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Multiple γ-crystallins of the mouse lens: Fractionation of mRNAs by cDNA cloning

(γ-crystallin mRNAs/amino acid sequences)

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ABSTRACT **cDNAs made from polyadenylylated RNAs of the mouse lens were cloned by the G·C tailing procedure in the bacterial plasmid pBR322. Four recombinant DNAs containing γ-crystallin sequences were identified by hybrid selection and translation. Sequence analysis of the *in vivo*-labeled γ-crystallin polypeptides that cofocused isoelectrically with the hybrid-selected translation products established that the four cloned cDNAs were derived from mRNAs encoding γ-crystallin polypeptides with similar NH_2 termini. The cDNA clones had different restriction maps and could discriminate among the different γ-crystallin mRNAs under stringent hybridization conditions. Under relaxed hybridization conditions, the cDNA clones cross-hybridized with all γ-crystallin mRNAs, and even slightly with β-crystallin mRNAs, as judged by *in vitro* translation. RNA blot hybridization showed that the mouse lens γ-crystallin mRNAs are 840 ± 100 nucleotides long. These data indicate that there are at least four similar γ-crystallin mRNAs and suggest (but do not establish) the existence of a closely related family of γ-crystallin genes.**

The crystallins are a group of structural proteins that constitute ≈90% of the soluble protein of the vertebrate lens (1, 2). There are four immunologically distinct classes of crystallins called α-, β-, γ-, and δ-crystallin. The mammalian lens lacks δ-crystallin, which is confined to birds and reptiles (3, 4). Each crystallin class is composed of a family of polypeptides that have related primary structures. The crystallin polypeptides are highly conserved evolutionarily (5) and are differentially synthesized during lens development (6).

The existence of multiple related polypeptides in each crystallin class has made it difficult to know which polypeptide is a primary gene product and which is posttranslationally derived from a precursor polypeptide. Numerous posttranslational changes, such as deamidation, cleavage, and oxidation, occur among the crystallins during maturation and aging of the lens (for review, see refs. 1, 2, and 7). One obvious approach to understanding the basis for the multiplicity of the crystallin polypeptides is to identify and fractionate their various mRNAs. This is most efficiently carried out by cloning the crystallin cDNAs. The identified crystallin cDNA clones can then be used for investigations on the crystallin genes.

This study concerns the γ-crystallins. These are the smallest of the crystallin polypeptides (M_r 18,000–20,000) and have similar immunological and structural properties (1, 5, 8–10). Protein sequence data indicate that there are at least three different genes for γ-crystallins in the bovine lens (11, 12). Here we show by analysis of cDNA clones and amino acid sequence data that four major γ-crystallin polypeptides of the 5- to 10-day-old mouse lens have similar but different mRNAs.

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MATERIALS AND METHODS

Materials. Avian myeloblastosis virus RNA-dependent DNA nucleotidyltransferase (reverse transcriptase) was obtained from J. W. Beard. *Escherichia coli* DNA polymerase I and bovine pancreatic DNase I were obtained from Boehringer Mannheim. Oligo(dT)-cellulose (type 7) was obtained from P. L. Biochemicals. Terminal deoxyribonucleotidyltransferase was obtained from Collaborative Research (Waltham, MA). dATP, dCTP, dGTP, TTP, nuclease S1, and antibiotics (ampillicin, tetracycline, and chloramphenicol) were obtained from Sigma. Restriction endonucleases and the bacterial plasmid pBR322 were purchased from Bethesda Research Laboratories and New England BioLabs. All radioactive isotopes and the rabbit reticulocyte cell-free lysate kit were purchased from New England Nuclear. An isoelectric focusing polyacrylamide gel kit (pH 3.5–9.5) was obtained from LKB and the nitrocellulose filters (0.45-μm pore size) were obtained from Millipore.

