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Electrophoretic Variation in Low Molecular Weight Lens Crystallins from Inbred Strains of Rats

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Analysis of rat lens soluble proteins by analytical isoelectric focusing detected two inherited electrophoretic differences in low molecular weight (LM) crystallins from inbred strains of rats (Rattus norvegicus). *The polymorphic lens crystallins were shown to be similar to a genetically variant LM crystallin, LEN-1, previously described in mice* (Mus musculus) *and encoded on chromosome 1, at a locus linked to* Pep-3 *(dipeptidase). Linkage analysis demonstrated that the rat crystallin locus was loosely linked to* Pep-3 *at a recombination distance of 38 ± 4.5 U. These data suggest the conservation of a large chromosomal region during the evolution of Rodentia and support the hypothesis that the γ-crystallins are evolving more rapidly than α- or β-crystallins.*

KEY WORDS: electrophoretic variation; lens crystallin; rat.

INTRODUCTION

The bulk of the vertebrate lens consists of terminally differentiated lens fiber cells composed primarily of lens-specific proteins, the crystallins. Three distinct classes of crystallins, designated α, β, and γ, each with characteristic molecular weights and subunit structures, occur in lenses from mammals (Harding and Dilley, 1976; Bloemendal, 1977). The monomeric γ-crystallins belong to the low molecular weight (LM) lens crystallins and represent about

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40% of the total protein mass in mouse and rat eye lenses (Ocken *et al.*, 1977; Wada *et al.*, 1981). Several chromatographic and electrophoretic forms of γ-crystallins have been detected in bovine, murine, and rat lenses (Slingsby and Croft, 1978; Skow, 1982; Ramaekers *et al.*, 1982). So far at least five closely related γ-crystallins have been identified in the rat (Dodemont *et al.*, 1981), and *in vitro* translation studies indicate at least four putative γ-crystallins in mice (Shinohara *et al.*, 1982).

Members of each crystallin class can be considered the products of a gene family that probably originated by duplication of an ancestral gene (Jones *et al.*, 1980; Driessen *et al.*, 1981; Inana *et al.*, 1983). Different mammalian species show a great homology among the crystallins of a given class (Bloemendal and de Jong, 1979; de Jong, 1981), suggesting that the proteins have been highly conserved during evolution. This would be consistent with the paucity of genetic variation, expressed as electrophoretic variants, in lens crystallins within species (Day and Clayton, 1973). It has been suggested, however, that the γ-crystallins might show relatively more variation due to single or tandem duplications of mature γ-crystallin sequences (Moormann *et al.*, 1982) and that the rate of molecular evolution of γ-crystallins would be slightly faster than the rates of α- and β-crystallins (Tomarev *et al.*, 1984). Recently, a heritable electrophoretic polymorphism in a γ-crystallin was detected by isoelectric focusing of lens proteins from inbred strains of mice (*Mus musculus*) (Skow, 1982). The genetic locus was designated *Len-1* and mapped to mouse chromosome 1 between the locus for isocitrate dehydrogenase (*Idh-1*) and that for dipeptidase (*Pep-3*). In this communication we describe genetic variation, also detected by isoelectric focusing, in similar γ-crystallins in the lenses of inbred strains of rats (*Rattus norvegicus*). Linkage data suggest that the chromosomal segment containing the γ-crystallin and the dipeptidase loci has been conserved during the evolution of Rodentia.

MATERIALS AND METHODS

Source of Animals and Tissues

Animals were obtained from the research colony of the Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania, or from the Comparative Medicine Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. The rats were killed by CO_2 asphyxiation, and lenses and livers were removed and frozen at −80°C until analysis. Immediately prior to isoelectric focusing, lenses were homogenized in distilled H_2O (1 lens/0.5 ml) and the insoluble material was removed by centrifugation at 12,000*g* for 10 min. Soluble lens

proteins were examined from at least two adult (aged 3 to 6 months) animals of each of 24 inbred strains of rats. Linkage tests were conducted using backcross animals (aged 2 to 3 months) produced from the matings (KGH × ACP)F_1 × ACP and (KGH × ACP)F_1 × KGH.

