Regulatory and Structural Genes for Lysozymes of Mice

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ABSTRACT

The molecular and genetic basis of large differences in the concentration of **P** lysozyme in the small intestine has been investigated by crossing inbred strains of two species of house mouse (genus *Mus*). The concentration of **P** in *domesticus* is about 130-fold higher than in *castaneus*. An autosomal genetic element determining the concentration of **P** has been identified and named the **P** lysozyme regulator, *Lzp-r*. The level of **P** in interspecific hybrids (*domesticus* × *castaneus*) as well as in certain classes of backcross progeny is intermediate relative to parental levels, which shows that the two alleles of *Lzp-r* are inherited additively. There are two forms of **P** lysozyme in the intestine of the interspecific hybrid—one having the heat stability of *domesticus* **P**, the other being more stable and presumably the product of the *castaneus P* locus. These two forms occur in equal amounts, and it appears that *Lzp-r* acts in *trans*. The linkage of *Lzp-r* to three structural genes (*Lzp-s*, *Lzm-s1*, and *Lzm-s2*), one specifying **P** lysozyme and two specifying **M** lysozymes, was shown by electrophoretic analysis of backcrosses involving *domesticus* and *castaneus* and also *domesticus* and *spretus*. The role of regulatory mutations in evolution is discussed in light of these results.

SINCE regulatory and structural mutations were first distinguished in the early 1960s, there has been discussion about the relative importance of these two kinds of mutations in evolution (WALLACE 1963; WILSON 1975; WILSON, CARLSON and WHITE 1977; MACINTYRE 1982; PAIGEN 1986; DICKINSON 1987). Direct evidence for the role of regulatory mutations comes from experiments with bacterial populations (WILSON, CARLSON and WHITE 1977; HALL 1982). Similarly, adaptation in cultured mammalian cells resulted in higher concentrations of specific proteins, but the mechanism was often gene amplification (SCHIMKE 1982). These lines of evidence lead one to ask whether mutations that change the concentration (as opposed to the structure) of specific proteins have played a major role in the adaptive evolution of multicellular organisms.

WILSON, CARLSON and WHITE (1977) cite 18 vertebrate examples in which the concentrations of proteins with known functions vary by at least an order of magnitude from one species to another. Some of these major differences in concentrations of enzymes have accompanied and apparently underlain major shifts in metabolism in the evolution of vertebrate groups. Recent evidence of instances where enzyme levels have risen at least 10-fold comes from studies on human intestinal lactase (POTTER *et al.* 1985), hominoid milk lipases (BLÄCKBERG *et al.* 1980), and ruminant stomach lysozymes (DOBSON, PRAGER and WILSON 1984). However, the genetic mechanisms by which these changes in enzyme concentration have come about are uncertain.

To examine the evolutionary role of regulatory mutations and gene amplifications, one must conduct comparative studies of protein concentration differences that are amenable to genetic and phylogenetic analysis. A recent approach has been to study gut epithelial enzymes whose levels differ among closely related species (DICKINSON 1980; DOBSON, PRAGER and WILSON 1984; WILSON 1985). In cases where the genetic mechanisms have been studied, little is known about the adaptive significance (DICKINSON 1987). Alternatively, DOBSON, PRAGER and WILSON (1984) have related the elevation of stomach lysozyme levels in ruminants to the evolution of the ruminant mode of digestion, but the mechanism has remained obscure. In this case, the evolutionary adaptation took place so long ago that the mutation responsible may have been obscured by later mutations. Contributing further to the difficulty of analyzing this case genetically is the fact that ruminants and nonruminants cannot be crossed.

We now draw attention to the value of analyzing regulatory evolution in house mice, which are the most genetically tractable group of vertebrate species. Moreover, there is no other group of multicellular species whose physiology is so well known. Furthermore, this group of species is especially suitable for evolutionary analysis because their phylogenetic relationships (FERRIS *et al.* 1983; BONHOMME *et al.* 1984) are also becoming well defined and their times of divergence are so recent.

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We have discovered enormous variation in lysozyme levels within the house mouse group of species (HAMMER *et al.* 1987). The genetic basis for such variation can thus be investigated. Two major types of lysozyme *c*, called **M** and **P**, occur in the mouse genus *Mus* (HAMMER *et al.* 1987). **M** and **P** are related by a gene duplication that took place around 50 million years ago. In that time they have diverged in structure and pattern of expression. **P** lysozyme has been detected chiefly in the small intestine, where it is probably produced by Paneth cells. In some mice, the intestinal concentration of **P** lysozyme is about 100 times higher than in other mice. Phylogenetic analysis suggests that the **P** level rose about 4 million years ago in the ancestor of the house mouse group of species. This large increase in concentration could be due to additive effects of mutations at numerous loci or to mutation at one locus. This paper gives evidence that the variation can be explained by mutation at a single locus having a *trans* regulatory function. Such studies of the genetic basis of differences in protein levels can contribute to the development of realistic models of the evolutionary process.

MATERIALS AND METHODS

Mice: The 37 strains examined represent three species of house mice. For each species, we list the source and strains. *Mus domesticus*: Jackson Laboratory (Bar Harbor, Maine)—A/HeJ, A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL6/J, C58/J, DBA/2J, IS/Cam/J, P/J, Peru/A, RIIIS/J, SF/Cam/J, SJL/J, SWR/J; Cancer Research, University of California, Berkeley—I; V. M. CHAPMAN (Roswell Park Memorial Institute, Buffalo, New York)—AT9, AT10 (both counted as C3H—see below); J. HILGERS (Netherlands Cancer Institute, Amsterdam)—ACR/A, BIM/A, DD/HeAf, FVB/NA, GRS/A, LTS/A, LIS/A, MAS/A, STS/A, TSI/A, 020/A; K. FISCHER LINDAHL (Basel Institute of Immunology, Basel, Switzerland)—NMRI/Navy, NZB/Bom, NZB/Ibm. *Mus castaneus*: V. M. CHAPMAN—casA; T. RODERICK (Jackson Laboratory)—CASA/RK. *Mus molossinus*: T. RODERICK—MOLC/RK, MOLD/RK, MOLE/RK; J. HILGERS—MOL3/JA; L. SKOW (National Institute of Environmental Health and Science, Triangle Park, North Carolina)—MOL/SK.

AT9 is a congenic line containing a *M. molossinus* allele of β-glucuronidase on a C3H background and AT10 is a congenic line containing a *M. musculus* allele of β-glucuronidase on a C3H background. For the purposes of this paper, both will be referred to as C3H.

The 60 wild *domesticus* mice studied here are a subset of those examined by HAMMER *et al.* (1987) and HAMMER (1984). Four of these mice were trapped in the wild and the remainder were laboratory descendants of wild-caught animals. Similarly, the laboratory descendants of wild-caught *castaneus* mice used in the present work are a subset of those reported on by HAMMER *et al.* (1987).

Preparation of tissue extracts: Animals were sacrificed by asphyxiation with carbon dioxide. Organs were removed and rinsed in ice cold 0.9% NaCl. The small intestine, consisting of the entire region between the pyloric valve of the stomach to the beginning of the cecum, was flushed with saline solution and slit open lengthwise. The contents of the intestine were washed away with cold saline. All tissues were then frozen at −20° for at least 24 hr before further processing. Tissues were homogenized, generally in a Dounce homogenizer, with two volumes (w/v) of 2% acetic acid or soaked overnight at 4° in two volumes (w/v) of 2% acetic acid plus 2% 2-phenoxyethanol. For extracts involving more than 5 g of tissue, an Omnimixer was used for the homogenization. Supernates, obtained after centrifugation at 13,000 × *g* for 15 min, were frozen, thawed, and recentrifuged. The final supernates were stored frozen at −20°.[2] Tissues from mice which had been stored frozen were treated the same way except that intestines were not opened or flushed of their contents.

Biochemical techniques: Previously described techniques (DOBSON, PRAGER and WILSON 1984; HAMMER 1984; HAMMER *et al.* 1987) were used for lysozyme purification, measurement of lysozyme activity, and nondenaturing electrophoresis; brief summaries of the methods are given here.