Construction of cDNA Clones. Polyadenylylated RNA (300 μg) was purified from 1,000 5- to 10-day-old mouse lenses (NIH general purpose mice) by oligo(dT)-cellulose chromatography as described (13); 100 μg of the RNA was reverse transcribed, made double-stranded with *E. coli* DNA polymerase I, and tailed with deoxycytidine at the 3′ ends by using terminal transferase and 1 μCi of [^{32}P]dCTP (1 Ci = 3.7 × 10^{10} becquerels) as a marker (14). The tailed double-stranded cDNAs were hybridized to pBR322 DNA cut by *Pst* I (in the ampicillin-resistant site), tailed with deoxyguanosine at the 3′ ends with terminal transferase and used to transform *E. coli* LE392. Transformed colonies were screened by resistance to tetracycline and sensitivity to ampicillin and by the colony-hybridization procedure (15), using [^{32}P]cDNAs derived from the polyadenylylated RNAs of the mouse lens. The plasmids were amplified in the presence of chloramphenicol at 100 μg/ml, phenol extracted, and purified by cesium chloride density-gradient centrifugation.

Restriction Endonuclease Analysis of the Cloned cDNAs. The purified plasmids containing the cDNAs were digested with *Pst* I, *Hae* III, *Hin*fI, *Bam*HI, *Bgl* I, or *Eco*RI under conditions suggested by the manufacturers. The digested DNAs were examined by electrophoresis on horizontal 1% or 1.5% agarose gels (16) or on a vertical 6% polyacrylamide gel (17).

Blot Hybridization of RNA. Total lens RNA was subjected to electrophoresis on 0.8% agarose/2.2 M formaldehyde gel (18) and blotted onto a nitrocellulose filter (16). After baking at 80°C for 2 hr at reduced pressure, the filters were soaked in hybridization mixture for 12 hr and then hybridized overnight at 45°C to nick-translated (19) probes labeled with [^{32}P]dCTP (2,000–3,000 Ci/mmol). The hybridization mixture (20 ml) was 4-fold Denhardt's solution (20)/90 mM Na citrate/0.9 M NaCl/

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50% deionized formamide/0.1% $NaDodSO_4$. The blot was washed twice at room temperature with 1.5 mM Na citrate/15 mM NaCl/0.1% $NaDodSO_4$/1 mM EDTA and twice with 10 mM Tris·HCl, pH 7.4/1 mM EDTA and autoradiographed with Kodak XAR5 x-ray film.

Hybridization Selection and Translation of mRNA. The recombinant plasmids were used for hybridization to total polyadenylylated RNA from the 5- to 10-day-old mouse lens described elsewhere (21), except that the ionic strength of the hybridization reaction mixtures and subsequent washings of the filters were varied as given below. All hybridization reactions were carried out in 65% deionized formamide/20 mM Pipes, pH 6.4/0.1% $NaDodSO_4$ containing yeast tRNA (0.1 mg/ml; extracted three times with phenol) at 50°C for 2 hr. Under low-stringency conditions, the hybridization mixture was adjusted to 60 mM Na citrate/0.6 M NaCl; under medium-stringency conditions, the mixture was adjusted to 15 mM Na citrate/0.15 M NaCl; and under high-stringency conditions, the mixture was adjusted to 1.5 mM Na citrate/15 mM NaCl. All filters were washed twice for 20 min each with 10 mM Tris·HCl, pH 7.4/0.1% $NaDodSO_4$/1 mM EDTA and twice for 20 min each with 10 mM Tris·HCl, pH 7.4/1 mM EDTA with the following modifications. Under low-stringency conditions, all washes were at room temperature; the first two washes contained 30 mM Na citrate/0.3 M NaCl and the second two washes contained 15 mM Na citrate/0.15 M NaCl. Under medium-stringency conditions, all washes were at room temperature; the first two washes contained 7.5 mM Na citrate/0.075 M NaCl and the second two washes were without additional salt. Under high-stringency conditions, the first two washes were at room temperature and with 1.5 mM Na citrate/0.15 M NaCl and the second two washes were at 45°C without added salt. The hybridized RNAs were eluted from the filters with 100 μl of sterile water containing 50 μg of yeast tRNA at 100°C for 1 min, extracted with phenol/chloroform/isoamyl alcohol (50:50:1), ethanol precipitated, and translated in a rabbit reticulocyte lysate containing 50 μCi of [^{35}S]methionine. The *in vitro*-synthesized products were subjected to isoelectric focusing at pH 3.5–9.5 in a polyacrylamide gel.

