Alternative Splicing of αA-Crystallin RNA

STRUCTURAL AND QUANTITATIVE ANALYSES OF THE mRNAs FOR THE αAα- AND αAαα-CRYS TALLIN POLYPEPTIDES

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(Received for publication, July 8, 1983)

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The eye lens contains a structural protein (α-crystallin) composed of two homologous primary gene products, αA2 and αB2. In certain rodents, there is another α-crystallin polypeptide, αAαα which is identical with αA2 except that it contains an additional peptide between residues 63 and 64 of the αA2 chain. The α-crystallin gene encodes the αAαα peptide in a separate 69-base pair exon, suggesting that the αA2 and αAαα mRNAs are derived by alternative RNA splicing. In the present study, we report the isolation of a cloned cDNA (pMaAααCr1) from a cDNA library constructed in the bacterial plasmid pBR322. The nucleotide sequence of pMaAααCr1 in the region of the insert peptide provides compelling evidence that the αAαα mRNA is derived from the same gene as the αA2 mRNA. S1 nuclease protection experiments, using a DNA fragment from pMaAααCr1, showed that the alternative splicing gives 5 to 10 times more αA2 than αAαα mRNA. This ratio is comparable to that of the respective polypeptides in the lens. We did not detect an age-related or a differentiation-related difference in the ratio of the αA2 to the αAαα mRNA or their polypeptides. These results indicate that when the αAα-crystallin gene is expressed in the lens, it splices the RNA sequences from the insert exon into functional mRNA 10 to 20% of the time.

Crystallins are the predominant soluble proteins in the lens of the vertebrate eye (1, 2). There are four main classes of immunologically distinct crystallins (α, β, γ, and δ). The α-crystallin of most animals is comprised of two primary gene products, αA and αB, which share 57% amino acid sequence homology in their polypeptide chains (3). The lens fiber cells synthesize relatively more of the αA2 polypeptide than do the lens epithelial cells, indicating differential regulation of the αA- and αB-crystallin genes during cellular differentiation (4, 5). The lenses of the rodent families Muridae (mouse, rat) and Cricetinae (hamster, gerbil) contain an additional αA-crystallin polypeptide, αAαα (6, 7). In rats, this polypeptide is identical with the αA2 chain except that it contains 22 amino acids inserted between residues 63 and 64 (6). By determining the nucleotide sequence of part of the murine αA-crystallin gene (8), we have shown that an intron separates codons 63 and 64 of the coding sequence for the αA2 polypeptide. This intron contains a 69-bp′ sequence encoding a 23-amino acid peptide homologous to the insert peptide of rat αAαα-polypeptide at 19 of 23 positions. Since only one αA-crystallin gene could be detected in the mouse genome by Southern blot analysis, it appeared that the mRNAs for both the αA2 and the αAαα-polypeptides are derived from the same αA-crystallin gene by alternative splicing of the RNA transcript.

In this study, we report the isolation of a cDNA clone, pMaAααCr1, for the mRNA encoding the αAαα-polypeptide. Nucleotide sequence analysis provides direct evidence that this mRNA is derived from the αA-crystallin gene in accordance with the proposal of alternative RNA processing. Previous studies have shown that the purified 14 S mRNA from the rat lens directs in vitro synthesis of 20 times more αA2 than αAαα polypeptide (9), suggesting that there are 20 times more αA2 mRNA than αAαα mRNA in the lens. It is possible, however, that the αA2 and αAαα mRNAs translate at different efficiencies. Synthesis of the two β-crystallin polypeptides demonstrates that the translational efficiency of very similar mRNAs can vary considerably under different ionic conditions (10). In order to estimate directly the ratio of the mRNAs for the αA2 and αAαα polypeptides, we have used an S1 nuclease protection assay. The results of these experiments showed that there is 5 to 10 times more αA2 than αAαα mRNA in the lens. This ratio is not affected during lens fiber cell differentiation or lens maturation.

