inbred strains of mice. The hybridizable mRNA is expressed as a percentage relative to that detected in the cerebellum of an 18-day-old B6 mouse. The 18-day-old B6 RNA is included on each blot, and its hybridization intensity is assigned a value of 100. Enzyme activity, expressed as μmoles of NADH oxidized/min/mg of protein, is taken from the data in Table 1. Error bars for the mRNA measurements represent standard deviations. The numbers of determinations for each strain are B6 (eight), B6 × B6.CAST)F_1 (four), B6.CAST (eight), CXBE (eight), CXBD (three), CXBJ (seven), CXBI (four), CXBK (eight), CXBH (seven), CXBG (three) and BALB/c (three). Each RNA preparation is made from a pool of 20 mice. Data for B6, CXBJ, CXBK, CXBE, CXBH and B6.CAST come from two separate RNa preparations; only one RNA preparation was available for the other strains. Statistical analysis of data by the Student-Newman-Keuls multiple range test indicates that CXBG, CXBH, CXBK and BALB/c are significantly different from CXBD, CXBE, B6.CAST and (B6 × B6.CAST)F_1s at $P = 0.05$, whereas CXBJ and CXBI are not significantly different from either group.

in which only those offspring with the GPDH-C allozyme had the activity level characteristic of BALB/c mice.

2. Presence of the *Gdc-1^c* allele is a necessary but not a sufficient condition for the high enzyme levels as evidence by the fact that strains within the intermediate class may have either the GPDH-C allozyme (CXBD and CXBK) or GPDH-B allozyme (CXBI, CXBJ and CXBE). This suggests a second property of the phenotype, namely, that a second locus is required with a BALB allele in order to have high levels of GPDH. These characteristics of GPDH expression in RI mice are consistent with data obtained from the earlier backcross analysis in which homozygous *Gdc-1^c* and *Gdc-1^b* mice expressed intermediate enzyme levels.

Segregation of GPDH phenotypes in crosses with the RI strains: From consider-

TABLE 3

SDPs of alleles at the Gdc-1 *locus and at a proposed regulatory locus among CXB recombinant inbred and progenitor strains of mice*

	B6.CAST	B6	D	E	G	H	I	J	K	BALB/c
Gdc-1 (chromosome *15*)[a]	D	B	C	B	C	C	B	B	C	C
Activity[b]	L	L	I	I	H	H	I	I	I	H
Regulatory[a]	B	B	B	C	C	C	C	C	B	C

[a] Letters designate the allele present in a given strain.
[b] Letters indicate the level of enzyme activity where L = low, I = intermediate and H = high.

ations of the backcross data and from the strain distribution patterns among the RI mice for the heat sensitivity phenotype and the activity level phenotypes, a two-locus model to explain cerebellar GPDH regulation has been devised to make allelic assignments for both the structural gene *Gdc-1* and the regulatory phenotype (Table 3).

The heat inactivation phenotype allows the unambiguous assignment of either a *b* or a *c* allele at the structural locus *Gdc-1*. The activity level SDP then would be a consequence of allelic distributions at two loci, the structural locus on chromosome *15* and a regulatory locus on an unknown chromosome. For those strains with a high activity level, the *c* allele at the structural locus could be accompanied by a *c* allele at the regulatory locus. Those strains with an intermediate level of activity could have either a *c* allele at the regulatory locus if a *b* allele is at the structural locus or a *b* allele at the regulatory locus if a *c* allele is at the structural locus. With this model we would expect the RI strains to vary in GPDH levels with the differences between them controlled by one locus in some cases and by two loci in other cases. Pairs of strains can be selected for comparison in which the differences are controlled by alternate alleles at only one locus, either the structural locus (CXBH *vs.* CXBJ) or the regulatory locus (CXBH *vs.* CXBD).

To construct a model for regulation of GPDH in the cerebellum the following points must be recognized:

1. The products of the *Gdc-1*^{b} and *Gdc-1*^{c} genes do not differ kinetically nor are they known to differ in any physiochemical property other than heat stability.

2. BALB/c cerebellum has an increased amount of GPDH enzyme activity, enzyme protein, translatable mRNA and hybridizable mRNA.

3. Genetic analysis with conventional backcrosses and RI strains indicates that there are genetic elements both unlinked and linked to the structural gene which result in an increase in the number of GPDH mRNA molecules. Therefore, a genetic element physically associated with *Gdc-1* on chromosome *15* modulates the production of mRNA. This could involve autoregulation by chemically different GPDH enzyme molecules, processing of nuclear RNA, differential rates of degradation of cytoplasmic RNA or differences in mRNA transcription. Thus, at this stage of development, the model includes the structural gene, *Gdc-1*, a regulatory element, *Gdcr-1*, which exhibits genetic linkage with *Gdc-1*, and an unlinked regulatory element, *Gdcr-2*.