Lysozyme **P** was purified 136-fold from the small intestine of 24 C58/J mice (HAMMER 1984; HAMMER *et al.* 1987) as follows: Tissue extracts were dialyzed against 10 mM ammonium acetate, pH 4.5, and applied to a 40-ml carboxymethyl cellulose column (CM-52, Whatman) preequilibrated with the same buffer. After washing with this buffer, the column was eluted with a linear gradient of 200 mM ammonium acetate, pH 4.5, to 600 mM ammonium acetate, pH 5.0, followed by elution with additional 600 mM ammonium acetate at pH 5.0. Lysozyme peaks were dialyzed against 10 mM ammonium acetate at pH 3.5, lyophilized, resuspended in 10 ml of 10 mM ammonium acetate at pH 4.5, and subjected to gel filtration on a 40-ml Bio-Gel A-0.5m (Bio-Rad) column preequilibrated with the same buffer and developed with 200 mM ammonium acetate, pH 5.0. Fractions containing lysozyme were pooled, lyophilized, and stored frozen at −20°. In the present work **P** lysozyme was partially purified (40-fold) by employing only the carboxymethyl cellulose chromatography following dialysis of tissue extracts.

Spectrophotometric assays of lysozyme activity were done at pH 7.0, ionic strength 0.066, with *Micrococcus luteus* as the substrate at a concentration of 0.25 mg/ml; from the specific activities determined for highly purified **M** and **P** lysozymes from *domesticus* (HAMMER *et al.* 1987), we calculate that 1 unit of activity in this assay is produced by about 0.17 μg of these lysozymes. Native gel electrophoresis was carried out at pH 4.3 in 10% polyacrylamide; lysozyme activity in gels was determined using *M. luteus* incorporated at a concentration of 0.5 mg/ml into a 7% polyacrylamide overlay gel at pH 7.0. Electrophoretic mobilities were calculated relative to poly-L-lysine taken as 100. On occasion (see RESULTS, *Biochemical basis of level differences*) native gel electrophoresis was done also at pH 8.9; where no pH is explicitly stated, the pH 4.3 system is being referred to in this report.

Intestinal lysozyme levels, on a per gram basis, from mice of several strains that were frozen for up to 2 years contained approximately 63% less activity than the freshly prepared intestines from the same strains (n = 7; SD = 16%). The large error is mainly due to different amounts of food remaining in the digestive tract. To adjust for the difference in weight of the frozen intestines, lysozyme levels were multiplied by a factor of 2.7.

To assess lysozyme thermostability, microcentrifuge tubes

[2] Protease inhibitors were not included during the preparation for the following reasons. Native lysozyme *c* with its four disulfide bridges is inherently rather resistant to proteolytic digestion; at the extraction pH of about 3, few proteolytic enzymes which are present in the tissues examined are active; and pepsin, which does exhibit optimal activity at low pH, is present at high levels only in the stomach, which was not considered in this work.

containing 1 unit of lysozyme activity in 20 μl of 0.05 M sodium phosphate buffer (pH 7.5) with bovine serum albumin (1 mg/ml) were placed in a preheated water bath. For purified lysozymes the temperature used was 63–64°, while that for tissue extracts was 66–67°.[3] Tubes were removed at time points for 60 min and immediately placed into an ice bath for 1 hr. After centrifugation at 13,000 × *g* for 15 min, lysozyme activity was measured in the spectrophotometric assay at room temperature.

Genetic crosses: Two kinds of interspecific hybrids involving *castaneus* (AT9 × casA and casA × AT10) were kindly supplied by V. M. CHAPMAN. The BALB/c × *spretus* F_1 hybrid and backcross progeny derived from the female F_1 crossed to BALB/c were provided by C. DAY and P. JONES (Stanford University, Stanford, California). The remaining crosses were carried out in our animal facility and all progeny were analyzed at 10 weeks of age. In the course of these studies, casA stopped breeding, which caused a shortage of males. Outbred *castaneus* males were therefore used instead for some crosses.

RESULTS

Intestinal lysozyme levels

Levels in laboratory strains: Figure 1 shows the wide range of lysozyme concentrations found by spectrophotometric assay in the small intestines of 30 strains of inbred *domesticus*, five strains of inbred *molossinus*, and two inbred strains of *castaneus*. Three distinct classes are apparent: 27 *domesticus* strains with high lysozyme levels (above 200 units per gram of tissue), five *molossinus* strains with moderate levels (from 114 to 144 units/g), and five *domesticus* and *castaneus* strains with low levels (between 6 and 14 units/g). It is not known whether the range of activity levels within the high class is due solely to genetic factors.

Figure 2 shows the separation of the **M** and **P** lysozymes from the small intestine of C58/J mice by ion-exchange chromatography on carboxymethyl cellulose. The **P** type accounts for about 97% and the **M** type for about 3% of the lysozyme. Electrophoretic comparisons of intestinal extracts of C58/J and C3H revealed the same proportions of **P** and **M** in the two strains. (The purification of C58/J lysozymes from the small intestine was done before selection of the strains for genetic analysis—see next paragraph.)

The *domesticus* C3H strain and the *castaneus* casA strain were selected as representatives of the high and low activity groups for further analysis. These strains were chosen because F_1 hybrids between them were already available from V. M. CHAPMAN (see MATERIALS AND METHODS, *Genetic crosses*). Furthermore, casA was the only low strain known when the genetic experiments were begun. Physiological factors that could contribute to the 34-fold difference in lysozyme levels between these two strains were considered. No evidence of activators or inhibitors of lysozyme was detected in mixing experiments. An environmental or dietary inducer seemed unlikely because mice of both strains have been maintained on identical diets for generations, and lysozyme levels in newborn high mice are higher than those in adult low mice.

Developmental profile: Figure 3 shows the intestinal lysozyme levels in *domesticus* and *castaneus* mice as a function of age. In *domesticus* the concentration goes up nearly 10-fold in the first 6 weeks after birth, and nearly doubles again by 20 weeks. In *castaneus*, the level stays low through 20 weeks, and has not been observed to increase in animals of this species who were over 1 yr of age. The rate of increase of intestinal lysozyme in *domesticus* parallels that for the number of Paneth cells per intestinal crypt (MÖLLER, KÜNNERT and GEYER 1970).

Biochemical basis of level differences: The low content of intestinal lysozyme in casA could reflect fewer enzyme molecules, less activity per molecule, or both. Nondenaturing electrophoresis was used to recognize and to quantify lysozyme by testing for enzyme activity and by protein staining. As described in detail by HAMMER *et al.* (1987), it was observed that in tissue extracts with high activity, the lytic bands seen in native pH 4.3 gels corresponded exactly with major protein bands detected by the Coomassie stain. By contrast, for tissue extracts with low activity the bands of lysozyme activity corresponded to minor protein bands or to regions in the gel with no detectable protein. Moreover, protein bands matching the lysozyme activity exhibited a violet fluorescence characteristic of lysozyme *c* (HAMMER *et al.* 1987). This same correspondence between lysozyme activity and protein bands was evident also after electrophoresis at pH 8.9 (*cf.* DOBSON, PRAGER and WILSON 1984).

The gel shown in Figure 4 illustrates these findings for intestinal extracts from C3H and casA. **P** lysozyme is a major protein band in C3H, while it appears to be absent from casA. The activity overlay shows that **P** is present in trace amounts in casA and has the same electrophoretic mobility as the enzyme in C3H. From this we infer that the difference in activity between these two strains is due mainly to a difference in the number of lysozyme molecules.

Intestinal extracts also contain low concentrations of two electrophoretically distinct forms of **M** lysozyme. The C3H form has a relative electrophoretic mobility of 68 and the casA form has a mobility of 73 (Figure 4). By ion-exchange chromatography, **M** ac-

[3] The destabilization of lysozyme by bovine serum albumin during heating appears to involve sulfhydryl-disulfide interchange as well as denaturation. The inactivation of pure **P** lysozyme and of crude intestinal extracts containing overwhelmingly the **P** type of lysozyme (both from C58/J mice, see Figures 1 and 2) was examined as a function of temperature; in all cases a constant amount of lysozyme activity (1 unit) in the presence of 1 mg/ml bovine serum albumin was employed. It was found that temperatures higher by 3° were necessary to achieve the same inactivation profile for lysozyme in crude extracts as for pure **P** lysozyme. Thus components present in the crude extracts may be inhibiting the destabilization of lysozyme by bovine serum albumin. For further discussion of the phenomena involved, see CUNNINGHAM and LINEWEAVER (1967) and HAMMER (1984).