EXPERIMENTAL PROCEDURES

Isolation of pMaAααCr1—The construction of a cDNA clone library from 5 to 10-day-old murine lenses has been described previously (11, 12). DNA from individual bacterial colonies was screened by the method of Grunstein and Hogness (13) using a subcloned 532-bp BamHI restriction fragment of XMcrACrl (a genomic clone of the 5′ half of the αA-crystallin gene (8)), containing the insert exon flanked by intron sequences. The 532-bp DNA fragment was isolated by electrophoresis in a 5% acrylamide gel containing bisacrylamide and detected with [32P]dCTP by nick translation (15). The nucleotide sequence of the 532-bp DNA fragment was determined using the method of Maxam and Gilbert (16). The polynucleotides were separated by polyacrylamide gel electrophoresis in 8 M urea and detected by autoradiography.

S1 Nuclease Protection Experiments—Lenses were removed from 1-day-old or 2-month-old mice. The anterior epithelial cell sheets and the fiber cell masses were separated under a dissecting microscope. RNA was obtained as previously described by phenol/chloroform/sodium dodecyl sulfate extraction (17). The DNA fragment was prepared from pMaAααCr1 by 5′ end labeling at the XmnI site with [γ-32P]ATP (ICN) and T4 polynucleotide kinase (P-L Biochemicals). A singly labeled DNA fragment extending to the Tacl site (nucleotide positions 519 and 1085) was used as a probe to detect RNA degradation products of the αAαα mRNA.
34:11) of pBR322 was purified by electrophoresis in soluble polyacrylamide gels (14). Specific activities obtained were approximately $5 \times 10^4$ cpm/μg DNA.

Total RNA (2 to 0.1 μg) was precipitated with ethanol in the presence of the labeled DNA fragment (20 ng) and dissolved in 30 μl of hybridization buffer (40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 mM NaCl, and 50% formamide) (18). There was at least a 3-fold molar excess of the labeled DNA. The reaction mixture was heated for 15 min at 75 °C and annealed for 4 h at 55 °C. S1 nuclease digestion was conducted by adding 0.3 μl of 0.28 M NaCl, 0.05 M Na acetate, pH 4.6, 4.5 mM ZnSO<sub>4</sub>, 20 μg/ml of denatured calf thymus DNA, and 100 to 10,000 units of S1 nuclease (Bethesda Research Laboratories) per ml. Incubation was for 30 min at 37 °C. Reactions were terminated by adding 50 μl of 5.0 M ammonium acetate and 0.1 M EDTA. Reaction products were obtained by phenol/CHCl<sub>3</sub> extraction and ethanol precipitation. Fragments were denatured by boiling in 95% formamide and subjected to electrophoresis on 8% polyacrylamide gels containing 8 μM urea. Following autoradiography, bands were quantified by scanning using a Beckman DUS spectrophotometer.

Analysis of Lens α-Crystallin Polypeptides—Lanes were homogenized by freezing and thawing in 100 mM Tris, pH 8.6, 150 mM NaCl, 10 mM EDTA, and 5 mM β-mercaptoethanol and centrifuged at 12,000 × g for 10 min at 4 °C. Native α-crystallin was purified from the adult lens homogenate by gel filtration chromatography on Sephadex G-200 (1, 2). When homogenates of 1-day-old or 2-month-old lenses were used, α-crystallin was immunoprecipitated using formalin-treated Staphylococcus aureus protein A (19). The α-crystallin polypeptides were resolved by electrophoresis in 15% polyacrylamide, 0.1% sodium dodecyl sulfate gels (20). The gels were stained with Coomassie R-250, destained, and scanned using a Beckman DUS spectrophotometer.

RESULTS

Identification and Partial Sequence of pMaα<sup>Macr</sup>1—In order to identify a cDNA clone for the αα<sup>Macr</sup> polypeptide, we screened the murine cDNA library with a 532-bp genomic clone containing the 69-bp sequence encoding the insert peptide. The sequences coding for the insert peptide in this genomic clone were surrounded by intron sequences of the αα<sup>Macr</sup> gene. Previous experiments (8) showed that this probe hybridizes to a 14 S RNA, which is the size of the mRNA for the αα<sup>Macr</sup> polypeptide. The cDNA clone pMaα<sup>Macr</sup>1 hybridized strongly to the probe.