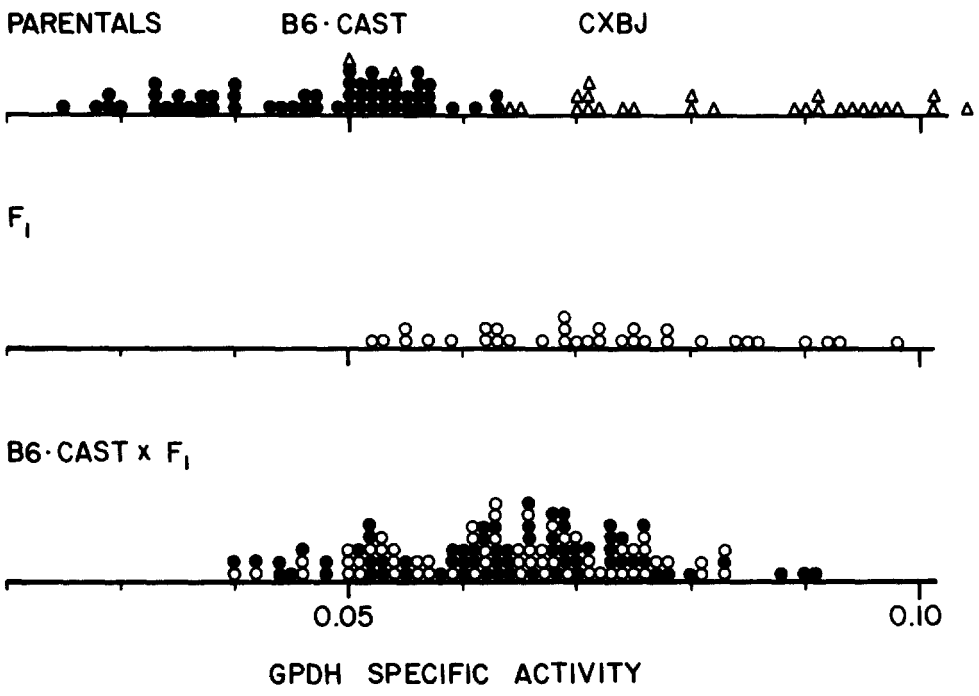

FIGURE 3.—Frequency distribution of GPDH in the cerebellum of parental strains, (B6.CAST × CXBJ)F_1 and in progeny of the backcross generation. Mice were 14 days of age. Symbols identify mice with GPDH-D (●), GPDH-B (△) and GPDH-BD (○).

With this model we can now make predictions about the association of an activity phenotype and a particular structural allele. According to the model the D, J and K strains with intermediate levels of GPDH enzyme and mRNA differ from the high strains (BALB/c, G, H) or the low strains (B6.CAST) by only one gene (Table 3). We would predict that a comparison of the segregation patterns for the structural genotypes and activity phenotypes among the backcross progeny of D (*Gdc-1*^{c}) and K (*Gdc-1*^{c}) crossed to B6.CAST (*Gdc-1*^{d}) should be indistinguishable from each other and that there should be a strong association between those animals carrying the *Gdc-1*^{c} allele and those with higher activity. In contrast, a comparable association between the *Gdc-1*^{b} allele and enzyme activity should not be observed in a cross of J (*Gdc-1*^{b}) to B6.CAST (*Gdc-1*^{d}).

The B6.CAST × (J × B6.CAST)F_1 backcross, not segregating for *Gdc-1*^{c}, can only test whether the frequency distribution will provide the two distinct modes expected for segregation at *Gdcr-2*. It is clear from the frequency distribution that the data do not support such a conclusion (Figure 3). Since the distribution of activity levels tends to lie between the two parental values, it is probable that more than one genetic factor is involved; however, as will be discussed later, the number of factors must be small. As expected, the presence of the *b* or *d* structural allele at *Gdc-1* has no influence on the level of GPDH activity in the cerebellum (Table 4).

TABLE 4

Association between structural gene phenotypes and GPDH activity phenotypes among 14-day-old offspring with alleles segregating at the Gdcr-2 *locus*

B6.CAST					(J × B6.CAST)F_1				
r-2	*r-1*	*Gdc-1*			*r-2*	*r-1*	*Gdc-1*		
b	*b*	*d*			*c*	*b*	*b*		
b	*b*	*d*			*b*	*b*	*d*		
		Gdc-1$^{d/d}$		*Gdc-1$^{b/d}$*		*Gdc-1$^{b/b}$*			*P*
		Mean	SD	Mean	SD	Mean	SD		
Parentals:									
B6.CAST, F_1, J		0.047 (58)	0.010	0.072* (33)	0.012	0.081* (26)	0.015		0.01
Backcross:									
B6.CAST × (J × B6.CAST)		0.064 (69)	0.011	0.064 (56)	0.011				0.816

The number of animals analyzed is given in parentheses.

* The numbers are not significantly different from each other, but both differ from 0.047 at $P = 0.01$.

Since the CXBD and CXBK strains both carry *Gdc-1^c* and express intermediate levels of GPDH activity, we expected that the frequency distribution of the GPDH activity phenotypes would be similar for the two strains and that the high activity phenotype would cosegregate with *Gdc-1^c*. The failure of the data to corroborate this provides major insights into the genetic mechanism controlling *Gdc-1* expression. First, the B6.CAST × (D × B6.CAST)F_1 backcross was virtually indistinguishable from the B6.CAST × (J × B6.CAST)F_1 backcross discussed above; the frequency distribution of the GPDH activity was intermediate with respect to the parental distribution (Figure 4), and there was no association between *Gdc-1^c* allele and the high activity phenotype (Table 5). This lack of correlation with the *Gdc-1^c* was contrary to the strain distribution pattern among the RI lines and the segregation patterns observed among the two locus crosses, *i.e*, B6.CAST *vs.* CXBH, CXBG and BALB/c. The data from this cross with D strain imply that the *Gdc-1^c* allele, isolated from *c* alleles at other unlinked loci, will not result in elevated levels of GPDH and that it is no different from the *Gdc-1^b* allele in its quantitative effects.