Amino Acid Sequence Analysis. Ten mouse lenses (5 to 10 days old) were incubated in 0.5–1.0 ml of modified medium 199 (21) containing 0.5–1 mCi of ^{35}S (500–1,000 mCi/mmol)- or ^{3}H (5–60 mCi/mmol)-labeled amino acids in plastic tissue culture dishes (Falcon Plastics; 35 mm × 10 mm) for 24 hr at 37°C in 5% CO_2/95% air. After culture, the lenses were homogenized in 10 mM 2-mercaptoethanol and centrifuged at 1,000 × *g* for 10 min at 4°C, and the supernatant fraction was subjected to isoelectric focusing on a polyacrylamide gel at pH 3.5–9.5. The γ-crystallin bands were stained with Coomassie brilliant blue R-250 and then electrophoretically extracted from the gel. The purified proteins were precipitated with 90% acetone at −20°C overnight, dissolved in water, and reprecipitated with acetone. Automated Edman degradations were carried out with an updated Beckman 890B sequencer, using 0.1 M Quadrol (program 060275). Horse heart myoglobin (1.4 mg) and ovalbumin (0.5 mg) were added as carriers. For samples containing multiple ^{3}H-labeled amino acids, radiolabeled phenylthiohydantoin amino acid derivatives were separated by HPLC (23) in the presence of unlabeled standard phenylthiohydantoin amino acid derivatives (3 nmol). Radioactivities were measured with an Analytic 81 liquid scintillation counter at efficiencies of 85% (^{35}S) and 49% (^{3}H).

RESULTS

Identification of Four Different Mouse γ-Crystallin cDNA Clones. Previous studies have shown that γ-crystallin constitutes 48% of the soluble protein of the mouse lens at 10 days after birth (24). Accordingly, we estimated that ≈50% of the protein synthesized *in vitro* from polyadenylylated RNA of the 5- to 10-day-old mouse lens is γ-crystallin, as judged by cell-free translation in a reticulocyte lysate followed by isoelectric focusing in a polyacrylamide gel (data not shown). The γ-crystallins synthesized in the reticulocyte lysate comigrated with authentic γ-crystallin polypeptides from the lens by isoelectric focusing and by $NaDodSO_4$/polyacrylamide gel electrophoresis.

After cloning the cDNAs synthesized from the mouse lens mRNAs, four plasmids containing γ-crystallin sequences were identified by a positive hybrid-selection translation test (21). The mRNAs hybrid selected by the individual clones were translated in a rabbit reticulocyte lysate supplemented with [^{35}S]methionine, and the polypeptides synthesized were characterized by isoelectric focusing in a polyacrylamide gel followed by autoradiography. The four clones we analyzed were called pMγ_1Cr1, pMγ_2Cr1, pMγ_3Cr1, and pMγ_4Cr1.

The results showed that the four γ-crystallin cDNA clones are similar but not identical (Fig. 1). The different β- and γ-crystallin polypeptides synthesized by total lens polyadenylylated RNA added to the reticulocyte lysate polypeptides are shown in lane A. α-Crystallins were also synthesized by the total mRNAs but are not shown due to smearing at the top of the gel by reticulocyte proteins and by precipitation of the α-crystallin polypeptides at their isoelectric point (25). Lanes B, C, and D show the proteins synthesized *in vitro* by the mRNAs selected by pMγ_1Cr1 at three different stringencies of hybridization. At the lowest stringency, this clone selected all the γ-crystallin mRNAs (lane B). A trace of β-crystallin mRNAs hybridized with pMγ_1Cr1 under these conditions, which is consistent with the 25% homology between amino acid sequences of the βBp- and γII-crystallin polypeptides of the calf lens (26). At an intermediate hybridization stringency, pMγ_1Cr1 did not hybridize to β-crystallin mRNAs and preferentially selected the mRNA encoding γ_1-crystallin (lane C). At the highest hybridization stringency, pMγ_1Cr1 hybridized almost exclusively to the mRNA for γ_1-crystallin (lane D). Similar results were obtained with the other three cDNA clones: pMγ_2Cr1, pMγ_3Cr1, and pMγ_4Cr1 hybridized selectively to the mRNAs for γ_2- (lane E), γ_3- (lane F), and γ_4- (lane G) crystallins, respectively, under our most stringent hybridization conditions.