Preliminary experiments established that pMα<sup>Macr</sup>1 contained a similar, but not identical, restriction map to pMaACr1, the cloned cDNA for the α<sub>A</sub> mRNA (11). The cDNA insert in pMα<sup>Macr</sup>1 was partially sequenced by the Maxam-Gilbert method (16) using the strategy shown in Fig. 1. The sequence of this portion of the pMα<sup>Macr</sup>1 is shown in Fig. 1. The nucleotide sequence in pMα<sup>Macr</sup>1 encoding the insert peptide is identical with that of the insert exon in the αα<sup>Macr</sup> gene, and the nucleotide sequences flanking the cDNA sequence encoding the insert are exactly the same as those in exon 1 and exon 2 of the αα<sup>Macr</sup> gene (8).

Previously we inferred the positions of the borders of the insert exon in the αα<sup>Macr</sup> gene by the presence of consensus splicing sequences (21) and by analogy of the encoded peptide to the known amino acid sequence of the rat αα<sup>Macr</sup> polypeptide (6). The nucleotide sequence of pMaα<sup>Macr</sup>1 shown in Fig. 1 confirms the positions of the borders of the insert exon. The sequence at the 3′ border of the insert exon of the αα<sup>Macr</sup> gene was of special interest since it deviated from the AGGT consensus sequence by being AGGC (see Fig. 1). In view of the importance of this 3′ splice junction, we show a direct comparison of sequence data for the α<sub>A</sub> and the αα<sup>Macr</sup> cDNAs at the region encoding the insert peptide. As shown in Fig. 2, the cDNAs share a sequence corresponding to exon 2. The cDNA sequences diverge at the point of alternative RNA splicing. The 3′ end of the insert exon sequence is clearly contiguous with the 5′ end of the exon 2 sequence in pMaα<sup>Macr</sup>1.

Relative Amounts of α<sub>A</sub> and α<sup>Macr</sup> mRNAs in the Murine Lens—We used an S1 nuclease assay to determine the relative amounts of the mRNAs for the α<sub>A</sub> and the αα<sup>Macr</sup> polypeptides in the lens. A DNA fragment derived from pMaα<sup>Macr</sup>1 was used for the S1 nuclease tests. Since the α<sup>Macr</sup> and the α<sub>A</sub> mRNAs share nucleotide sequences derived from exons 1 and 2 of the αα<sup>Macr</sup> gene, the αα<sup>Macr</sup> cDNA should hybridize efficiently to both mRNAs. A DNA fragment from pMα<sup>Macr</sup>1 containing exon 1, insert exon, 40 nucleotides of exon 2, and pBR322 sequences adjacent to the PstI cloning site was 5′ end-labeled at the XmnI site in exon 2. Annealing of this DNA fragment to α<sub>A</sub> and αα<sup>Macr</sup> mRNA is shown diagrammatically in Fig. 3. Two size classes of DNA fragments were protected from S1 nuclease digestion by hybridization with lens RNA (Fig. 3). A smaller series of DNA fragments, 40-45 bases in length, resulted when the DNA probe annealed to the mRNA for the α<sub>A</sub> polypeptide. This mRNA lacks sequences encoding the insert peptide. Thus, the sequences encoding the insert peptide in the DNA fragment will not hybridize to the α<sub>A</sub> mRNA and will remain sensitive to S1 nuclease. We believe that the multiplicity of the smaller bands is due to the inability of S1 nuclease to remove consistently all unpaired nucleotides from the DNA loop in the hybrid. The positions of the S1 nuclease attack lie within the insert loop of the DNA fragment of the RNA-DNA hybrid, as judged by comparison on gels with the sequence of the labeled DNA fragment (data not shown). The larger size class of DNA fragments resistant to S1 nuclease was approximately 200 nucleotides in length. These resulted from hybridization with the αα<sup>Macr</sup> mRNA. In this case, since a perfect cDNA-RNA

![Fig. 1.](http://www.jbc.org/DownloadedFrom)

**Fig. 1.** The partial nucleotide sequence of pMaα<sup>Macr</sup>1 is identical with exon 1, insert exon, and exons 2 of the αα<sup>Macr</sup> gene (8). Nucleotide sequence of the borders of these regions of the gene are shown beneath the schematic representation of the gene. The arrows at the bottom show the strategy used to determine the sequence of the insert peptide coding region of pMaα<sup>Macr</sup>1.
FIG. 2. Comparison of the nucleotide sequence of pMaACr2 and pMaA"Cr1 shows the 3' border of the insert exon. DNA fragments labeled at the XmnI site were partially degraded (16) and displayed by polyacrylamide gel electrophoresis. The regions of identity with the αA-crystallin gene are indicated.

match was formed, only the pBR322 vector sequences in the DNA fragment were digested by S1 nuclease. The generation of both the smaller and larger DNA fragments by S1 nuclease was dependent on the addition of lens RNA to the annealing reaction (Fig. 3).

