Regulatory and Structural Genes for Lysozymes of Mice

Michael F. Hammer\(^1\) and Allan C. Wilson

Department of Biochemistry, University of California, Berkeley, California 94720

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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 (Blackberg 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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\(^1\) Present address: Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544.
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, 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


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-phenoxethanol. For extracts involving more than 5 g of tissue, an Omniflexer 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°. 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 5.5, 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°.
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°C, while that for tissue extracts was 66–67°C. 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 X streptus F1 hybrid and backcross progeny derived from the female F1 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 F1 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 (Møller, Könnerz, 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-

Control of lysozyme concentration by a single locus was confirmed in progeny of the backcross to the C3H parent and in the F2 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 F1 and F2 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-rd allele and casA carries Lzp-rc (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 F1 X C3H backcross.

M lysozymes: The 68 and 73 electromorphs of M lysozyme produced by the C3H and casA parents were followed in interspecific cross, backcrosses, and F2 animals (Figure 5). The F1 had equal amounts of both forms; in the progeny of the backcross to C3H and casA and in the F2 generation, the 68 and 73 forms segregated in a Mendelian fashion. The slow C3H allele will be called Lzm-s6*, and the fast casA allele will be called Lzm-s83. The backcross to outbred castaneus gave more complicated results which will be discussed below.

**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 F1 hybrids to casA and outbred castaneus segregated into two approximately equal classes, with phenotypes similar to those of the casA parent and the F1 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 F2 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 F1 and F2 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-rd allele and casA carries Lzp-rc (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 F1 X C3H backcross.
Mouse Lysozyme Genes

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 x 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 (el%) of E lysozyme was detected in the P lysozyme preparation; E is another type of lysozyme 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) (C) and castaneus (P) 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, F1 sons of C3H × casA would have been expected to have only the 68 form of M, which was not the case. Similarly, for example, among the progeny of the F1 × 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 F1 with C3H and in the F2 mice, the distinction between P levels in progeny of the intermediate (F1 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 F2 cross, one mouse showed an activity of 93 units/g, a low heterozygous value, but was homozygous at the M structural locus (Lzm-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 F1 animals to casA, only 15 mice were scored, and none gave evidence for recombina-
Segregation of intestinal lysozyme levels in progeny of crosses between high (C3H) and low mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Class</th>
<th>No. of mice</th>
<th>Mean</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental types</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inbred domesticus (C3H)</td>
<td>High</td>
<td>10</td>
<td>474.9</td>
<td>27.1</td>
</tr>
<tr>
<td>Inbred castaneus (casA)</td>
<td>Low</td>
<td>5</td>
<td>13.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Randomly bred castaneus</td>
<td>Low</td>
<td>10</td>
<td>16.5</td>
<td>3.2</td>
</tr>
<tr>
<td>Interspecific hybrid F₁ (C3H × casA)</td>
<td>Intermediate</td>
<td>6</td>
<td>224.4</td>
<td>33.4</td>
</tr>
<tr>
<td>Backcross types</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F₁ × C3H</td>
<td>High</td>
<td>38</td>
<td>495.9</td>
<td>30.5</td>
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<tr>
<td></td>
<td>Intermediate</td>
<td>40</td>
<td>288.6</td>
<td>18.1</td>
</tr>
<tr>
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<td>Intermediate</td>
<td>10</td>
<td>245.5</td>
<td>18.4</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>5</td>
<td>12.0</td>
<td>2.4</td>
</tr>
<tr>
<td>F₁ × castaneus</td>
<td>Intermediate</td>
<td>43</td>
<td>205.5</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>35</td>
<td>14.8</td>
<td>0.8</td>
</tr>
<tr>
<td>F₂⁺ [(casA × C3H) × (casA × C3H)]</td>
<td>High</td>
<td>21</td>
<td>404.6</td>
<td>48.6</td>
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<tr>
<td></td>
<td>Intermediate</td>
<td>30</td>
<td>195.1</td>
<td>14.1</td>
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<tr>
<td></td>
<td>Low</td>
<td>16</td>
<td>7.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* F₁ × C3H backcross and F₂ 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).