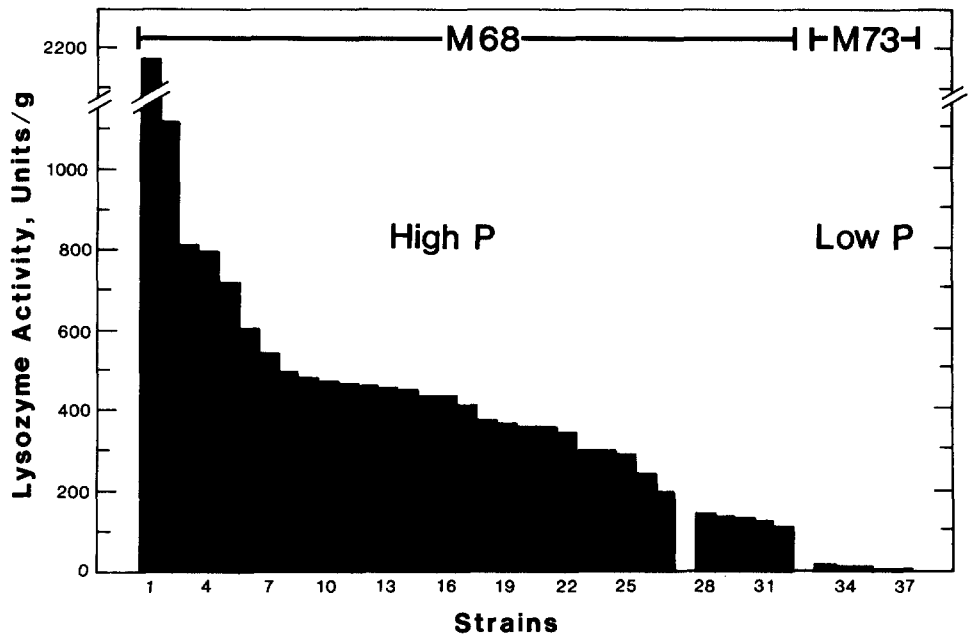

FIGURE 1.—Levels of lysozyme in the small intestines of 37 strains of mice representing three species. The level of intestinal lysozyme was measured in the spectrophotometric assay at pH 7.0. **M68** and **M73** refer to the electrophoretic forms of the **M** lysozyme present in these strains. Strains are designated by numbers. High **P** level—(1) NMRI/Navy, (2) RIIIS/J, (3) NZB/Bom, (4) AKR/J, (5) BALB/cJ, (6) ACR/A, (7) C58/J, (8) LTS/A, (9) LIS/A, (10) C3H (C3H/HeJ + AT9), (11) MAS/A, (12) DBA/2J, (13) A/J, (14) SWR/J, (15) C57BL/6J, (16) NZB/Ibm, (17) IS/Cam/J, (18) DD/HeAf, (19) SJL/J, (20) I, (21), A/HeJ, (22) TSI/A, (23) BIM/A, (24) 020/A, (25) SF/Cam/J, (26) P/J, (27) FVB/NA; moderate **P** level—(28) MOLC/RK, (29) MOL/SK, (30) MOLD/RK, (31) MOL3/JA, (32) MOLE/RK; and low **P**—(33) casA, (34) GRS/A, (35) STS/A, (36) CASA/RK, (37) Peru/A. Strains 28–32 are *M. molossinus*, strains 33 and 36 are *M. castaneus*, and the remainder are *M. domesticus*. Mice from strains 1–3, 6, 16, 22, and 31 had been stored frozen at −20° and the lysozyme levels adjusted as described in MATERIALS AND METHODS.

counted for about 3% of the total intestinal lysozyme in C58/J (Figure 2) and for a similar percent in C3H (see text above, *Levels in laboratory strains*). In contrast, activity measurements from electrophoretic gels show that **M** accounts for approximately 75% of the casA intestinal lysozyme (Figure 4). Assuming that the **P** molecule has the same specific activity in C3H and casA, we calculate, as follows, that the concentration of **P** lysozyme in the C3H intestine is about 130 times higher than in the casA intestine:

$$\frac{\mathbf{P}(\text{C3H})}{\mathbf{P}(\text{casA})} = \frac{\text{total(C3H)} \times \text{fraction } \mathbf{P}(\text{C3H})}{\text{total(casA)} \times \text{fraction } \mathbf{P}(\text{casA})}$$

$$= \frac{475 \times 0.97}{14 \times 0.25} = 132.$$

Mendelian inheritance of mouse lysozyme loci

P lysozymes: The inheritance of the **P** level difference was tested in a cross between C3H and casA. Interspecific hybrids expressed an intermediate level of intestinal lysozyme (Table 1). As shown in Figure 5 and Table 1, progeny of the backcrosses of F_1 hybrids to casA and outbred *castaneus* segregated into two approximately equal classes, with phenotypes similar to those of the casA parent and the F_1 hybrid. A single locus with additive inheritance could thus be the major determinant of intestinal lysozyme concentration. Control of lysozyme concentration by a single locus was confirmed in progeny of the backcross to the C3H parent and in the F_2 generation (Figure 5, Table 1). The method of FALCONER (1960) estimates the number of loci determining a quantitative difference using the mean parent values and the F_1 and F_2 variances (see LUSIS and PAIGEN 1975). The number of loci involved in the C3H by casA cross is estimated to be 0.83, consistent with the hypothesis of a single locus. This locus will be referred to as the **P** lysozyme regulator, *Lzp-r*. C3H carries the *Lzp-r*^{d} allele and casA carries *Lzp-r*^{c} (with *d* and *c* derived from *domesticus* and *castaneus*, respectively). That *Lzp-r* is autosomal, not *X*-linked, can be concluded from the observation of sons with high **P** levels and no sons with low **P** levels among the progeny of the F_1 × C3H backcross.

M lysozymes: The 68 and 73 electromorphs of **M** lysozyme produced by the C3H and casA parents were followed in the interspecific cross, backcrosses, and F_2 animals (Figure 5). The F_1 had equal amounts of both forms; in the progeny of the backcross to C3H and casA and in the F_2 generation, the 68 and 73 forms segregated in a Mendelian fashion. The slow C3H allele will be called *Lzm-s*^{68}, and the fast casA allele will be called *Lzm-s*^{73}. The backcross to outbred *castaneus* gave more complicated results which will be discussed below.

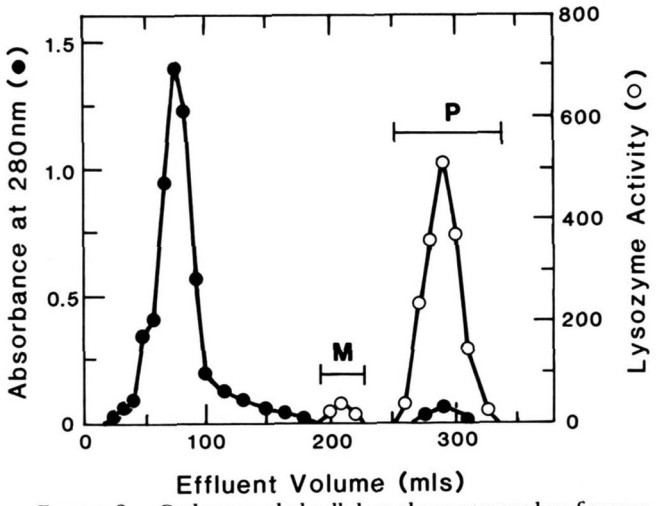

FIGURE 2.—Carboxymethyl cellulose chromatography of mouse intestinal lysozymes. A dialyzed extract (125 ml) prepared from the small intestines of 24 C58/J mice was applied to a Whatman CM-52 column (7 × 2.7 cm, 40 ml) equilibrated with 10 mM ammonium acetate, pH 4.5. The column was washed with equilibration buffer until the absorbance of the column effluent at 280 nm was zero. Lysozyme activity, measured in units/ml in the spectrophotometric assay, was not detected during the loading or washing of the column. Lysozyme was eluted with a linear gradient of 120 ml of 200 mM ammonium acetate (pH 4.5) and 120 ml of 600 mM ammonium acetate (pH 5.0); thereafter more of the 600 mM buffer was used to elute the column. The flow rate was 24 ml/hr, and 2-ml fractions were collected. The positions of **M** and **P** lysozymes are indicated. The carboxymethyl cellulose chromatography completely separated **M** and **P** lysozymes from one another, as judged from electrophoresis in native gels at pH 4.3. A trace (<1%) of **E** lysozyme was detected in the **P** lysozyme preparation; **E** is another type of lysozyme *c* present in very small amounts in many tissues of mice and other rodents (HAMMER *et al.* 1987).