The S1 nuclease assay was used to determine the ratio of mRNAs for the αA2 and αA"m polypeptides in the lenses of mice of different ages and in different regions of the lens. Lenses from 1-day-old or 2-month-old mice were examined. Total RNA was extracted from the whole lenses, the lens epithelial cells, or the lens fiber cells. The S1 nuclease assay shown in Fig. 3 was conducted on each sample. The amount of DNA protected from S1 digestion by virtue of being hybridized to the mRNA was quantified by scanning the autoradiograms. The results, presented in Table I, show that the ratio of αA"m mRNA to αA2 mRNA ranged from 0.11 to 0.26 in these tests.

Relative Amounts of αA2 and αA"m Polypeptides in the Murine Lens—Previous studies have indicated that there is considerably more of the αA2 polypeptide than of the αA"m polypeptide in the rat lens (6). This is demonstrated for the murine lens in the gels shown in Fig. 4. Purified α-crystallin was obtained from the lens of adult mice by gel filtration chromatography on Sephadex G-200 (1, 2). The α-crystallin polypeptides were fractionated by electrophoresis in a 15% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and visualized by staining. The results showed appreciably more αA2 than αA"m polypeptide in the α-crystallin preparation. The relative amounts of αA2 and αA"m polypeptides in total lens homogenates of 1-day-old and 2-month-old mice were also examined by immunoprecipitation with α-crystallin antiserum followed by polyacrylamide gel electrophoresis (Fig. 4). Scans of the stained gels indicated that the ratio of the αA"m to the αA2 polypeptides was 0.10 to 0.12 (Table 1). These results agree well with the relative amounts of αA"m and αA2 mRNAs present in the lens.

DISCUSSION

Protein sequencing studies (6, 7) first suggested that the mRNAs for the αA2 and αA"m polypeptides were neither products of different alleles nor of separate genes. Our recent examination of the αA-crystalline gene (8) showed that it indeed contains coding information for the αA"m insert peptide within an intervening sequence. Since evidence for only a single αA-crystalline gene was obtained by Southern blotting, we proposed that both the αA2 and αA"m mRNAs were generated by alternative RNA processing. The present investigation strongly supports this proposal. Nucleotide sequencing studies of cDNAs showed that both the αA"m and αA2 mRNAs have sequences identical with those of exon 1 and exon 2 of the αA-crystalline gene; the mRNA for the αA"m polypeptide contains, in addition, a sequence identical with the insert exon of this gene. We conclude that both mRNAs are derived from the same αA-crystalline gene.

The nucleotide sequence in pMaA"mCr1 encoding the insert peptide defines the insert exon of the gene. The borders of this region may provide a clue to the relatively infrequent use of this region as an exon. The nucleotides at the junctions of the insert exon in the α-crystalline gene are similar to the consensus sequences (AGGT) of the intron-exon borders in other genes (21), with the exception that the 3' end of the insert exon is flanked by GC rather than the highly conserved GT. Alteration of the GT dinucleotide flanking the 3' ends of exons affects its use as a splice site in other systems (22-24).
Fig. 3. Single-stranded nuclease (S1) analysis of the mRNAs for the \(\alpha A_2\) and \(\alpha A'^{\text{ins}}\) polypeptides. Lens RNA was annealed to a DNA fragment derived from pMaA'^{\text{ins}}Cr1 DNA; the fragment was labeled at its 5' end at an \(X_{mni}\) site (see text). The hybrid molecules (shown schematically) were digested with S1 nuclease, and the products were displayed by polyacrylamide gel electrophoresis. No bands were present when lens RNA was omitted from the annealing reaction. The position of the denatured DNA fragment is shown by the arrow. Higher molecular weight species are probably due to incomplete denaturation of the DNA fragment.