The K × (K × B6.CAST)F_1 backcross, on the other hand, resulted in a frequency distribution of the offspring which was consistent with major control by one locus for approximately equal numbers of animals are present in the frequency distribution with activity levels that span the range of K and (K × B6.CAST)F_1 parents (Figure 5). Furthermore, a strong correlation was present between the high activity phenotype and *Gdc-1^c* (Table 6). However, because a substantial number of backcross progeny are GPDH-CC but have a level of enzyme activity expected for GPDH-CD, it suggests that the association between *Gdcr-1* and *Gdc-1* is weaker than would be expected if *Gdcr-1* was either within or closely flanking the structural gene.

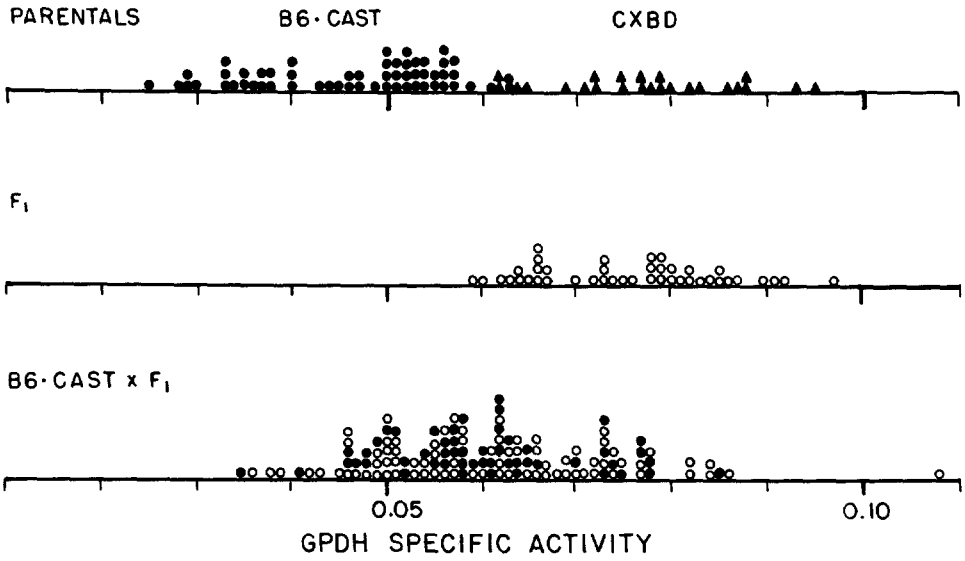

FIGURE 4.—Frequency distribution of GPDH in the cerebellum of parental strains (B6.CAST × CXBD)F_1 and in the progeny of the backcross generation. Mice were 14 days of age. Symbols identify mice with GPDH-D (●), GPDH-C (▲) and GPDH-CD (○).

TABLE 5

Association between structural gene phenotypes and GPDH activity phenotypes among 14-day-old offspring with alleles segregating at the Gdcr-2 *locus*

B6.CAST					(D × B6.CAST)				
r-2	*r-1*	*Gdc-1*			*r-2*	*r-1*	*Gdc-1*		
b	*b*	*d*			*c*	*b*	*c*		
—	—	—			—	—	—		
b	*b*	*d*			*b*	*b*	*d*		
		Gdc-1$^{d/d}$		*Gdc-1$^{c/d}$*		*Gdc-1$^{d/c}$*			*P*
		Mean	SD	Mean	SD	Mean	SD		
Parentals:									
B6.CAST, F_1, D		0.047	0.010	0.076*	0.009	0.073*	0.013		0.01
		(58)		(42)		(30)			
Backcross:									
B6.CAST ×		0.060	0.010	0.061	0.013				0.751
(D × B6.CAST)		(53)		(76)					

Numbers in parentheses are numbers of animals.

* The numbers are not significantly different from each other, but both differ from 0.047 at $P = 0.01$.

One interpretation, reconciling the results of the B6.CAST × (D × B6.CAST)F_1 backcross analysis with these results, is that the single locus controlling the activity levels between CXBK and B6.CAST is unlinked to *Gdc-1*. Moreover, the product of the *c* allele at this regulatory locus preferentially interacts with the *c* allele at the *Gdc-1* locus. Accordingly, the genotypes in the