Analytical Isoelectric Focusing

Isoelectric focusing, using a LKB Multiphor system in narrow (*p*H 6.5–8.5)- or broad (*p*H 3.5–10)-gradient polyacrylamide gels purchased precast from LKB, was conducted as previously described (Skow, 1982). After focusing was completed, the gels were immersed for 30 min in a fixative solution containing 0.14 M sulfosalicylic acid and 0.7 M trichloroacetic acid in 30% methanol. Gels were then rinsed briefly in a 25% ethanol:15% acetic acid solution and stained for 60 min in a 0.1% solution of Coomassie brilliant blue R-250 dissolved in the ethanol:acetic acid solution. Gels were destained in the solvent alone.

Linkage Test

Dipeptidase (PEP-3) phenotypes of the backcross progeny were determined in kidney homogenates. One kidney from each animal was homogenized in 2 vol of ice-cold H_2O, and the supernatant collected after centrifugation at 100,000*g* for 15 min. The supernatants were applied to cellulose acetate plates (Titan III, Helena Laboratories) which had been equilibrated in 50 mM Tris–glycine buffer, *p*H 8.6. Electrophoresis was conducted at 300 V (1.5 mA/plate) for 20 min in Tris–glycine buffer. Dipeptidase activity was visualized by pouring a 1% agar solution containing a histochemical stain (Harris and Hopkinson, 1976) over the surface of the plate and incubating at 37°C for 1 hr. A 2× staining solution contained 5 mg L-aminoacid oxidase, 2.5 mg horseradish peroxidase, 10 mg L-lysyl-leucine, 4 mg $MnCl_2$, and 5 mg *o*-dianisidine in 10 ml 0.2 M phosphate buffer, *p*H 7.6. The stain solution was mixed with an equal volume of 2% agar (60°C) and immediately poured over the cellulose acetate plates.

Gel Filtration of Rat and Mouse Lens Crystallins

F344/N rats (aged 3 to 4 months) or DBA/2J mice (aged 2 to 3 months) were killed by CO_2 asphyxiation, and lenses from 10 rats or 20 mice collected in 3 ml of ice-cold 50 mM NH_4HCO_3:3 mM β-mercaptoethanol, *p*H 7.1. The lenses were homogenized and centrifuged at 10,000*g* for 10 min. The supernatant (containing 200–300 mg protein) was loaded on a Sephadex G-75SF or G-200SF (Pharmacia) column (90 × 2.5 cm) at 4°C and eluted with 50 mM

NH_4HCO_3:3 mM β-mercaptoethanol, *p*H 7.1. The flow rate of the column was 25 ml/hr; 2-ml fractions were collected, and the absorbances were measured at 280 nm. The column was calibrated with the proteins bovine serum albumin (BSA), ovalbumin, carbonic anhydrase, α-lactalbumin, and myoglobin (Sigma). The void volume (V_0) was measured with blue dextran. The crystallin fractions were concentrated and used for electrophoretic and immunological studies.

Preparative Isoelectric Focusing of the Rat γ-Crystallins

Rat lens crystallin supernatant was prepared as for gel filtration, and the protein fractions were separated by isoelectric focusing in an Ultrodex gel (LKB). The gel fractions containing the crystallins were collected with a spatula, passed over a Sephadex G-10 (Pharmacia) column (10 × 2.5 cm), and eluted with 50 mM NH_4HCO_3:3 mM β-mercaptoethanol, *p*H 7.1, to remove ampholytes from the samples (Skow, 1982). Fractions of 1.5 ml were collected, and the proteins were identified by absorbance at 280 nm, analytical isoelectric focusing, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

The molecular weights and subunits of the isolated crystallin fractions were determined by SDS-PAGE, according to the method of Laemmli (1970), on 15% (w/v) acrylamide gels with 0.735 M Tris–HCl (*p*H 8.9) and 0.1% SDS. The gels were polymerized with 0.01% ammonium persulfate and 0.01% TEMED, and the electrode buffer was 0.2 M glycine, 25 mM Tris–HCl (*p*H 8), and 0.1% SDS. Proteins were dissolved in 10 μl of sample buffer (1.5 μg/μl) containing 2% SDS, 0.375 M Tris–HCl buffer (*p*H 8), and 5% β-mercaptoethanol and were boiled for 5 min before adding 2 μl of 0.1% bromophenol blue in 20% sucrose. Standard proteins were from the MW-SDS-70 Kit (Sigma). The gels were run for 5 hr at 30 mA, fixed, and stained with silver stain according to the manufacturer's procedure (Bio-Rad).