Table I

<table>
<thead>
<tr>
<th>Age</th>
<th>Source</th>
<th>(\alpha A'^{\text{ins}}/\alpha A_2) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mRNAs(^a)</td>
</tr>
<tr>
<td>1-day-old</td>
<td>Total lens</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Epithelia</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Fibers</td>
<td>0.26</td>
</tr>
<tr>
<td>2-month-old</td>
<td>Total lens</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Epithelia</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Fibers</td>
<td>0.17</td>
</tr>
<tr>
<td>Adult</td>
<td>Total lens</td>
<td>0.10</td>
</tr>
</tbody>
</table>

\(^a\)Determined by the S1 nuclease assay.
\(^b\)Determined by scanning sodium dodecyl sulfate-polyacrylamide gels.

The effect of mutations in this dinucleotide on splicing should be interpreted with caution, however, since the second exon of the chicken \(\alpha^3\)-globin gene has a GC flanking its 3' end and yet is a functional splice site (25). It would be interesting if the lowered expression of the \(\alpha A'^{\text{ins}}\)-globin gene (compared with the \(\alpha^3\)-globin gene) in the chicken was related to a reduced splicing efficiency at this site.

The present investigation demonstrates that the murine lens contains 5 to 10 times more \(\alpha A_2\) mRNA than \(\alpha A'^{\text{ins}}\) mRNA. This finding is consistent with \textit{in vitro} translation experiments of rat (9) and mouse\(^2\) lens RNA and with the observation that there is 5 to 10 times more of the \(\alpha A_2\) polypeptide than of the \(\alpha A'^{\text{ins}}\) polypeptide in the lens. The \(\alpha A'^{\text{ins}}/\alpha A_2\) ratios of mRNAs and polypeptides remain the same as lens epithelial cells differentiate into fiber cells or as the lens matures. We cannot rule out the possibility that the ratio of \(\alpha A'^{\text{ins}}\) and \(\alpha A_2\) mRNAs can vary to suit the requirements of aspects of lens morphogenesis which we have not examined.

The constant ratio of the \(\alpha A'^{\text{ins}}\) and \(\alpha A_2\) mRNAs originating by alternative splicing contrasts with the controlled alternative RNA splicing that occurs in the \(\alpha\)-amylose (26), immunoglobulin (27, 28), and calcitonin (29) genes. In these cases, alternative RNA processing allows the gene to tailor its prod-

\(^2\)C. R. King and J. Piatigorsky, unpublished data.
ucts in different cells or under different conditions in a highly regulated fashion.

It is not known whether the αAimm peptide serves a special function within the lens distinct from that of the αA polypeptide. The importance of the function of the αA2 polypeptide is indicated by the highly conserved nature of this polypeptide (30). No amino acid sequence differences are found between mouse and rat αA2-crystallin polypeptides (11, 30). By contrast, only 19 of 23 residues are identical between mouse and rat αA2-polypeptides (11, 30). By comparison of gene and protein structures (32), it appears that the insert peptide of the αA2-crystallin gene is a neutral evolutionary event. On the other hand, the insert peptide may cause significant advantageous properties to the lens of these animals. Further studies of surface loops in the tertiary structure of the proteins have indicated that the αAimm chain associates efficiently into the characteristic oligomers of α-crystallin.

It has been suggested that each exon within a gene may possess information for a functional domain (31) or structural unit (32) of the protein it encodes. This has recently been supported by comparison of the gene and protein structures of a murine β-crystallin polypeptide (33). In addition, comparison of gene and protein structures has indicated that the locations of introns within genes correlate with the positions of surface loops in the tertiary structure of the proteins (34). The insert peptide of the αAimm chain may not greatly affect the protein structure. This would suggest that the development of the ability to alternatively splice the RNA transcripts of the αA-crystallin gene is a neutral evolutionary event. On the other hand, the insert peptide may cause significant changes in the α-crystallin structure and provide novel, advantageous properties to the lens of these animals. Further studies on the isolated αA2 and αAimm polypeptides are necessary to resolve these problems. Such investigations may be facilitated by use of their cDNAs.

Acknowledgments—We thank Deborah Carper for providing the anti-α-crystallin antiserum, Drs. John Nickerson and James Hawkins for critically reading the manuscript, and Dawn Sickles for expert secretarial assistance.

REFERENCES

Alternative splicing of alpha A-crystallin RNA. Structural and quantitative analyses of the mRNAs for the alpha A2- and alpha Ains-crystallin polypeptides.

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