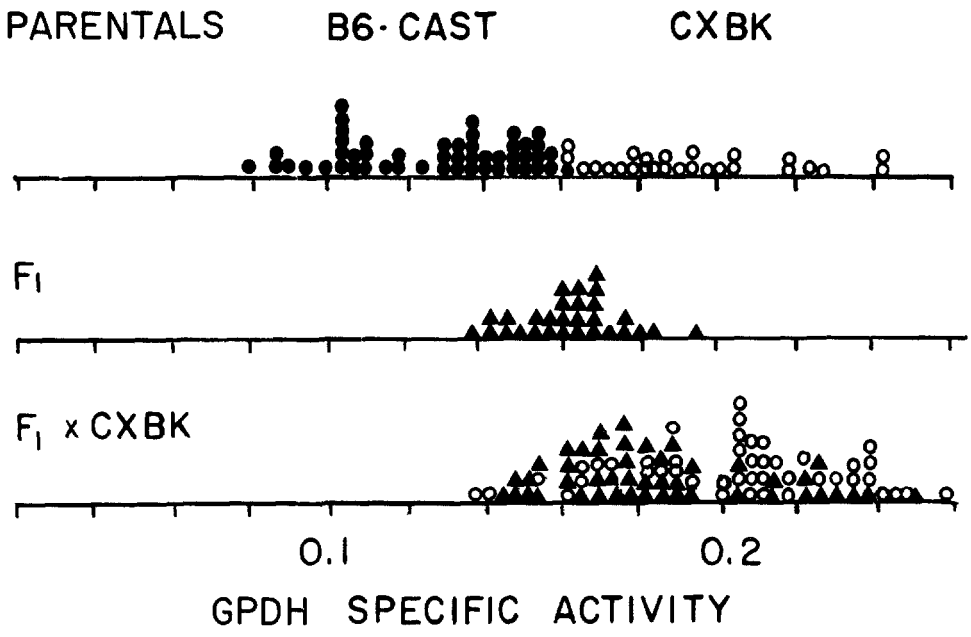

FIGURE 5.—Frequency distribution of GPDH in the cerebellum of parental strains (B6.CAST × CXBK)F_1 and in the progeny of the backcross generation. Symbols identify mice with GPDH-D (●), GPDH-C (○) and GPDH-CD (▲).

TABLE 6

Association between structural gene phenotypes and GPDH activity phenotypes among 18-day-old offspring with alleles segregating at the Gdcr-1 *locus*

	K				(K × B6.CAST)F_1				
r-2	*r-1*	*Gdc-1*			*r-2*	*r-1*	*Gdc-1*		
b	*c*	*c*			*b*	*b*	*d*		
—	—	—			—	—	—		
—	—	—			—	—	—		
b	*c*	*c*			*b*	*c*	*c*		
		$Gdc\text{-}1^{d/d}$		$Gdc\text{-}1^{c/d}$		$Gdc\text{-}1^{c/c}$			*P*
		Mean	SD	Mean	SD	Mean	SD		
Parentals:									
B6.CAST, F_1, K		0.125	0.023	0.161	0.012	0.192	0.023		0.01
		(50)		(29)		(26)			
Backcross:									
K × (K × B6.CAST)				0.182	0.025	0.200	0.026		0.001
				(58)		(50)			

The numbers of animals analyzed are given in parentheses.

K × (K × B6.CAST)F_1 backcross progeny and their postulated rank order with respect to GPDH activity are: $r\text{-}1^{c/c}Gdc\text{-}1^{c/c} > r\text{-}1^{c/c}Gdc\text{-}1^{c/d} = r\text{-}1^{b/c}Gdc\text{-}1^{c/c} > r\text{-}1^{b/c}Gdc\text{-}1^{c/d}$. All mice would have the $r\text{-}2^{b/b}$ genotype in this cross.

Crosses similar to those carried out for the D, J and K RI strains were also carried out using BALB/c and H and G RI strains. The frequency distributions

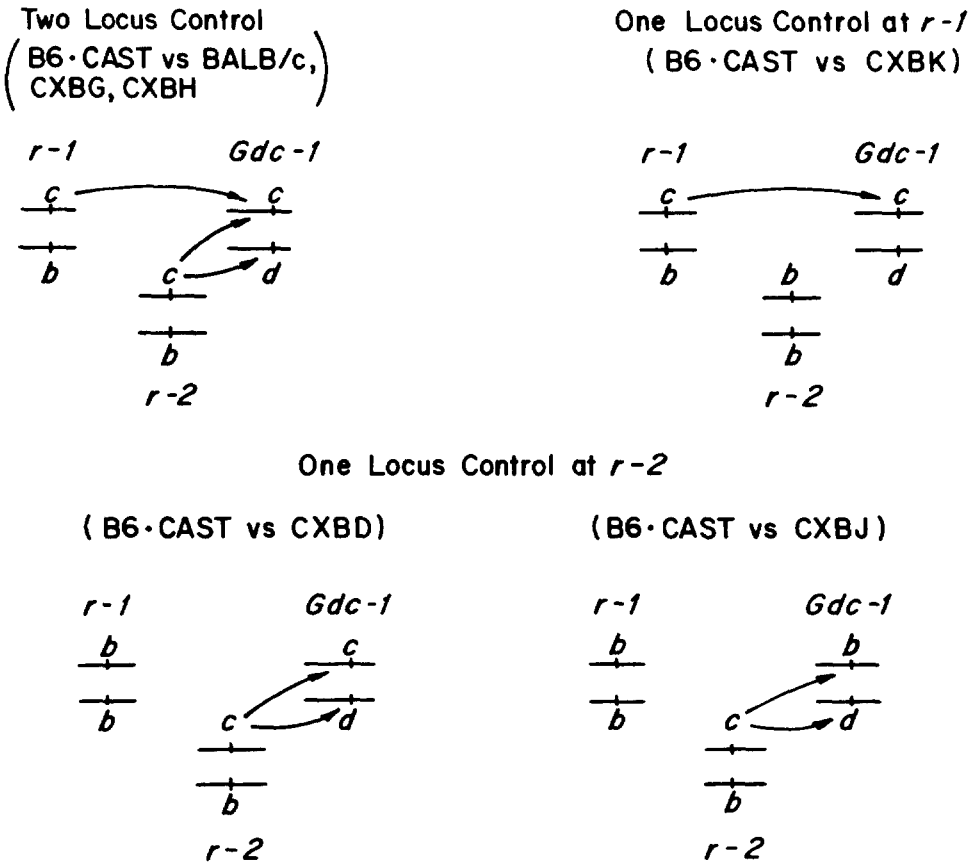

FIGURE 6.—Model for the genetic control of *Gdc-1* in the cerebellum. *r-1* = *Gdcr-1*, *r-2* = *Gdcr-2*.

of phenotypes in these segregating generations were similar to the distributions observed earlier (KOZAK 1972), namely, that two loci control GPDH levels and there is a strong involvement of the *Gdc-1*^{c} allele. Since no additional information is available from these crosses that is not already present in Table 1, the data are not presented.