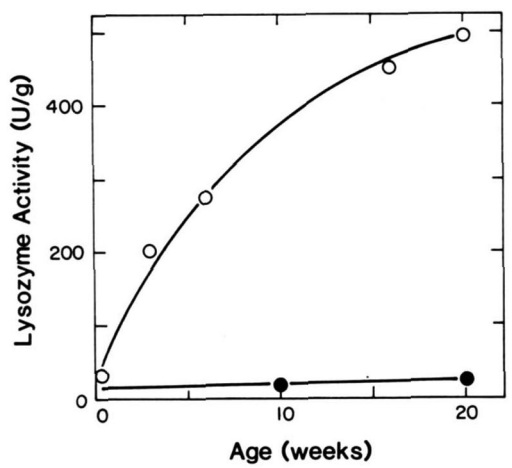

FIGURE 3.—Intestinal lysozyme level as a function of age. The intestinal lysozyme level was measured in siblings from consecutive litters of three to six mice from a single pair of *domesticus* (Morocco) (○) and *castaneus* (●) mice. Each point represents the mean activity from each litter.

The results of the crosses just described also enabled us to conclude that *Lzm-s* is on an autosome. If *Lzm-s* were on the *X* chromosome, F_1 sons of C3H × casA would have been expected to have only the 68 form of **M**, which was not the case. Similarly, for example,

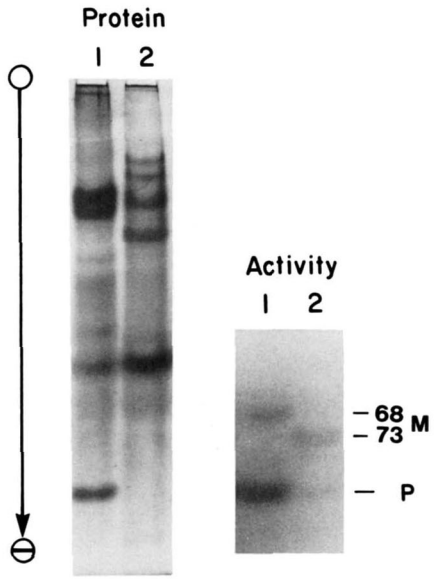

FIGURE 4.—Electrophoresis of lysozymes in intestinal extracts from *domesticus* and *castaneus* mice. Extracts (50 μl) from *domesticus* C3H (lane 1) and *castaneus* casA (lane 2) were subjected to electrophoresis at pH 4.3 in a 10% polyacrylamide gel. The left panel shows the result of staining the gel for protein and the right panel shows the lysozyme activity overlay. The numbers refer to the electrophoretic mobilities of **M** lysozymes relative to poly-L-lysine. **P** lysozyme has a mobility of 84 on this scale.

among the progeny of the F_1 × C3H backcross some sons with only the 73 electromorph would have been expected, and no such animals were observed, even though 78 mice were scored (Figure 5); furthermore, male as well as female progeny of this cross which were heterozygous for **M** lysozyme were observed. We note also that none of the lysozyme genes described in this report could reside on the *Y* chromosome, since all are expressed in females as well as males.

Linkage of P and M: In the backcross mice produced by crossing the F_1 with C3H and in the F_2 mice, the distinction between **P** levels in progeny of the intermediate (F_1 phenotype) and the high (C3H phenotype) classes was slightly obscured by their overlapping distributions (Figure 5). On the basis of the electrophoretic mobility of the **M** lysozymes of the progeny, mice were sorted into two classes (Figure 5 and Table 1). In most progeny the loci determining **P** level and **M** electrophoretic mobility appeared to remain closely linked; however, a few individuals may have had recombinant phenotypes. For example, among the progeny of the F_2 cross, one mouse showed an activity of 93 units/g, a low heterozygous value, but was homozygous at the *M* structural locus ($Lzm\text{-}s^{68/68}$). Because the mice were sacrificed in the process of being scored, it is impossible to determine if recombination had occurred or if these mice represent extremes in the expression of **P** lysozyme.

In the backcross of F_1 animals to casA, only 15 mice were scored, and none gave evidence for recombina-

TABLE 1

Segregation of intestinal lysozyme levels in progeny of crosses between high (C3H) and low mice

Mice	Class	No. of mice	Lysozyme activity (units/g)	
			Mean	Standard error
Parental types				
Inbred *domesticus* (C3H)	High	10	474.9	27.1
Inbred *castaneus* (casA)	Low	5	13.5	1.5
Randomly bred *castaneus*	Low	10	16.5	3.2
Interspecific hybrid				
F_1 (C3H × casA)	Intermediate	6	224.4	33.4
Backcross types				
F_1 × C3H[a]	High	38	495.9	30.5
	Intermediate	40	288.6	18.1
F_1 × casA	Intermediate	10	245.5	18.4
	Low	5	12.0	2.4
F_1 × *castaneus*	Intermediate	43	205.5	7.7
	Low	35	14.8	0.8
F_2[a]				
[(casA × C3H) × (casA × C3H)]	High	21	404.6	48.6
	Intermediate	30	195.1	14.1
	Low	16	7.8	0.9

[a] F_1 × C3H backcross and F_2 mice were sorted into the high and intermediate categories by considering also the type(s) of **M** lysozyme present (see Figure 5 and RESULTS in text).

tion. More progeny were scored in the backcross to outbred *castaneus* (Table 1), but the **M** electrophoretic phenotypes were complicated by unequal amounts of 68 and 73 (see below). Yet, the cosegregation of these phenotypes with intermediate and low levels of **P** was consistent with close linkage.

Heat denaturation of P lysozyme

F_1 hybrids and the *cis-trans* test: Two types of **P** lysozyme can be distinguished in each F_1 hybrid by heat denaturation. When lysozyme from intestinal extracts was heated at 66.5° for 60 min, the activity of C3H lysozyme decayed rapidly and after 20 min less than 10% of the original activity remained. In contrast, F_1 lysozyme initially decayed rapidly and later more slowly such that about 25% of the original activity remained after 45 min. Table 2 shows the residual activities in C3H and F_1 hybrids after 45 min of heating. The levels of **P** lysozyme were too low in casA to measure heat stability by the spectrophotometric assay. Using gel electrophoresis and the activity overlay method, estimates of residual activity suggest that the casA **P** lysozyme is more stable to heat than is the C3H **P** lysozyme.

This difference in the rate of decay of intestinal lysozyme between C3H and the F_1 may be the result of (1) an intrinsic structural difference between lysozymes, (2) an effector molecule that stabilizes lysozyme in the F_1 or destabilizes it in C3H, or (3) an elevated level of the more heat-stable **M** lysozyme (HAMMER *et al.* 1987) in the F_1. The last alternative was discounted on the basis of quantitative protein ectrophoresis. As shown in Figure 6, the majority of activity remaining in the F_1 after heating was due to **P** lysozyme, and not due to an elevation of **M**. [Figure 6 also emphasizes the heat lability of **P** lysozyme in C3H mice, which in heated extracts (lane 2) is essentially undetectable with the Coomassie stain.] The second possible explanation, an effector molecule, seems unlikely for two reasons: when the C3H and F_1 extracts were mixed prior to heating, the decay kinetics were additive. Furthermore, the difference in heat stability was still apparent after 40-fold purification of the lysozymes from the F_1 and C58/J, a strain containing a **P** lysozyme identical in heat stability to that of C3H. As shown in Figure 7, the purified C58/J lysozyme is heat labile, while the purified F_1 has a heat-stable component comprising about 40% of the total lysozyme level.