Heat denaturation of P lysozyme

**F₁ hybrids and the cis-trans test:** Two types of P lysozyme can be distinguished in each F₁ hybrid by heat denaturation. When lysozyme from intestinal extracts was heated at 66.5°C for 60 min, the activity of C3H lysozyme decayed rapidly and after 20 min less than 10% of the original activity remained. In contrast, F₁ 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 F₁ 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 F₁ may be the result of (1) an intrinsic structural difference between lysozymes, (2) an effector molecule that stabilizes lysozyme in the F₁ or destabilizes it in C3H, or (3) an elevated level of the more heat-stable M lysozyme (HAMMER et al. 1987) in the F₁. The last alternative was discounted on the basis of quantitative protein electrophoresis. As shown in Figure 6, the majority of activity remaining in the F₁ 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 F₁ 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 F₁ 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 F₁ 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 F₁ 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 F₁ hybrid and C3H. Figure 8 is a kinetic analysis showing that 16 mice of the intermediate activity class (F₁ 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-
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°C (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>
<thead>
<tr>
<th>Mice</th>
<th>Initial lysozyme level</th>
<th>No. of mice</th>
<th>Mean±</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>domesticus</em> (C3H)</td>
<td>High</td>
<td>4</td>
<td>1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>F1 hybrid (C3H × casA)</td>
<td>Intermediate</td>
<td>4</td>
<td>27.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Backcross (F1 × C3H)</td>
<td>High</td>
<td>37</td>
<td>4.8</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>39</td>
<td>27.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* See Figure 7 for a study of heat denaturation as a function of time for purified P lysozyme from *domesticus* and F1 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 F1 mice using gel electrophoresis and an activity overlay.

Average percentage of initial activity remaining after heating for 45 min at 66.5°C.

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 F1 and C3H parents, suggest that the heat stability of P is determined by a single locus, presumably the structural locus for P.
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 \times 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...
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 and wild mice. Among inbred strains there are only two types, low P—M73 and high P—M68. These two types were also the most common among wild mice. Yet, low P among wild mice, suggesting that recombination has occurred between Lzp-r and Lzm-s. 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 F1 X 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 homozygous for the 73 form at the other locus.

The two-locus model was tested by typing the progeny of a cross between C3H and casA and the progeny of crosses between these F1 hybrids (C3H X 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

---

**TABLE 3**

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

<table>
<thead>
<tr>
<th>P level*</th>
<th>M mobility</th>
<th>No. of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>73</td>
<td>Inbred</td>
</tr>
<tr>
<td>Low</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>73/68</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>73/68</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>73/71</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>68/66</td>
<td></td>
</tr>
</tbody>
</table>

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

**TABLE 4**

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

<table>
<thead>
<tr>
<th>Parental types</th>
<th>Mice</th>
<th>73</th>
<th>73(68)</th>
<th>73/68</th>
<th>(73)68</th>
<th>68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outbred castaneus</td>
<td></td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inbred castaneus (casA)</td>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inbred domesticus (C3H)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Interspecific hybrid</td>
<td>F1 (C3H X casA)</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Backcross types</td>
<td>F1 X casA</td>
<td>5</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F1 X C3H</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>F1 X castaneus 73/68</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>26</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>F1 X castaneus 73/68*</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>73</th>
<th>73(68)</th>
<th>73/68</th>
<th>(73)68</th>
<th>68</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suggested</td>
<td>73-73</td>
<td>73-73</td>
<td>68-68</td>
<td>73-68</td>
<td>68-68</td>
</tr>
<tr>
<td>genotype</td>
<td>73-75</td>
<td>75-68</td>
<td>73-68</td>
<td>68-68</td>
<td>68-68</td>
</tr>
<tr>
<td>73 alleles</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>68 alleles</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
in Table 4. When the castaneus with equal amounts of 68 and 73 was crossed to the F1, 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 F1. 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 F1 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 ( Paige 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-r 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-r 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, \(Lzp^{-}\) would influence a 130-fold increase in cell proliferation relative to \(Lzp^{+}\). 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 influenced (PAIGEN 1979; LUSIS et al. 1983). In the case of \(\beta\)-glucuronidase, however, a regulator that is trans-acting and lies within the \(\beta\)-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, ElA, 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 regulatory allele, as has been observed for a regulatory gene, ElA, in adenovirus (KOVESDI, REICHEL and NEVINS 1986).

An alternative hypothesis is that high intestinal levels of \(P\) lysozyme have arisen independently on several 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 (GECHELLER, FAVERSHAM and COHN 1978), histidine deacetylase in the kidney (MARTIN et al. 1984), and \(\alpha\)-
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 (Navudu 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 a-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 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, Carlsson 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, Carlsson and White 1977; Wilson 1985).

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LITERATURE CITED


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