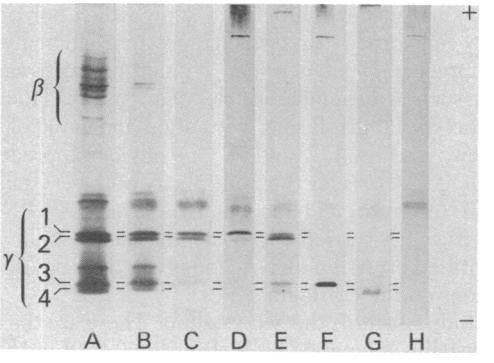

FIG. 1. Autoradiograms of isoelectric focusing gels (pH 3.5–9.5) of *in vitro*-synthesized products of 5- to 10-day-old mouse lens γ-crystallin mRNAs purified by hybrid selection to pMγ_1Cr1, pMγ_2Cr1, pMγ_3Cr1, and pMγ_4Cr1. Polypeptides were synthesized by total mRNAs (lane A); by mRNAs selected by low-, medium-, and high-stringency hybridization to pMγ_1Cr1 (lanes B–D); by mRNAs selected by high-stringency hybridization to pMγ_2Cr1 (lane E); by mRNAs selected by high-stringency hybridization to pMγ_3Cr1 (lane F); by mRNAs selected by high-stringency hybridization to pMγ_4Cr1 (lane G); and by mRNAs selected by high stringency to a nitrocellulose filter without added DNA (lane H).

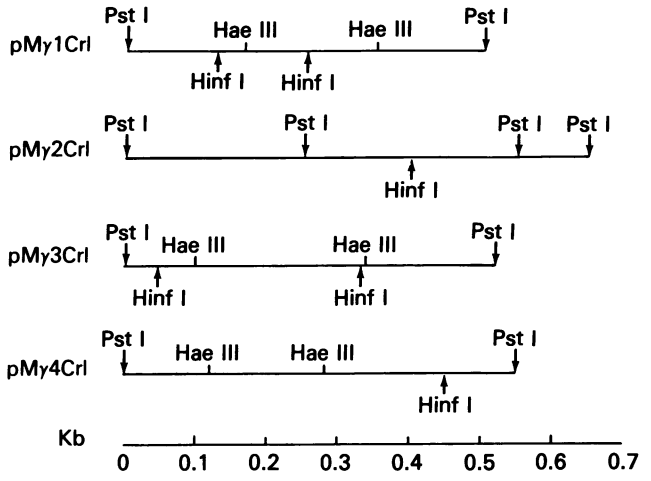

FIG. 2. Partial restriction maps of four cloned γ-crystallin cDNAs. Sites that produce DNA fragments of <50 base pairs would not have been detected in these tests. The exact order of sites yielding very small DNA fragments may be incorrect. pMγ_2Crl has at least five *Hae* III sites, yielding a series of DNA fragments 50–150 base pairs long.

Although we do not yet know the orientation of the cloned cDNAs in the plasmids, treatment with restriction endonucleases demonstrated that the four γ-crystallin cDNA clones have different sequences (Fig. 2). All four inserted cDNAs can be excised from pBR322 with *Pst* I; however, only pMγ_2Crl has internal *Pst* I sites. pMγ_2Crl has five *Hae* III sites that are not shown in Fig. 2 because of the difficulty in orienting this relatively large number of sites within the cloned cDNA. The other three clones have two *Hae* III sites each. All four clones have one or two *Hinf*I sites, but in different relative positions. None of the clones have sites for *Bam*HI, *Bgl* I, or *Eco*RI.

RNA Blot Hybridization. We have examined the mouse lens γ-crystallin mRNAs by blot hybridization using nick-translated pMγ_1Crl and pMγ_3Crl as probes to determine the sizes of the γ-crystallin mRNAs. A broad RNA band of 840 ± 100 nucleotides hybridized to the labeled pMγ_1Crl (Fig. 3). A similar result was obtained with pMγ_1Cr3 (data not shown). Since these experiments used low-stringency hybridization conditions and medium-stringency washing conditions, some cross-hybridization of the cDNA probes to the different γ-crystallin mRNAs will have occurred. These results indicate that the smallest of our cloned cDNAs (pMγ_1Crl, ≈500 base pairs) contains ≈60% of the mRNA sequences and that the largest cloned cDNA (pMγ_2Crl, ≈650 base pairs) contains ≈77% of the mRNA sequences. Since ≈500 nucleotides are required to code for a γ-crystallin polypeptide (11), each of the cloned cDNAs must contain all of the putative 3′ untranslated sequences and some of the coding sequences of the mRNA.