The combined evidence suggests that C3H expresses a homogeneous population of heat-labile **P** lysozyme and the F_1 expresses about equal amounts of both heat-labile and heat-stable **P** lysozymes. The implication is that *Lzp-r* acts *trans*, causing intermediate expression of both the C3H **P** lysozyme and the casA **P** lysozyme.

Heat denaturation in backcross progeny: Heat denaturation was used to characterize the **P** lysozyme of backcross progeny obtained in the cross between the F_1 hybrid and C3H. Figure 8 is a kinetic analysis showing that 16 mice of the intermediate activity class (F_1 phenotype) had two forms of **P** lysozyme: a fast decaying one, and a more stable one which accounted for the survival of about 25% of the initial activity after 45 min of heating. By contrast, intestinal lyso-

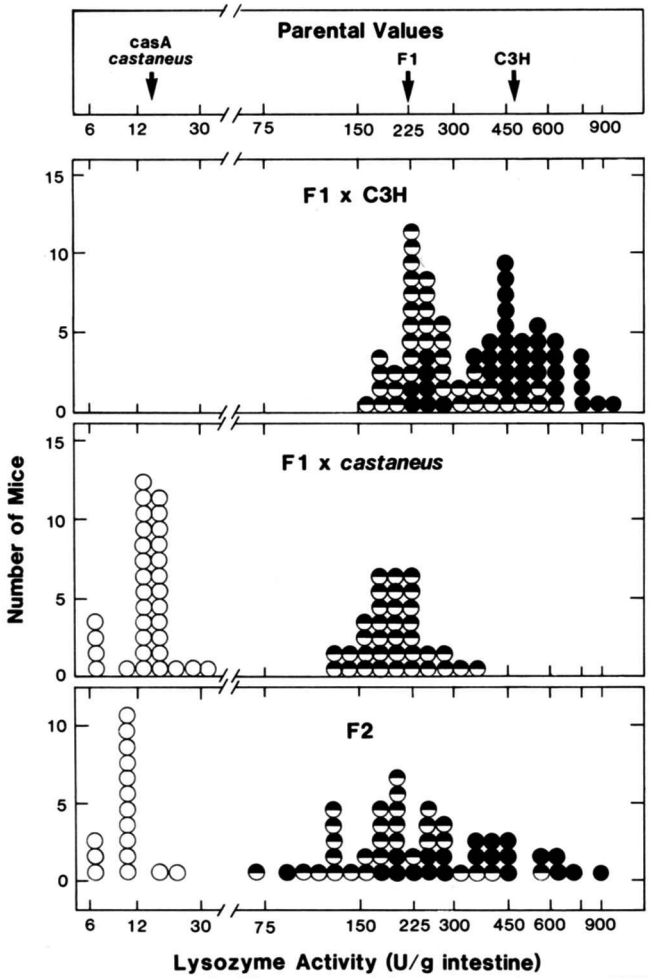

FIGURE 5.—Intestinal lysozyme activity in crosses between C3H and *castaneus*. Progeny were sacrificed at 10 weeks of age and intestinal extracts were assayed for lysozyme activity with the spectrophotometric method. Each circle represents the average activity of two assays from a single mouse. In the top and bottom panels, the open circles refer to mice with the 73 form of **M** lysozyme, the half-filled circles refer to mice with both the 73 and 68 forms, and filled circles refer to mice with only the 68 form. In the middle panel, the open circles refer to mice with the 73(68) or 73 **M** phenotypes and half-filled circles refer to mice with the 73/68 or (73)68 **M** phenotypes (see Tables 4 and 5). Ten additional mice resulting from the type of cross plotted in the middle panel here are not shown due to difficulty in scoring the ratios of the 73 and 68 forms of **M** lysozyme. However, these individuals are included in Table 1 (see also Table 4).

zymes from 12 progeny of the high activity class (C3H phenotype) were exclusively of the heat-labile type. The remaining backcross progeny were scored by measuring the surviving lysozyme activity at a single time point, *viz.* after 45 min of heating at 66.5° (Table 2). The mean percent surviving activity for the high class was 4.8 ± 0.6 and that for the intermediate class was 27.7 ± 1.4. In addition, the heat-stability properties of **P** lysozymes that were purified approximately 50-fold from an individual from the intermediate class and an individual from the high class were unaltered. Thus the small amounts of **M** lysozyme present in the

TABLE 2

Heat stability of lysozyme from intestinal extracts of mice from the cross between C3H and casA and from backcross mice[a]

Mice	Initial lysozyme level	No. of mice	Surviving activity (percent) Mean[b]	Surviving activity (percent) Standard error
domesticus (C3H)	High	4	1.7	0.8
F_1 hybrid (C3H × casA)	Intermediate	4	27.0	6.5
Backcross (F_1 × C3H)	High	37	4.8	0.6
	Intermediate	39	27.7	1.4

[a] See Figure 7 for a study of heat denaturation as a function of time for purified **P** lysozyme from *domesticus* and F_1 hybrid mice and Figure 8 for an analogous kinetic study of the lysozyme in intestinal extracts of backcross mice. The 28 mice studied in Figure 8 are included among the 76 backcross mice in this table. See Figure 6 for an evaluation of lysozyme heat stability in C3H and F_1 mice using gel electrophoresis and an activity overlay.

[b] Average percentage of initial activity remaining after heating for 45 min at 66.5°.

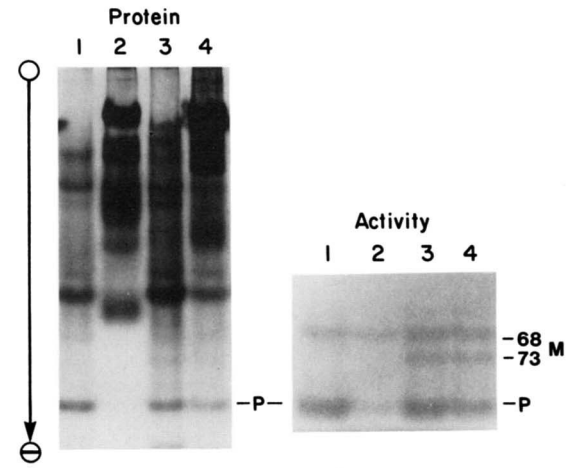

FIGURE 6.—Electrophoretic evaluation of the heat stability of **P** and **M** lysozymes from C3H and the interspecific hybrid (C3H × casA). Unheated and heated intestinal extracts from a C3H and an F_1 hybrid mouse were fractionated in a 10% polyacrylamide gel at pH 4.3. The unheated extracts (50 μl) are shown in lanes 1 (C3H) and 3 (hybrid). The same amount of each extract was lyophilized, resuspended in sodium phosphate buffer (pH 7.5) containing bovine serum albumin (1 mg/ml), and heated at 66.5° for 1 hr. The heated extracts from C3H and the F_1 are in lanes 2 and 4, respectively. The left panel shows the gel stained for protein, and the right panel shows the activity overlay. Two inferences emerge from these results. First, **P** lysozyme present in the hybrid (lane 4) is more heat stable than the **P** lysozyme in C3H (lane 2); see protein and activity panels. Second, the heat stability of the **M** lysozymes in the two types of mice is comparable (see activity panel). See Table 2 and also Figures 7 and 8 for a quantitative assessment with the spectrophotometric assay of lysozyme susceptibility to heat denaturation.

experiments just described (Figure 8) do not affect our interpretation.