Structural alleles expressed in heterozygous mice: The genetic analysis with the RI mice, in particular from the crosses with the D and K RI mice, has contributed further to the development of the model for gene control which is illustrated in Figure 6. Whereas we originally thought that *Gdcr-1* was linked to *Gdc-1*, the additional backcross data, presented above, now suggest that the two regulatory loci controlling *Gdc-1* expression in the cerebellum are in fact unlinked genetically. One locus, *Gdcr-1*, is characterized by enhanced preferential production of GPDH mRNA from *Gdc-1*^{c} by the *Gdcr-1*^{c}, whereas the other locus(i), *Gdcr-2*, acts in *trans*. If this model is correct, then one can predict the proportions of allozymes that will be present in the (B6 × B6.CAST)F$_1$-*Gdc-1*$^{b/d}$, (CXBD × B6.CAST)F$_1$-*Gdc-1*$^{c/d}$, (CXBJ × B6.CAST)F$_1$-

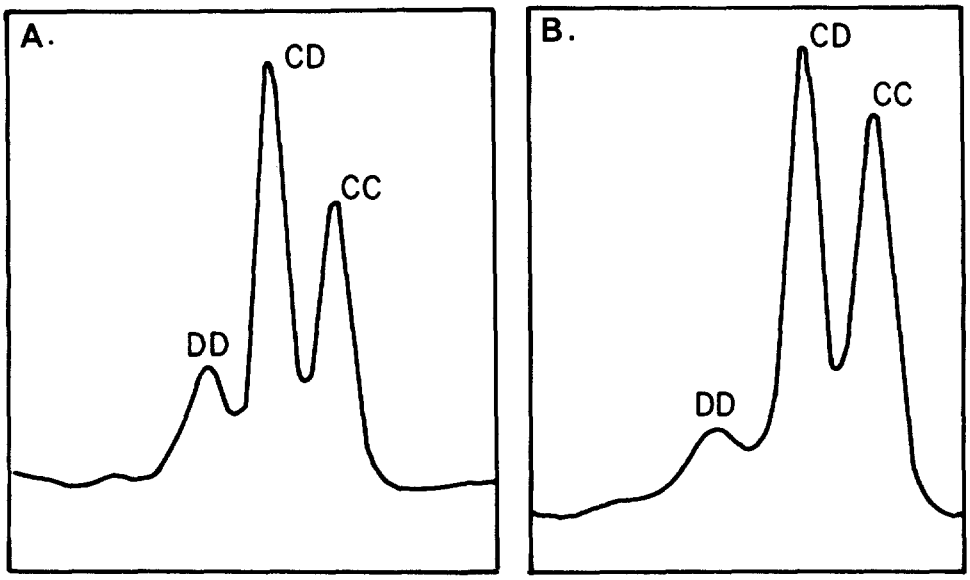

FIGURE 7.—Cellulose acetate electrophoresis of GPDH allozymes in the cerebellar postmitochondrial supernatant fraction of 15-day-old (CXBD × B6.CAST)F_1 (A) and (CXBH × B6.CAST)F_1 (B) mice. Profiles of stained regions on the electrophoresis plate were generated by scanning plates with the Helena Quick Scan.

Gdc-1$^{b/d}$, (CXBH × B6.CAST)F_1-*Gdc-1*$^{c/d}$ and (CXBK × B6.CAST)F_1-*Gdc-1*$^{c/d}$ progeny.

To interpret the results of this experiment, it is important to realize that (B6 × B6.CAST)F_1-*Gdc-1*$^{b/d}$ mice preferentially express the *b* allele in a cerebellar-specific, developmentally dependent manner even though these F_1 mice and the *Gdc-1*$^{b/b}$ and *Gdc-1*$^{d/d}$ mice do not differ in the levels of GPDH protein or mRNA. The basis for this effect is not understood, however, because B6, B6.CAST and F_1 have indistinguishable levels of hybridizable mRNA, it is likely that the mechanism is acting at the translational level. Consequently, in order to assess the affects of a *c* allele at *Gdcr-1* and *Gdcr-2* a comparison of the relative production of allozymes must be made with reference to the (B6 × B6.CAST)F_1 since these mice have a developmentally dependent alteration in the manner of GPDH-B and GPDH-D subunits they express.