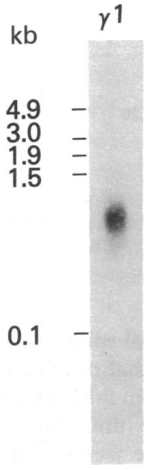

FIG. 3. Blot hybridization of mouse lens γ-crystallin mRNAs. Twenty micrograms of total RNA from 5- to 10-day-old mouse lens was subjected to electrophoresis in a denaturing agarose gel, transferred to a nitrocellulose filter, and hybridized to nick-translated pMγ_1Crl under low-stringency conditions; the blot was washed under medium-stringency conditions. The RNA markers were 28S [4.9 kilobases (kb)], 18S (1.9 kb), 23S (3.0 kb), and 16S (1.5 kb) ribosomal RNAs from the mouse lens and from *E. coli* and 4S (0.1 kb) RNA from yeast.

Partial Amino Acid Sequences of the NH_2 Termini of the Four γ-Crystallins. To confirm that the four translation products being examined are γ-crystallins and to test their similarity to each other, partial amino acid sequences were determined for the corresponding individual polypeptides isolated from the lens. The four γ-crystallin bands labeled with one or two amino acids (glycine, lysine, phenylalanine, tyrosine, cysteine, serine, or leucine) were electrophoretically eluted from a polyacrylamide gel after isoelectric focusing and then subjected to automated Edman degradation.

In almost every case, Edman degradation of the radiolabeled polypeptides indicated that they were homogenous preparations; i.e., the yield of radiolabeled amino acids at successive steps was equal to the known repetitive yield obtainable with the instrument (91–93%). This is shown by the degradations of the four [^{3}H]serine-labeled γ-crystallin polypeptides (Fig. 4 *Left*). However, in the degradations of [^{35}S]cysteine-labeled γ_3- and γ_4-crystallins (Fig. 4 *Right*), the high yields of radioactivity at positions 15 and 22 indicated that there was more than one polypeptide in these bands. Indeed for γ_3-crystallin, both tyrosine and phenylalanine were detected at position 6 and both glycine and serine were at position 10 (Figs. 4 *Left* and 5). Since the remainder of the sequence data for the γ_3- and γ_4-crystallin bands indicated homogeneity, it appears that the two (or more) polypeptides that must be present in these bands are extremely similar in amino acid sequence. At present, we cannot distinguish which polypeptide in these bands is encoded in pMγ_3Crl and pMγ_4Crl.

The summary of the partial amino acid sequences of the polypeptides in the four mouse γ-crystallin bands is shown in Fig. 5; for comparison, the sequence of γII-crystallin of the bovine lens (11) is also given. The four mouse and one bovine γ-crystallins are clearly similar at the positions examined. The differences found among the mouse γ-crystallin polypeptides are as follows: at position 15, γ_1 and γ_2 lack cysteine, which is present in γ_3 and γ_4; at position 20, γ_1 and γ_2 lack serine, which is present in γ_3 and γ_4; at position 22, γ_1 and γ_2 lack cysteine, which is present in γ_3 and γ_4; at position 23, γ_1 and γ_2 contain serine, which is absent from γ_3 and γ_4. No differences were found between γ_1 and γ_2 or between γ_3 and γ_4. Thus, γ_1 and γ_2 appear to be more closely related to each other than to γ_3 and γ_4.