Rabbit Anti-Mouse Crystallin Polyclonal Antibodies

The five purified mouse lens crystallin fractions prepared by gel filtration on Sephadex G-75SF were dialyzed against phosphate-buffered 0.9% saline (PBS; *p*H 7.0) and the protein concentrations were adjusted to 200 μg/ml. One hundred micrograms protein in 0.5 ml PBS was emulsified with 0.5 ml Freund's incomplete adjuvant (Sigma) and injected subcutaneously in the dorsum of female New Zealand white rabbits (8 weeks old). Weekly booster

injections were given starting 1 month after the first injection, and the rabbits were sacrified 9 weeks after the first immunization. Serum was obtained from blood collected by cardiac puncture, and the IgG fraction precipitated in 18% Na_2SO_4. The IgG fractions were dialyzed against PBS for 48 hr at 4°C.

Ouchterlony Double-Diffusion Assay

The antisera and the IgG fractions were analyzed by the Ouchterlony double-diffusion assay (Ouchterlony, 1968) in 1% agarose gels in Tris-buffered 0.9% saline, *p*H 7.2 (Bio-Rad immunodiffusion tablets). The immunodiffusion plates were sealed and incubated at room temperature until precipitin lines developed (usually overnight).

Western Blotting

The purified crystallin proteins were isoelectrofocused on broad (*p*H 3.5–10)-gradient polyacrylamide gels (LKB) and electrophoretically transferred to nitrocellulose filters (BA85 Schleicher & Schuell) (Towbin *et al.*, 1979) in a Bio-Rad Trans-blot cell for 3 hr at 40 V, 0.3 A, as described by the manufacturer. The electrode buffer was 0.8% acetic acid. Filters were immersed in 4% BSA in TBS (10 mM Tris-base, 0.9% NaCl, *p*H 7.4) for 2 hr and kept at 4°C overnight. After a 30-min wash in TBS the filters were sealed in plastic bags and incubated with the first antibody (monospecific for one of the crystallin classes) for 2 hr at 37°C. The filters were washed for 30 min in TBS before peroxidase-conjugated goat anti-rabbit antibody (Cappel) was added, and the incubation was continued for 2 hr at room temperature with moderate shaking. The binding of the antibody to the proteins was detected with the *o*-dianisidine reaction.

RESULTS

Two electrophoretic phenotypes were observed for lens crystallins among 20 inbred strains of rats (Fig. 1). Both phenotypes showed variation only in crystallins with basic ($pI \geq 8.0$) isoelectric points, indicating that the polymorphism was in the LM crystallins, probably in the γ-crystallin class. In each strain examined, the electrophoretic differences were seen as a doublet of crystallin bands so that samples from strains with the phenotypes LEN-1A, LEN-2A demonstrated a pair of crystallins with an isoelectric point higher than that of the corresponding doublet of crystallins found in samples from strains designated LEN-1B, LEN-2B. The distribution of crystallin phenotypes among inbred strains of rats is given in Table I. Electrophoretic patterns of lens proteins from (KGH × ACP)F_1 hybrid rats were indistinguishable from