These two classes of **P** defined by their heat stability, with approximately equal numbers and mean surviving activities similar to the F_1 and C3H parents, suggest that the heat stability of **P** is determined by a single locus, presumably the structural locus for **P**

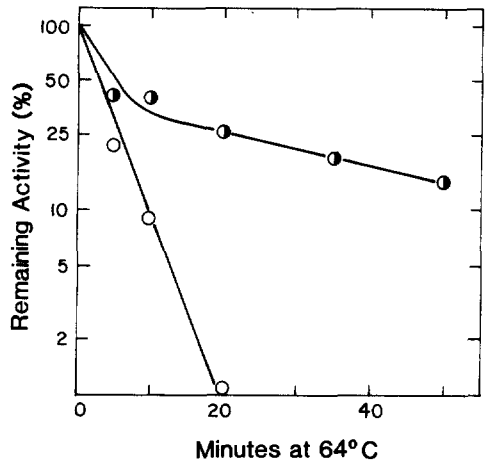

FIGURE 7.—Kinetics of heat denaturation of purified **P** lysozyme from C58/J (O) and the interspecific hybrid C3H × casA (◑). Lysozyme was purified about 40-fold from intestinal extracts by carboxymethyl cellulose chromatography. Aliquots containing 1 unit of lysozyme activity were lyophilized, resuspended in denaturation buffer, heated at 64° for up to 50 min, chilled, and assayed for lysozyme activity (*cf.* MATERIALS AND METHODS). The curve shown for C58/J has a single component, due to a heat-labile **P** lysozyme. The two-component decay of the curve for the hybrid, in turn, is attributable to two allelic forms of **P** lysozyme: a heat-labile form derived from C3H and a more heat-stable form derived from casA.

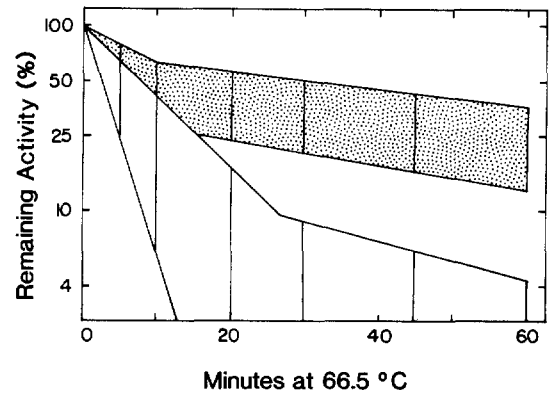

FIGURE 8.—Kinetics of heat denaturation of the lysozyme in intestinal extracts of backcross mice (F_1 × C3H). Aliquots containing about 1 unit of lysozyme activity were lyophilized, resuspended in denaturation buffer, heated at 66.5°, chilled, and assayed for lysozyme activity. The ranges of the percentage of the surviving activity in 12 progeny from the class with a high lysozyme level (unstippled) and in 16 progeny from the class with an intermediate level (stippled) are plotted as a function of the time heated. See Table 2 for a comparison (after 45 min of heating) of surviving lysozyme activity in C3H and F_1 as well as backcross mice. In the stippled region the lysozyme activity observed is due principally to two forms of **P** lysozyme, one of which is heat labile and one of which is heat stable; thus a two-component decay is observed, as in the upper curve of Figure 7. The activity observed in backcross mice initially having a high lysozyme level (unstippled region) is inferred to be due mainly to a single component, a heat-labile **P** lysozyme. [Small amounts of **M** lysozymes are also present in this assay and may contribute slightly to the more heat-stable component. However, in the mice considered here the amount of **M** lysozyme present is generally unlikely to exceed 10% of the total lysozyme, for the following reasons: Figure 2 and gel electrophoretic tests show that in *domesticus* mice like C3H **M** accounts for about 3% of the intestinal lysozyme; the absolute levels of **M** in C3H and casA are comparable (*cf.* RESULTS, *Biochemical basis of level differences*); from the values just mentioned, it can be inferred that in mice in the category shown in the stippled region, 6% of the lysozyme is **M**. Furthermore, the evidence presented here, though indirect, suggests that *castaneus* **P** may have a half-life similar to the 23 min at 64° reported for *domesticus* **M** (HAMMER *et al.* 1987). Finally, we note (Table 2) that in C3H after 45 min at 66.5° the percentage of the surviving activity is 1.7 ± 0.8, which can be attributed to **M** lysozyme [since *domesticus* **P** has a half-life at 64° of only 2 min (HAMMER *et al.* 1987 and Figure 7)]; a similar value of 4.8 ± 0.6% (Table 2) is seen in backcross mice in the high category, where **M** but not **P** is postulated to be active after 45 min of heating. From this last value and the discussion above, we can suggest that in backcross mice in the intermediate category (stippled region) after 45 min about 10% of the initial activity may be expected due to the survival of **M**, which contrasts with the observed average of 27.7 ± 1.4% and the range of 15–46% (Table 2 and this figure). Thus the two-component upper (stippled) region is due primarily to two forms of **P**, and the two-component lower region is due to one form of **P** and to residual **M**.]

lysozyme. Furthermore, the cosegregation of heat stability and **P** level suggests that *Lzp-r* and the *P* structural locus, *Lzp-s,* are either the same genetic locus or are closely linked. In the backcross there were no apparent recombinants between thermostability and **P** level, allowing us to estimate that the maximal recombinational distance between *Lzp-r* and *Lzp-s* is 4%, or 8000 kilobase pairs of DNA, at the 95% confidence level (DIZIK and ELLIOTT 1977; LUSIS *et al.* 1983).

Other linkage studies

Cross to *spretus*: The linkage of *Lzp-s* and *Lzm-s* was confirmed in a cross between *spretus* and *domesticus*. In the electrophoretic mobility of both **M** and **P** lysozymes, *spretus* differs from *domesticus*: the *spretus* used in this study produce an **M** lysozyme with an electrophoretic mobility of 62 and a **P** lysozyme with an electrophoretic mobility of 82 (HAMMER 1984). Twenty-one progeny from female hybrids (BALB/c × *spretus*) crossed to BALB/c males were scored for **M** and **P** mobility, and only double heterozygotes (*62/68, 82/84*) and double homozygotes (*68/68, 84/84*) were found. If these two structural loci were unlinked, we would have expected the progeny observed as well as progeny with (*62/68, 84/84*) and (*68/68, 82/84*).

The results of the *domesticus-spretus* work reinforce also the autosomal location of the **P** and **M** lysozyme genes: males as well as females were among the 14 doubly heterozygous backcross individuals, and no progeny of this backcross with only the 62 **M** and 82 **P** lysozymes were seen.

Variation in natural populations: Table 3 summarizes the variation in **M** and **P** found among inbred strains of *domesticus* and among descendants of *domesticus* mice trapped in the wild. For the descendants of wild-caught mice, the frequency of the 68 allele was 0.42 and that of high **P** was 0.33 (grouping intermediate and high classes). These frequencies contrast

TABLE 3

Covariation between the level of P lysozyme and the electrophoretic mobility of M lysozyme among inbred and wild *domesticus* mice

P level[a]	M mobility	No. of individuals	
		Inbred[b]	Wild
Low	73	3	26
Low	68	0	9
Low	73/68	0	5
High	73	0	3
High	68	27	11
High	73/68	0	4
High	73/71	0	1
High	68/66	0	1

[a] High and low levels of **P** are defined as >100 units/g and <100 units/g, respectively (see also Figure 1).
[b] Number of strains (see Figure 1).

with those found in lab strains, which are 0.90 for both. Table 3 also allows us to examine covariation between the level of **P** and the electrophoretic mobility of **M** lysozyme among inbred mice and wild mice. Among inbred strains there are only two types, low **P—M**73 and high **P—M**68. These two types were also the most common among wild mice. Yet, low **P—M**68 and high **P—M**73 haplotypes also occurred among wild mice, suggesting that recombination has occurred between *Lzm-s* and *Lzp-r*. The mechanism causing linkage disequilibrium is not known because the 60 mice examined were from several populations.