DISCUSSION

Our results show that at least four of the major γ-crystallin polypeptides of the mouse lens are encoded by similar but distinct mRNAs. A previous study using cloned rat lens cDNAs also indicated cross-hybridization among the γ-crystallin mRNAs by a hybrid-selection translation test (27). In view of the similarity of the primary structures of the γ-crystallin polypeptides (8–10, 12), it is possible that differences in the putative 3′ untranslated region of the mDNAs contribute to the ability of the mouse γ-crystallin cDNA clones to select their complementary mRNA

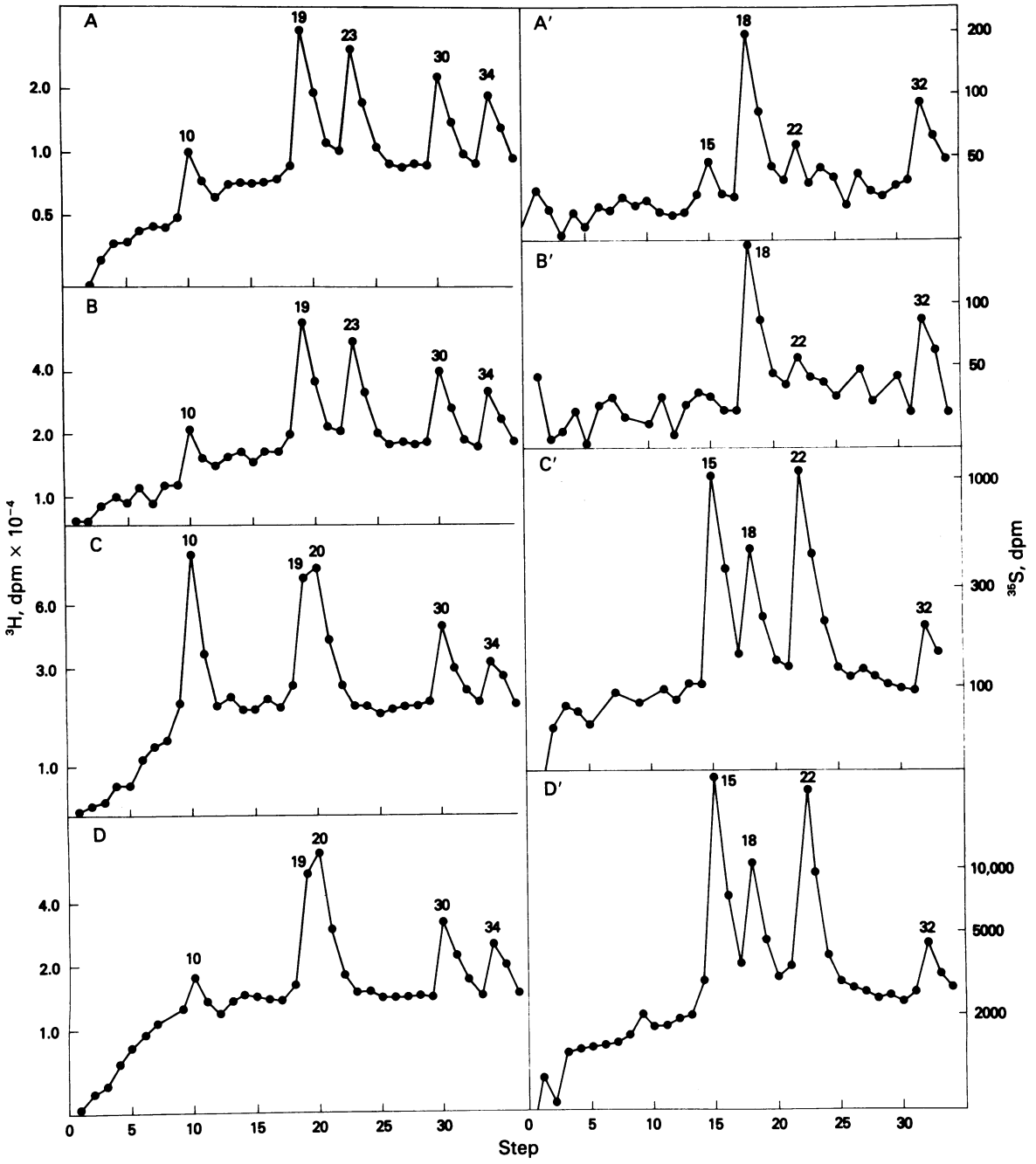

FIG. 4. Amino acid sequence analyses of mouse γ-crystallins labeled with [^{3}H]serine (*Left*) or [^{35}S]cysteine (*Right*). (*A* and *A'*) γ_1-Crystallin. (*B* and *B'*) γ_2-Crystallin. (*C* and *C'*) γ_3-Crystallin. (*D* and *D'*) γ_4-Crystallin. The radioactivity in the thiazolinone obtained at each step of the Edman degradation is presented as a semilogarithmic plot. The radioactivity at position 10 in [^{3}H]serine-labeled γ_1, γ_2, and γ_4 is probably due to biosynthetic conversion to glycine.