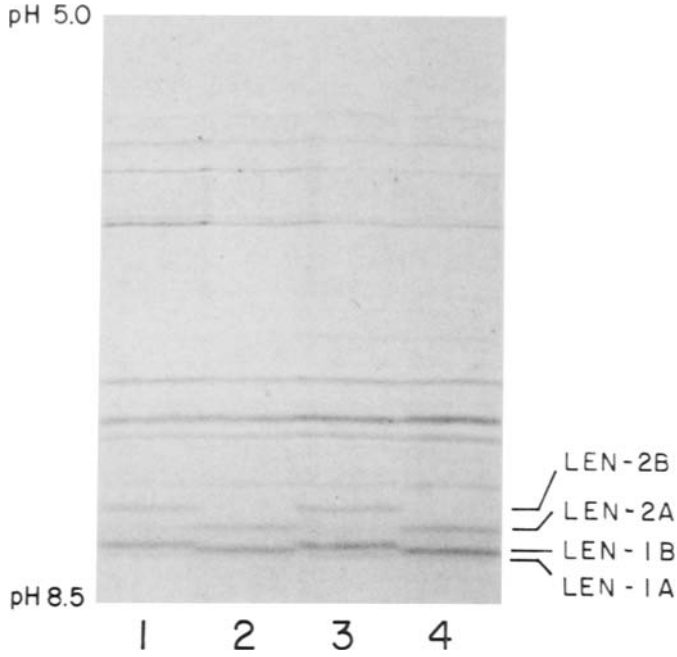

Fig. 1. Isoelectric focusing phenotypes observed in soluble lens proteins among inbred strains of rats. Genetically determined variation was observed among components of the LM crystallins with a $pI \geq 8.0$ and consisted of an electrophoretic shift in a pair of crystallins such that the doublet LEN-1A, LEN-2A (Lane 2, KGH; Lane 4, ALB) has a slightly higher pI than the corresponding pair, LEN-1B, LEN-2B (Lane 1, ACP; Lane 3, MNR).

Table I. Distribution of LEN-1 and LEN-2 Phenotypes Among 20 Inbred Strains of Rats[a]

Phenotype	Strains
LEN-1A, LEN-2A	ALB, BBD, BI, DA, KGH, LEW, NBR, WF, YO
LEN-1B, LEN-2B	ACP, AUG, BIL, BN, BUF, F344, KAGL, MNR, MR, PVG, WKA

[a]The LEN-1A, LEN-2A form migrates more cathodally than LEN-1B, LEN-2B. Strain designations have been described by Festing (1979) and by Gill *et al.* (1983).

those from mixtures of the parental samples, showing that the electrophoretic differences were heritable and the polymorphic crystallins exist in monomeric form. The electrophoretic properties of the rat crystallin variants are similar to the properties of a polymorphic γ-crystallin in mice designated LEN-1, which is encoded by a gene linked to the dipeptidase (*Pep-3*) locus (Skow, 1982). Genetic variants of dipeptidase have been described in rats (Womack and Cramer, 1980); therefore, a linkage test was conducted among backcross animals to determine whether *Len-1,2* and *Pep-3* were also linked in rats. Analysis of the transmission of the crystallin variants in 71 progeny from (KGH × ACP)F_1 × ACP or (KGH × ACP)F_1 × KGH backcross matings determined that the variation was inherited as a single autosomal Mendelian trait without dominance. Data from the linkage experiment are presented in Table II and indicate a loose linkage of rat *Len-1,2* and *Pep-3*, with a recombination percentage of 38 ± 4.5.

Fractionation of rat lens proteins by Sephadex G-200SF column chromatography produced five protein peaks corresponding to the α, β_H, β_{L1}, β_{L2}, and LM components reported by others (Bloemendal, 1981; Ocken *et al.*, 1977; Uchiumi *et al.*, 1983; Zigler and Sidbury, 1974). In contrast, the mouse crystallins were eluted as three fractions (Fig. 2A). Fractionation by Sephadex G-75SF column chromatography resolved both the rat and the mouse lens proteins into five components (Fig. 2B). The rat α- and β_H-crystallins eluted together in the void volume, β_L eluted as the second peak, and the LM crystallins were incompletely resolved into three components. Isoelectric focusing of peak fractions from the three components under the LM region revealed that the crystallins in each fraction were electrophoretically distinct, with basic isoelectric points. A single crystallin with a *p*I ~ 6.8 was observed in fraction III, similar to the crystallin in the corresponding fraction from mouse lens. Two crystallins were observed in fraction IV, with a *p*I of 8.2–8.3, identical to those crystallins demonstrating genetic variation.