The relative proportions of GPDH-C to GPDH-D subunits present in the cerebellum of *Gdc-1*$^{c/d}$$F_1$ mice at 15 days of age are determined from allozyme patterns illustrated in Figure 7. Since GPDH-B and GPDH-C are not distinguishable electrophoretically, similar patterns are obtained for *Gdc-1*$^{b/d}$$F_1$ mice. Four types of F_1 animals were analyzed in this manner: those in which no difference in enzymes or mRNA are present in the parentals (B6 and the B6.CAST), those with a difference in both enzyme and mRNA levels which is controlled by one locus (CXBD and CXBJ *vs.* B6.CAST) and those with a difference in enzyme and mRNA levels which is controlled by two loci (CXBH *vs.* B6.CAST). The data in Figure 8 plus additional data, tabulated in Table 7, demonstrate that F_1 hybrids between B6.CAST and CXBD, CXBJ and B6 are indistinguishable in the production of enzyme subunits and that hybrids

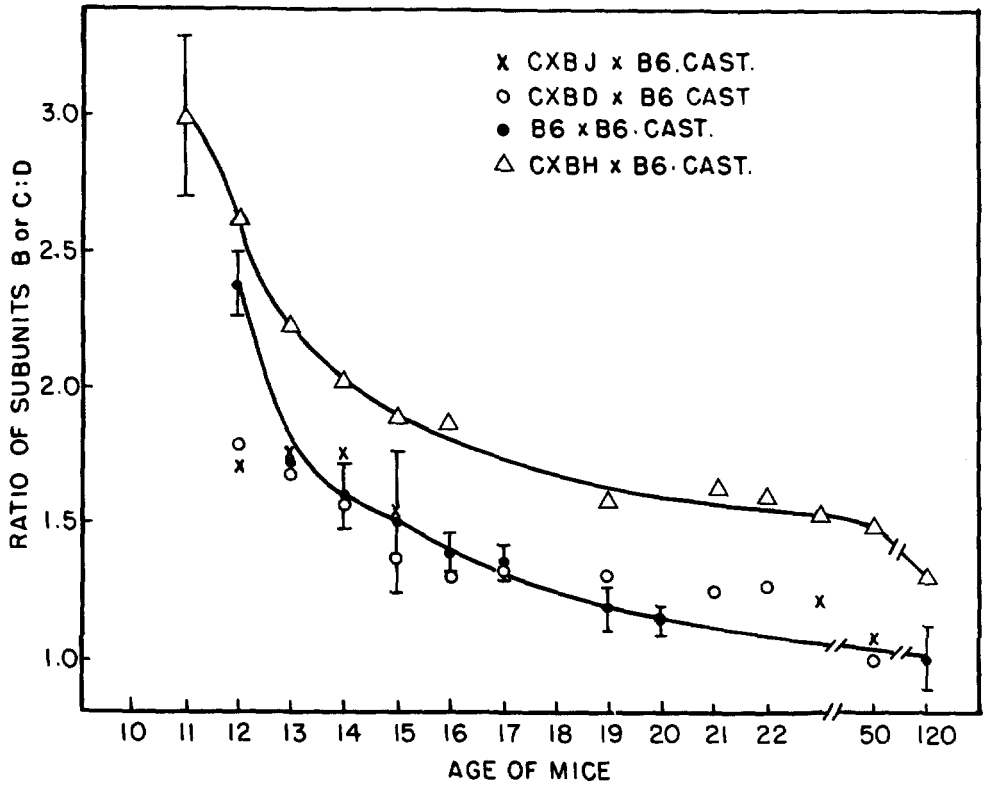

FIGURE 8.—Developmental profile illustrating the variation in the proportion of GPDH subunits derived from alternative structural alleles in a series of hybrid animals. B6.CAST chromosomes carry Gdc-1^d, CXBJ and B6 chromosomes carry *Gdc-1^b* and CXBD and CXBH chromosomes carry *Gdc-1^c*.

TABLE 7

Relative production of GPDH subunits in 14-day-old mice heterozygous at the Gdc-1 *locus*

F_1 cross	Subunit composition	Ratio of subunits[a] B or C / D
B6 × B6.CAST	BD	1.60 ± 0.12 (21)
CXBD × B6.CAST	CD	1.59 ± 0.34 (14)
CXBJ × B6.CAST	BD	1.74 ± 0.25 (11)
CXBK × B6.CAST	CD	2.34 ± 0.13 (12)
CXBH × B6.CAST	CD	2.28 ± 0.12 (12)

[a] Ratio is given as the mean ± the standard deviation. The numbers of animals analyzed are given in parentheses.

with CXBH and CXBK have greater numbers of GPDH-C subunits produced than do the other strains. If these data are considered together with the data on mRNA levels (Figures 1 and 2), it is probable that increased synthesis of the GPDH-C protein subunits observed in the (CXBH × B6.CAST)F_1 and

(CXBK × B6.CAST)F_1 mice is determined by enhanced production of mRNA from the *Gdc-1*^{c} gene. Although enhanced production of mRNA is also observed with CXBD and CXBJ mice as compared to B6.CAST, it appears that, since the relative production of allozymic subunits in the F_1s between CXBD, CXBJ and B6 and B6.CAST are all similar, the alleles that are increasing the amount of mRNA in the CXBD and CXBJ mice must be acting on both structural alleles in an F_1 hybrid.

An additional aspect of this expression of the *Gdc-1* locus is that the genes affecting quantitative variations are being expressed only in the cerebellum, even though the same structural locus is expressed in most other tissues of the mouse. Evidence of this tissue specificity was presented in Figure 1 where differences exist in the amount of hybridizable GPDH mRNA among the RI lines in the cerebellum but not in the cerebral cortex. Consistent with this data is the evidence that at 11 days of age the subunit ratio in the cerebellum of (CXBH × B6.CAST)$F_1^{c/d}$ mice is 2.93 ± 0.34 (n = 8), whereas in the cerebral cortex this ratio is 1.19 ± 0.12 (n = 8). Clearly, the enhanced expression of the *Gdc-1*^{c} allele is restricted to the cerebellum.