Linkage to other loci: The progeny of the F_1 × C3H backcross were scored for 14 enzyme markers by J. WOMACK (Texas A&M University). Similarly, 14 recombinant inbred lines derived from BALB/cHeA and STS/A (HILGERS and ARENDS 1985) were examined for 76 markers by J. HILGERS (Netherlands Cancer Institute). Linkage of *Lzp-r*, *Lzp-s* and *Lzm-s* to other loci was not demonstrated in either of these studies, which together considered markers mapped to 16 of the 19 autosomes.

Evidence for linked, duplicate M loci

Every one of 10 descendants of a randomly bred population of wild-caught *castaneus* contained both the 68 and 73 forms of **M** lysozyme in their kidneys (Table 4). By contrast, all casA examined have only the 73 form and all C3H have only the 68 form. The chances of making the observation described for the wild *castaneus* are less than one in a thousand if these forms are segregating alleles at one locus.

An alternative model is allelic variation at duplicate loci for **M** lysozyme (Table 5). Consistent with this model, the relative amounts of the two forms of **M** were not constant in the outbred *castaneus*. According to both activity testing and protein staining of electrophoretic gels, three individuals expressed the 73 and 68 forms equally (Figure 9), and are designated 73/68 in Tables 4 and 5. In the remaining seven individuals, the 73 form was more abundant than the 68 form (Figure 9), and individuals with this phenotype are designated as 73(68) in Tables 4 and 5. One can interpret the 73/68 individuals as being homozygous for the 73 form at one locus and homozygous for the 68 form at the other locus. Likewise, the 73(68) individuals may be heterozygous for the 73 form at one locus and homozgyous for the 73 form at the other locus.

TABLE 4

Relative amounts of two forms of M lysozyme in kidney extracts of *domesticus* and *castaneus* mice and their hybrids

Mice	No. of mice				
	73	73(68)	73/68	(73)68	68
Parental types					
Outbred *castaneus*	0	7	3	0	0
Inbred *castaneus* (casA)	5	0	0	0	0
Inbred *domesticus* (C3H)	0	0	0	0	10
Interspecific hybrid					
F_1 (C3H × casA)	0	0	6	0	0
Backcross types					
F_1 × casA	5	0	10	0	0
F_1 × C3H	0	0	40	0	38
F_1 × *castaneus* 73/68[a]	0	21	0	26	0
F_1 × *castaneus* 73(68)	6	6	4	5	0

The phenotype 73(68) indicates that 73 is present in greater amount than 68; 73/68 denotes equal amounts of 73 and 68; (73)68 indicates that the amount of 68 exceeds that of 73 (see Table 5).
[a] Ten mice were not included due to difficulty in scoring. The intestinal lysozyme levels of these individuals are, however, included in Table 1.

TABLE 5

Two-locus model for five phenotypes and six genotypes of M lysozymes in *domesticus* and *castaneus* mice

	73	73(68)	73/68	(73)68	68
Phenotype					
Suggested genotype	73-73 / 73-73	73-73 / 73-68	73-73 / 68-68 / 73-68 / 73-68	73-68 / 68-68	68-68 / 68-68
73 alleles / 68 alleles	4 / 0	3 / 1	2 / 2	1 / 3	0 / 4

The two-locus model was tested by typing the progeny of a cross between C3H and casA and the progeny of crosses between these F_1 hybrids (C3H × casA) and four types of mice—C3H, casA and the two sorts of outbred *castaneus* individuals, 73/68 and 73(68). As shown in Table 4, each of the four crosses gives a different result. Backcrosses to the inbred parental lines, casA and C3H, give results consistent with either the one-locus or two-locus model.

We draw particular attention to the last two crosses

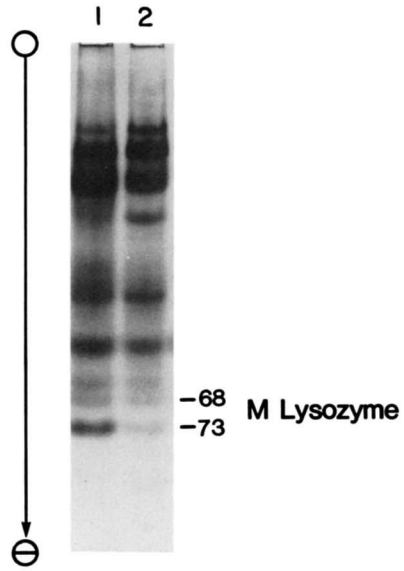

FIGURE 9.—Electrophoresis at pH 4.3 of kidney extracts (50 μl) from outbred *M. castaneus*. The gel was assayed for activity with the overlay method (not shown) and stained for protein. Lane 1, an individual with a prevalence of the 73 form of **M** lysozyme, phenotypic designation 73(68); lane 2, an individual with equal amounts of both the 68 and 73 forms, phenotypic designation 73/68.

in Table 4. When the *castaneus* with equal amounts of 68 and 73 was crossed to the F_1, two types of offspring were produced in almost equal numbers: those with a prevalence of the 73 form and those with a prevalence of the 68 form. This is inconsistent with a single-locus model, which would predict three progeny classes: homozygous mice with only the 68 form, heterozygous mice with equal amounts of both, and homozygous mice with only the 73 form. The two-locus hypothesis was further supported by the results of the cross between *castaneus* with a prevalence of the 73 form and the F_1. Four types of progeny were produced: mice with a prevalence of the 68 or 73 form, mice with an equal amount of both forms, and mice with only the 73 form (Tables 4 and 5).

Besides supporting the two-locus model, the results in Table 4 imply that these two loci are linked. This implication is most evident from the absence of progeny with skewed ratios of the 73 and 68 forms (*i.e.*, the 73(68) and (73)68 phenotypes) in the backcrosses between the F_1 and either the casA or C3H parents.

In summary, the results just presented are consistent with a model where **M** lysozyme is determined by two, linked loci, *Lzm-s1* and *Lzm-s2*, each of which carries the 73 and/or 68 allele. The inbred casA mice appear to be homozygous at both loci for the 73 form. By contrast, C3H mice are homozygous at both loci for the 68 form. Outbred *castaneus*, in turn, are either heterozygous at one locus and homozygous at the other for the 73 form, or homozygous at each locus for alternative forms.

DISCUSSION

Models of gene regulation: We have assumed that *Lzp-r* is a regulatory locus; however, we have not excluded the possibility that it is identical to the *P* structural locus. Either model must explain the results of the crosses between *domesticus* (high **P**) and *castaneus* (low **P**) mice: (1) *additivity* (the level is intermediate in heterozygotes), (2) *trans-action* (both parental forms are expressed in heterozygotes), and (3) *linkage* (factors affecting level and heat stability of **P** cosegregate in backcross progeny). We discuss these two models, and, for simplicity, present the less likely model first.

1. 1 structural locus/2 alleles: This model assumes that there is one structural gene for **P** lysozyme and that both heat stability and enzyme level can be explained by allelic variation at this locus. The implication is that a mutation in the structural gene causes a difference in the final concentration of lysozyme in Paneth cells as well as a heat stability difference. To explain *trans*-action, this model also assumes that lysozyme can regulate its own expression. Examples of this type of model include tubulin (PITTENGER and CLEVELAND 1985) and the simian virus 40 A gene (RIO *et al.* 1980), where autoregulatory mechanisms control the expression of tubulin and of the SV40 T antigen, respectively.

2. 1 structural locus/2 alleles; 1 regulatory locus/2 alleles: According to this model, **P** lysozyme is controlled by two separate, linked loci. Alleles of *Lzp-s* code for heat-stable and labile lysozymes. Alleles of *Lzp-r* presumably cause differential rates of synthesis or degradation of **P** lysozyme or its precursors. The *trans*-acting sites are presumed to release diffusible regulatory signals. All cases of mammalian *trans*-acting regulators are reported to give additive inheritance (PAIGEN 1979, 1986). This is also the case for *Lzp-r*. Two possible explanations involve a rate-limiting model and a competition model. In the rate-limiting model, $Lzp\text{-}r^d$ produces a 130-fold higher level of diffusible regulatory signal. Its concentration would be rate-limiting for the final level of **P**, so that a heterozygote would produce half as much **P**. In the competition model, both alleles of *Lzp-r* produce equal amounts of structurally different signals. These signals would then compete for modulation sites. Once bound, $Lzp\text{-}r^d$ would act 130-fold more effectively. Equal numbers of both signals binding to modulation sites produce the additive effect. In this model, the number of binding sites would determine the final concentration of **P**.