Table II. Frequency of Gametic Genotypes Transmitted from the F_1 Parent to 71 Progeny from (KGH × ACP)F_1 × ACP or (KGH × ACP)F_1 × KGH Backcross Matings

	Genetic locus		
	Len-1,2	*Pep-3*	*N*
Parental	a	a	24
	b	b	20
Recombinant	a	b	14
	b	a	13
Total			71

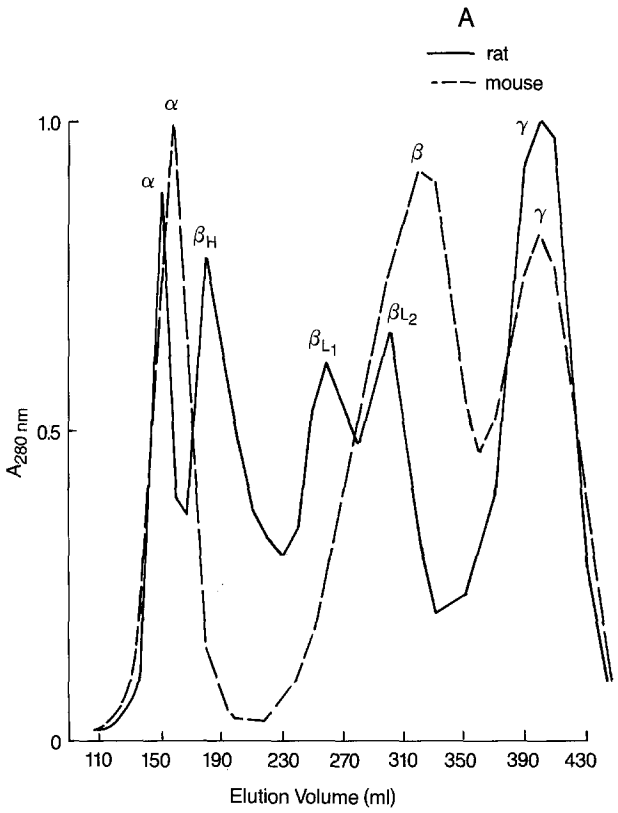

Fig. 2. (A) Elution profiles of rat (F344/N) and mouse (DBA/2J) soluble lens proteins after gel filtration on Sephadex G-200SF. The LM crystallins are eluted in the last fraction. (B) Elution profiles of rat (F344/N) and mouse (DBA/2J) soluble lens proteins after gel filtration on Sephadex G-75SF. The LM crystallins are eluted in fractions III, IV, and V.

Fraction V contained a crystallin with a *p*I ~ 7.5. Additional purification of γ-crystallins from peaks IV and V was obtained by preparative isoelectric focusing.

Estimation of molecular weights of the crystallins in Sephadex G-75SF fractions III, IV, and V was obtained by denaturing gel electrophoresis (SDS-PAGE). Crystallins within each fraction migrated as single bands with molecular weights consistently lower than calculated from the elution profiles after Sephadex G-75SF chromatography (Table III). The SDS-PAGE data are consistent with observations made by others (Liem-The and Hoenders, 1974; Coghlan and Augusteyn, 1977; Ocken *et al.*, 1977), while the gel filtration data may reflect the ellipsoid conformation of the γ-crystallin molecule (de Jong, 1981).

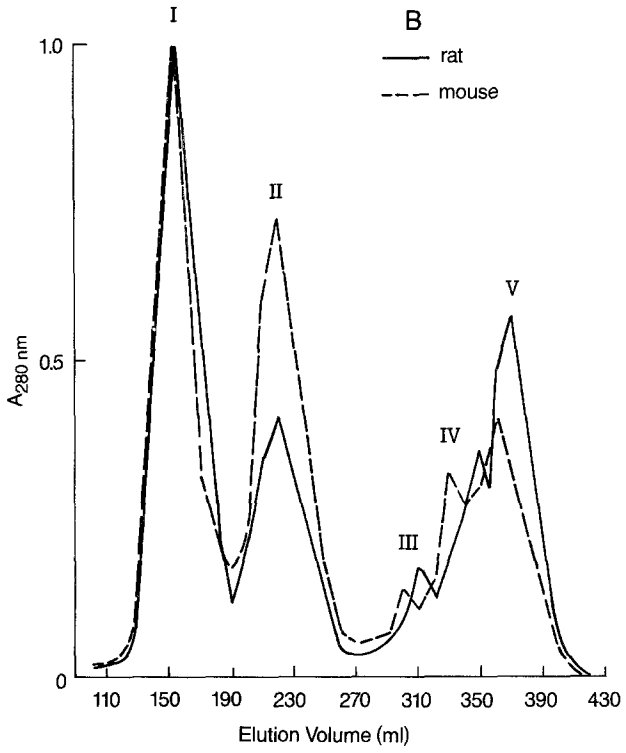

Fig. 2. Continued.