DISCUSSION

Our initial analysis of the inheritance of GPDH activity levels in the cerebellum indicated that the 2.5- to 3-fold difference observed between inbred strains of mice was controlled by the interaction of two or more major genes. Although a difference of this magnitude is not large, particularly for determining Mendelian modes of inheritance, the fact that the trait was largely restricted to GPDH expression during its most active phase of cerebellar development indicated that possibly one could identify tissue-specific regulators that interacted with the structural gene to control expression during development. Other characteristics of the GPDH system in mice also encouraged us to attempt to unravel the mode of inheritance in the cerebellum.

1. Expression of the *Gdc-1* locus in the cerebellum, observed predominantly in a single cell type, the Bergmann glial cell, depends on interactions between Bergmann glial cells and Purkinje cell neurons (KOZAK and FISHER 1984; FISHER 1984). Thus, this *Gdc-1* gene has provided an important model system to investigate the mechanisms by which neuronal glial interactions modulate gene expression. If genes, in addition to the structural gene, are involved in the regulation of GPDH expression, the possibility exists that the products of these genes are acting at the level of neuronal-glial cell interactions or that the activation of their expression is mediated by these interactions.

2. The *Gdc-1* gene whose genetic regulation is being analyzed in the cerebellum is one of the two known structural genes for GPDH; the other gene, *Gdc-2*, located on chromosome *9*, is distinguished from *Gdc-1* by the fact that enzyme products of each gene differ structurally and by the fact that *Gdc-1* is generally expressed in adult differentiated tissues, whereas *Gdc-2* is expressed in embryonic and fetal tissues (KOZAK and JENSEN 1974; KOZAK, BURKART and HJORTH 1982). Accordingly, these genes, with their variable expression during development and their dependence on neuronal-glial interactions, provide a

model system for the analysis of fundamental processes in neurobiological development.

The particular aspect of GPDH expression that this investigation addresses is the identification of cerebellar specific regulatory genes by use of Mendelian genetic analysis. The genes controlling GDPH expression in the developing cerebellum are found in three genetically separable loci. One locus, the structural locus *Gdc-1*, is located on chromosome *15*; the other two loci, genetically separable but with unknown locations, have regulatory functions. The characteristics of these regulatory loci, called *Gdcr-1* (*r-1*) and *Gdcr-2* (*r-2*), are discussed below.

The central finding emerging from the genetic analyses is that two regulatory elements interacting with the structural gene, *Gdc-1*, are necessary for the control of GPDH mRNA levels. This conclusion was first suggested by the B6.CAST × (B6.CAST × CXBD)F_1 backcross in which animals were identified with a GPDH-CD allozymic composition and an activity level indistinguishable from the B6.CAST parent; the net result is that the presence of the *Gdc-1^c* allele was not a sufficient determinant in the quantitative variation in GPDH enzyme levels. Additionally, in the CXBK × (CXBK × B6.CAST)F_1 backcross, the frequency distribution of enzyme activity levels and allozymic phenotypes suggested that the genes controlling these phenotypes were unlinked.

If the *Gdc-1^c* allele does not, by itself, determine differences in the amount of GPDH protein, then we are faced with explaining how it is that in mouse strains like CXBK, CXBH, CXBG and BALB/c the presence of a *Gdc-1^c* allele had a strong influence in determining GPDH levels. To explain this phenomenon, we hypothesized that the regulatory locus *r-1*, which is unlinked to *Gdc-1*, has a *c* allele in the above strains that interacts preferentially with the *c* allele at the *Gdc-1* locus to enhance production of GPDH mRNA. The evidence for this hypothesis is principally derived from a comparison of the backcross with B6.CAST and CXBD to the backcross with B6.CAST and CXBK. Both the CXBD and CXBK have intermediate levels of enzyme activity and both carry the *Gdc-1^c* allele; but although the backcross with CXBD showed no association of the activity phenotype with the *Gdc-1^c* allele, the cross with the CXBK showed a very strong correlation between the activity phenotype and the *Gdc-1^c* allele. It follows from this logic that, if CXBK mice carry a *r-1^c* allele which preferentially enhances expression of the *Gdc-1^c*, then, the CXBD mice must also carry a BALB/c-derived allele that increases or enhances the level of GPDH mRNA but does so by increasing expression at both the *c* and *b* or *d* alleles. This interpretation is strengthened by the results of the cross with CXBJ. This RI line has intermediate levels of enzyme similar to CXBK and CXBD; however, it carries the *Gdc-1^b* allele, with a segregation pattern of a particular structural allele and activity phenotype that is indistinguishable from that observed with the CXBD, *i.e.*, random segregation of the structural gene and activity phenotypes. Accordingly, we think that CXBD and CXBJ each carry a *c* allele at a second regulatory region *r-2* and that this locus acts *trans*. These interpretations are corroborated by the experiment with allozymic patterns (Figure 8). Each cross in which cosegregation occurs be-

tween the *Gdc-1^c* structural allele and the high activity phenotype has a greater proportion of GPDH-C subunits compared to the (B6 × B6.CAST)F_1 control, whereas those crosses showing random segregation do not exhibit this enhanced GPDH-C/D or GPDH-B/D subunit ratio. The model for this system of genetic control is illustrated in Figure 6.