under stringent conditions for mRNA·cDNA hybridization. Differences in the 3′ translated region of the γ-crystallin mRNAs (included in our clones) must also be present since studies on the bovine (28, 29) and haddock (30) lens have shown that there is heterogeneity at the COOH termini of the γ-crystallin polypeptides. Moreover, amino acid sequence data on bovine lens γ-crystallins reveal that there are also differences throughout these polypeptide chains (12). We have shown in this study that there are differences in the NH_2-terminal region of the mouse γ-crystallin polypeptides as well. Thus, it is likely that the γ-crystallins form a family of closely related genes that can now be examined directly by use of the cDNA probes. It is also possible that multiple γ-crystallin mRNAs are generated from a few genes by differential splicing.

Our data, as well as that of others, indicate that there are more than four γ-crystallin mRNAs. *In vitro* translation of total mRNAs from the mouse (this study) and rat (25) lens showed more than seven putative γ-crystallin polypeptides that are resolved by isoelectric focusing in a polyacrylamide gel. In addition, the present amino acid sequence data suggest that some of these γ-crystallin bands contain more than one γ-crystallin polypeptide with different primary structures (and thus different mRNAs). Two-dimensional polyacrylamide gel electrophoresis of *in vitro* translation products of total rat lens mRNAs shows the presence of at least five γ-crystallin polypeptides (27). Earlier experiments on accumulated proteins in the lens have

	1				5					10					15					20				25					30				
MOUSE γ1	GLY	LYS	–	–	PHE	TYR	–	–	–	(GLY)	PHE	–	(GLY)	–	–	TYR	–	CYS	SER	–	–	–	SER	–	*	–	–	TYR	PHE	SER	–	CYS	–	SER
MOUSE γ2	GLY	LYS	–	–	PHE	TYR	–	–	–	GLY	PHE	–	GLY	–	–	TYR	–	CYS	SER	–	–	–	SER	–	LEU	–	–	TYR	PHE	SER	–	CYS	–	SER
MOUSE γ3	GLY	LYS	–	–	PHE	TYR/PHE	–	–	–	GLY/SER	PHE	–	GLY	–	CYS	TYR	–	CYS	SER	SER	–	CYS	–	–	LEU	–	–	TYR	PHE	SER	–	CYS	–	SER
MOUSE γ4	GLY	LYS	–	–	PHE	TYR	–	–	–	GLY	PHE	–	GLY	–	CYS	TYR	–	CYS	SER	SER	–	CYS	–	–	LEU	–	–	TYR	PHE	SER	–	CYS	–	SER
BOVINE II	GLY	LYS	ILE	THR	PHE	TYR	GLU	ASP	ARG	GLY	PHE	GLN	GLY	HIS	CYS	TYR	GLN	CYS	SER	SER	ASN	ASN	CYS	LEU	GLN	PRO	PRO	TYR	PHE	SER	ARG	CYS	ASN	SER

FIG. 5. Partial amino acid sequences of four authentic γ-crystallin polypeptides purified by isoelectric focusing. –, Positions that do not have any of the seven labeled amino acids used (glycine, lysine, phenylalanine, tyrosine, leucine, cysteine, and serine); *, leucine was not determined in this polypeptide. Homologies are boxed with dark lines. In γ_3-crystallin, two amino acids were found at positions 6 and 10, indicating two polypeptides in this band. The assignment of glycine-10 and -13 in γ_1-crystallin is tentative.

resolved 5–16 γ-crystallin polypeptides in different species (see ref. 5). In the mouse, isotachophoresis experiments suggest that there may be as many as 16 γ-crystallin polypeptides (31).

In addition to being useful for investigations on the γ-crystallin genes, we anticipate that the ability of the γ-crystallin cDNAs to discriminate among the γ-crystallin mRNAs will be useful for studies on lens development and maturation. A number of reports have indicated differential synthesis of the γ-crystallin polypeptides in the lens (32–35) and this may now be examined at the level of mRNAs.

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