In the double-diffusion and Western immunoassays, there was evidence of common antigenic determinants on rat and mouse LM crystallins. The IgG fraction purified from antisera of rabbits immunized with mouse fraction III, IV, or V from Sephadex G-75SF reacted identically to rat or mouse crystallins of the appropriate type. IgG to mouse fraction III reacted only with rat or mouse fraction III. IgG to either mouse fraction IV or mouse fraction V showed essentially complete cross-reactivity with both fraction IV and fraction V from rat and mouse (Fig. 3).

DISCUSSION

Lens crystallins have been the subject of extensive biochemical and molecular studies (Bloemendal, 1981; de Jong, 1981), but little information has been obtained concerning the genetics of the crystallin genes. It is known from biochemical and molecular analyses that mammals have at least two α-crystallin genes and six or seven genes in each of the β- and γ-crystallin gene families (Piatigorsky, 1984), but the chromosomal locations of the

Table III. Molecular Weights (M_r) of LM Lens Crystallins of Rat (F/344N) or Mouse (DBA/2J), Determined by Gel Filtration on Sephadex G-75SF and with SDS-PAGE

	Sephadex G-75SF		
Fraction	V_e/V_0	M_r	SDS-PAGE, M_r
III	2.01	35,000	23,000
IV	2.23	30,000	22,000
V	2.36	26,000	21,000

crystallin genes remain unknown. This deficiency is due primarily to the apparent absence of genetic variation which could serve as genetic markers for the crystallin genes. Only minor electrophoretic differences were observed among the crystallins of representative mammalian species (Day, 1972; Zigler and Sidbury, 1974; Ocken *et al.*, 1977). A search for electrophoretic variation

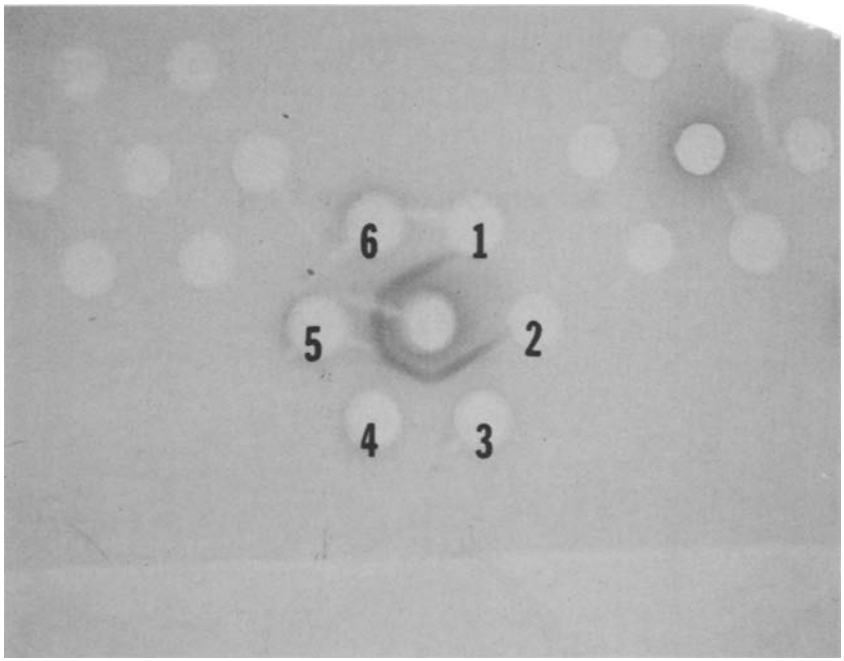

Fig. 3. Ouchterlony double-diffusion assay of rat (F344/N) LM crystallins separated by gel filtration on Sephadex G-75SF (fractions III–V). Lens crystallins in fractions IV (Wells 3 and 4) and V (Wells 5 and 6) reacted identically to antibody raised against mouse fraction IV crystallins, while no reaction could be detected with crystallins in fractions III (Wells 1 and 2).

among 14 inbred strains of mice and four inbred strains of rats was fruitless (Day and Clayton, 1973).