Although the *Gdc-1^c* allele cannot by itself determine the level of GPDH production, it must still possess properties that enable it to respond to a distal *r-1^c* regulatory allele in a manner quantitatively distinct from the *Gdc-1^d* allele. Presumably, this property is determined by DNA sequence differences between the *Gdc-1^d* and *Gdc-1^c* genes. At this time the molecular basis for the interaction between *Gdc-1* and *r-1* is completely unknown. This type of preferential interaction between specific alleles at two unlinked loci has been reported previously. An unlinked locus preferentially acting on a specific allele at the structural locus for erythrocyte antigens controlled the timing of their appearance in the red blood cells of mice during early development (BOUBELIK *et al.* 1975). The interpretation of this genetic variability in erythrocyte antigens has subsequently been questioned by the finding that the appearance of these antigens as detected by immunological methods was a reflection of the difference in the availability of the antigens to interaction with the test antibodies rather than differences in the amount of membrane-bound antigen (BOUBELIK and LENGEROVA 1977). Based on evidence that more than one locus was controlling the levels of β-galactosidase, BERGER, BREEN and PAIGEN (1979) also postulated that interactions between loci proximal and distal to the β-galactosidase structural gene controlled developmental variation in the liver. Other loci unlinked to the structural locus which modulate the production of enzyme have generally been observed to act *trans* (ABRAHAM and DOANE 1978; MARONI and LAURIE-AHLBERG 1983), whereas those that are linked to the structural gene act *cis* (LUSIS *et al.* 1980; PETERSON and WONG 1978). An exception to these generalities is the developmental variation in liver β-glucuronidase expression in mice which has both *cis*- and *trans*-acting components (LUSIS *et al.* 1983).

How strong is the evidence for the existence of the two loci *r-1* and *r-2*? The evidence that *r-1* is a single locus is reasonably strong. First, the segregation pattern in the backcross between CXBK and (CXBK × B6.CAST)F_1 was that expected for a single codominant locus; in addition, the effects of *r-1* are not found in the CXBD strain, which would be an improbable occurrence if the phenotype of *r-1* was determined by multiple genetic factors. The effects of the *r-1 c* allele are also observed in CXBG and CXBH strains; however, we cannot evaluate the genotypes in CXBE, CXBJ or CXBI because these strains carry a *b* allele at *Gdc-1*. The argument that *r-2* is also a single locus is not strong since the segregating patterns in the backcrosses derived from the CXBD, CXBJ and B6.CAST parents do not support such a conclusion. On the other hand, since the *r-2^c* phenotype does not appear to be present in the CXBK strain, it is likely that it is controlled by very few genes, possibly one major gene.

Based on the above caveats, one can construct a revised SDP for the three

TABLE 8

SDPs of alleles at Gdc-1, Gdcr-1 *and* Gdcr-2 *loci among CXB recombinant inbred and progenitor strains of mice*

	Strain								
Gene	B6	D	E	G	H	I	J	K	BALB/c
Gdc-1	B	C	B	C	C	B	B	C	C
Gdcr-1	B	B	(B,C)	C	C	(B,C)	(B,C)	C	C
Gdcr-2	B	C	(C,B)	C	C	(C,B)	(C,B)	B	C

loci in Table 8. Specific assignments of a *b* or *c* allele at the *r-1* and *r-2* loci in CXBE, CXBI and CXBJ cannot be made because, as indicated above, these strains carry the *Gdc-1*^{b} allele. The value of these SDPs is, however, that *r-1* has patterns that are concordant with, and, therefore, possibly linked to, prealbumin-1 (*Pre-1*), two polypeptide polymorphisms in liver, *Lth-1* and *Ltw-2*, detected by two-dimensional gel electrophoresis, and three minor histocompatibility genes, *H-17*, *H-34* and *H-38* (TAYLOR 1981). Since the latter three loci exist as BALB/ alleles congenic on C57BL/6J backgrounds, it is possible that a congenic strain exists for the *r-1* locus. Similarly, *r-2* has SDPs that suggest possible linkage to esterase-1 (*Es-1*), β-galactosidase (*Bgl*) and the minor histocompatibility genes *H-18* and *H-35* (TAYLOR 1981).

The mechanism by which regulatory genes modulate structural gene expression in a tissue-specific and developmentally dependent manner is poorly understood. Recently, attention has been directed to the possibility that short stretches of DNA sequence called enhancers, located in and around the structural gene, may be involved in tissue-specific expression of a gene (BANERJI, OLSON and SCHAFFNER 1983; GILLIES *et al.* 1983; WALKER *et al.* 1983). Since there is also evidence that diffusible regulatory proteins are involved in the action of enhancer sequences (EMERSON and FELSENFELD 1984), one can easily construct a model for GPDH expression in the cerebellum that is dependent on a mutable enhancer-like system. Combined structural and functional studies of the *Gdc-1* region together with genetic analysis of the *r-1* regulatory region can lead to additional insights into the control of development in the cerebellum of the mouse.

I would like to acknowledge the excellent technical assistance of DEBRA BOISSONNEAULT, DONNA BURKART, ANN PLAUD and ULRIKE STADLER, also the invaluable discussions with DONALD BAILEY, MICHAEL KOTARSKI, EDWARD BIRKENMEIER and WILLIAM DOVE and the assistance from ROBERT COLLINS for the statistical treatment of the data. This research was supported by National Institutes of Health grant HD06712.

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Communicating editor: R. E. GANSCHOW