It is also possible that *Lzp-r* controls lysozyme concentration at the cellular level. For example, the locus could code for a difference in the number of export sites in the Paneth cell, thus causing differences in the number of intracellular molecules of **P** lysozyme. Alternatively, *Lzp-r* may determine the number of Paneth cells per crypt, as is suggested by the correlation in development of the number of Paneth cells per

intestinal crypt and the level of intestinal lysozyme (*cf.* RESULTS, *Intestinal lysozyme levels—Developmental profile*). In this case, *Lzp-r*^{d} would influence a 130-fold increase in cell proliferation relative to *Lzp-r*^{c}. A *trans* effect would result from equal activity of **P** genes in high and low mice. Preliminary evidence (S. ERLANDSEN, personal communication) suggests that *domesticus* and *castaneus* mice do not differ in the number of Paneth cells per intestinal crypt, and so we do not favor this possibility.

To date there have been few precedents for proximal *trans*-acting regulators in mammals. In most published cases, *trans*-acting regulators had been mapped to sites distant from the structural genes they influened (PAIGEN 1979; LUSIS *et al.* 1983). In the case of β-glucuronidase, however, a regulator that is *trans*-acting and lies within the β-glucuronidase gene complex has been reported (HERRUP and MULLEN 1977; MEREDITH and GANSCHOW 1978; LUSIS *et al.* 1983). The maximal distance between this structural gene and its regulator was 0.3 centimorgan. In contrast, there are now several other regulatory genes known that act in *trans* at a distance by way of diffusible signals that are not products of the structural gene that is regulated (LUSIS *et al.* 1983; KILLARY and FOURNIER 1984). These observations suggest that proximal *trans*-acting regulators also produce diffusible signals, as has been observed for a regulatory gene, E1A, in adenovirus (KOVESDI, REICHEL and NEVINS 1986).

Persistent regulatory polymorphism? The results presented here suggest that a single locus may account for the variation in intestinal levels of **P** lysozyme reported by HAMMER *et al.* (1987) for species in the house mouse group. The hypothesis that a regulatory mutation arose once in the species that was ancestral to the seven commensal species is conveyed in the evolutionary tree shown in Figure 10. Rather than going to fixation, the mutant state persisted as a polymorphism in three descendant species (*domesticus, molossinus,* and *hortulanus*). In four other descendant species, fixation of either the mutant (*musculus, spretus*) or wild-type (*castaneus, abbotti*) regulatory allele apparently took place. We can ask if the time elapsed since the regulatory polymorphism arose agrees with the expectations of the neutral theory. For a selectively neutral mutation, it takes on the average $4N_e$ generations until fixation (KIMURA and OHTA 1969), where N_e is the long-term effective population size. Using the N_e value for *domesticus* and *musculus* estimated by WILSON *et al.* (1985) and assuming that the generation time for mice is 0.5 year, the mean time for random fixation is 1.5 million years. The survival of the **P** lysozyme regulatory polymorphism for 3–4 million years is thus in the realm of possibility without strong selection.

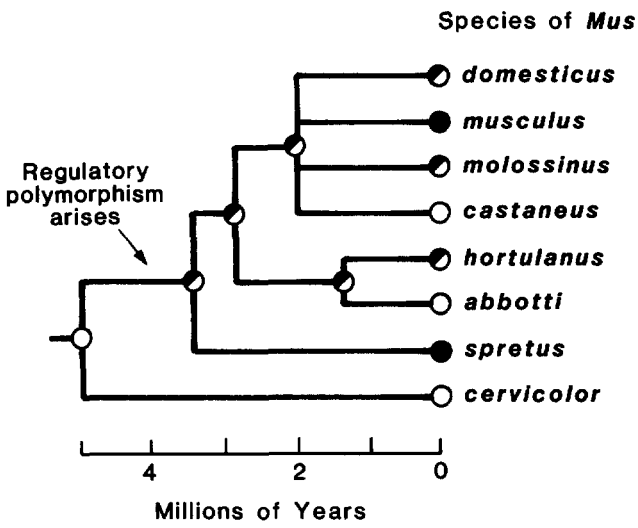

FIGURE 10.—Evolutionary tree for eight species of mice showing **P** lysozyme levels. The branching order and time scale are based on SAGE (1981), FERRIS *et al.* (1983), BONHOMME *et al.* (1984) and references therein and on unpublished data. The top seven species are in the house mouse species group and *Mus cervicolor* is an outgroup. The circles indicate **P** lysozyme levels in the small intestine (HAMMER *et al.* 1987) as follows: low (○), high (●), both high and low levels found within the species (◐).

An alternative hypothesis is that high intestinal levels of **P** lysozyme have arisen independently on numerous occasions in this group of species. Consistent with this possibility, there is evidence for an additional regulatory mutation in a population of *domesticus* mice from Azrou, Morocco. In this case the inheritance of high levels of **P** appears to be dominant rather than additive (HAMMER 1984). Furthermore, inbred strains of *molossinus* express moderate rather than high levels of **P** in the small intestine (Figure 1). Further genetic analyses by interspecific crosses and artificial insemination are possible between strains representing all members of the house mouse group (see BONHOMME *et al.* 1984). By investigating dominance relationships, *cis* and *trans* action, number of loci and linkage relationships of alleles and loci affecting **P** expression, it should be possible to find out how many mutations account for the diversity in lysozyme levels in this group of species.

Adaptive value of gene regulation: We conclude by considering how our results relate to other studies on intraspecific variation of protein levels in mammals. There seem to be two categories of genetic variation of protein levels within species of mice. Our survey of the mouse literature revealed about 30 cases in which the levels varied by only a factor of two or three (FELDER 1980; BULFIELD, HALL and TSAKAS 1984; JAMES *et al.* 1986) and four cases where protein concentration differed by 10-fold or more. These four proteins are renin in the submaxillary gland (WILSON *et al.* 1981), an immunoglobulin light chain (GECKELER, FAVERSHAM and COHN 1978), histidine decarboxylase in the kidney (MARTIN *et al.* 1984), and α-

fetoprotein in serum (OLSSON, LINDAHL and RUOSLAHTI 1977; BELAYEW and TILGHMAN 1982). Although a few of the systems with small differences in concentration showed polygenic inheritance (NAYUDU and MOOG 1967; KOZAK 1972; HUTTON 1971), most were due to alleles at a single locus. Similarly, all of the systems with large differences were shown to be controlled by alleles at a single locus.

That both small and large differences in protein levels can be adaptive has been shown in humans. On the one hand, adaptive evolution in populations exposed to malaria can be mediated by small changes in hemoglobin levels caused by the deletion of one out of two α-globin genes (FLINT *et al.* 1986). On the other hand, we see a more than 10-fold elevation in the intestinal concentration of lactase in adults in dairy-farming populations (POTTER *et al.* 1985).

Allelic variation at the regulatory locus for mouse **P** lysozyme gives another example of large differences in enzyme concentration. The magnitude of the concentration difference may be as great as in cases of bacterial populations adapting to new carbon sources in laboratory experiments. In these cases, a regulatory mutant often is selected that has high concentrations of an enzyme with weak activity on the new compound because of chance resemblance between the compound and the normal substrate. By virtue of having perhaps 100 times more of the enzyme than the wild-type bacteria do, the mutant can metabolize the new compound at a biologically significant rate (examples reviewed by WILSON, CARLSON and WHITE 1977). The observation that, in both mammals and bacteria, such differences in enzyme concentration are controlled by a single locus implies that major adaptive genetic changes can occur in one step. Mutations of this sort may help to account for the uncoupling of molecular evolution and organismal evolution observed in numerous taxonomic groups (WILSON, CARLSON and WHITE 1977; WILSON 1985).

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