The scarcity of electrophoretic variation among the crystallins is due, in part, to their conservative evolution but also to the fact that these proteins are poorly resolved by conventional electrophoresis. Two heritable polymorphisms in the LM crystallins of mice were detected by analytical isoelectric focusing but not by zone electrophoresis (Skow, 1982; Skow *et al.*, 1985). This report describing heritable electrophoretic variants in rat LM crystallins documents another instance in which crystallin polymorphism detected by isoelectric focusing was apparently not detectable by conventional electrophoresis (Day and Clayton, 1973).

The concordant inheritance of the two pairs of electrophoretic variants of the LM crystallins in rats, as reported in this study, is consistent with the known organization of the γ-crystallin gene family. Five γ-crystallin genes have been identified, in head-to-tail array, within 50 kb of DNA isolated from a rat cosmid library (Schoenmakers *et al.*, 1984), thereby confirming that the γ-crystallins are inherited as a tightly linked multigene complex or haplotype. Genetic polymorphisms occurring among the γ-crystallin genes would demonstrate essentially complete linkage disequilibrium. An alternative hypothesis for the failure to separate the two pairs of electrophoretic variants by genetic recombination is that only one genetic polymorphism exists and that the additional variation arises as post-translational modifications of the variant proteins. At present we cannot exclude this possibility, although we are confident that the observed variation does not arise as a technical artifact since similar electrophoretic patterns are observed from fresh and stored samples. Age-related post-translational modifications of crystallins can produce electrophoretic variants (Hoenders and Bloemendal, 1981) but cannot account for the polymorphisms reported here since the variation was observed in the linkage experiments which utilized sets of littermate animals analyzed at identical ages.

Several lines of evidence support our conclusion that the polymorphic crystallins observed in rats are homologous to the crystallin encoded by *Len-1* in mice. In both species, the LM crystallins are fractionated into three components by Sephadex G-75SF column chromatography, and the variant proteins are located in the middle component. Size estimates calculated from M_r values for column chromatography or denaturing gel electrophoresis were similar for both rat and mouse LEN-1 crystallins. Most importantly, antisera raised against mouse LEN-1 reacted specifically with purified rat LEN-1 and other LM crystallins in Sephadex G-75SF fraction V but not with an LM crystallin found in fraction III. Previous studies in mice have shown that the predominant crystallin in fraction III has an amino acid composition more similar to that of the β-crystallins than to that of the crystallins in fractions IV and V (Skow *et al.*, 1985).

The estimated recombination percentage of 38 ± 4.5 between *Len-1,2* and *Pep-3* in rats is almost twice the value reported for the *Len-1–Pep-3* interval in mice (Skow, 1982) and may reflect divergence by chromosomal rearrangement, an apparently common event in the evolution of the family Muridae (Viegas-Pequignot *et al.*, 1983). Conservation of linkage with variable recombination frequencies has also been reported for the *Gpi-1–Hbb* pair in deer mice, mice, and rats. A recombination frequency of 16% in *Peromyscus* (Snyder, 1980) is lower than the 25–30% in both mice and rats, seemingly in support of the internal rearrangement hypothesis of Viegas-Pequinot *et al.* (1983). There are presently relatively few gene markers in rats, and it is not yet possible to determine whether the order of other homologous loci in this chromosome region has been rearranged relative to that in the mouse. It should also be noted that recombination frequencies often vary greatly along the same chromosomal segments in different strains and between sexes of the same strains (Skow, 1981). Therefore, the differences in recombination frequencies reported here for the *Len-1,2–Pep-3* interval in rats and mice do not necessarily indicate a chromosomal rearrangement between these species.

The conservation of *Len-1,2–Pep-3* linkage between rats and mice is consistent with the high level of conservation observed for other linkage groups in these species. Of the nine known linkage groups in rats, five are conserved between rats and mice, apparently in identical gene order (see O'Brien *et al.*, 1985).

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