Volume 10 Number 9 1982 Nucleic Acids Research

Organization of δ-crystallin genes in the chicken

Kunio Yasuda, Hisato Kondoh, T.S.Okada, Noboru Nakajima and Yoshiro Shimura

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

Received 12 February 1982; Revised and Accepted 13 April 1982

ABSTRACT

Double-stranded DNA was synthesized from δ-crystallin mRNA prepared from lens fibers of 15-day-old chick embryos and cloned at the *Pst* I site of the plasmid pBR322. Using the cloned cDNA and single-stranded cDNA as hybridization probes, a number of genomic DNA fragments containing δ-crystallin gene sequences have been cloned from the partial and complete *Eco*RI digests of chick brain DNA. One of the clones from the partial digests contains a DNA fragment that consists of four *Eco*RI fragments of 7.6 kb, 4.0 kb, 2.6 kb, and 0.8 kb. The gene sequences reside in the (5')7.6 kb - 0.8 kb - 4.0 kb(3') fragments. Electron microscopy has provided evidence that the cloned DNA fragment includes the entire gene sequences complementary to δ-crystallin mRNA except for the 3' terminal poly(A) tail, and that the δ-crystallin gene is interrupted by at least 13 intervening sequences. Another clone contains a genomic fragment that consists of two *Eco*RI fragments of 3.0 kb and 11 kb. The DNA fragment in the latter clone represents a different δ-crystallin gene, as judged by restriction endonuclease mapping and by electron microscopy.

INTRODUCTION

δ-Crystallin, a major structural protein of the ocular lens of avians and reptilians (1,2), has been shown to be a useful index for studying differential gene expression in cell differentiation (3-6). Chicken δ-crystallin is a polymeric protein of about 200,000 daltons, which can be resolved by gel electrophoresis into two protein molecules of 50,000 and 48,000 daltons (7).

Recently, Bhat and Piatigorsky have cloned a chicken δ-crystallin cDNA that represents some 60% of δ-crystallin mRNA (8). Using the cloned cDNA as a hybridization probe, several genomic DNA fragments containing δ-crystallin gene sequences have also been cloned (9,10). Through the analyses of these clones, the presence of at least two non-allelic δ-crystallin genes has been indicated (9,10). It should be pointed out, however, that all of the genomic clones thus far described contain only portions of the δ-crystallin genes and none of the clones carries the entire sequences of the genes.

In this paper, we report on the molecular cloning of various genomic DNA

0305-1048/82/1009-2879$2.00/0

fragments containing chicken δ-crystallin gene sequences. One of the clones includes a whole δ-crystallin gene. The organization of the δ-crystallin gene has been extensively characterized. In addition, structural analyses of these genomic DNA clones have revealed the presence of at least two non-allelic genes for δ-crystallin in the chicken genome, thereby confirming the finding of the previous workers.

MATERIALS AND METHODS

Preparation of δ-Crystallin mRNA. Lenses were removed from the eyes of 15-day-old white Leghorn chick embryos. The capsules and adhering epithelia were removed under a dissecting microscope. About 1,000 fiber masses were thawed in an equal volume of 1 mM ethylenediaminetetraacetate (EDTA), 10 mM Tris-HCl (pH 8.0), 0.3 M NaCl, and 0.5% Nonidet P-40, and homogenized with a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C to remove nuclei. The supernatant fraction was made 0.5% in sodium dodecylsulfate (SDS) and extracted repeatedly with phenol/chloroform/isoamyl alcohol (25/24/1), and RNA was precipitated with ethanol. δ-Crystallin mRNA was purified by repeated chromatography on oligo(dT)-cellulose columns, followed by sucrose gradient (5-20%) centrifugation. δ-Crystallin mRNA was identified by *in vitro* translation assay using the reticulocyte lysate system (New England Nuclear), followed by SDS-polyacrylamide gel electrophoresis.

Synthesis of cDNA. δ-Crystallin cDNA labeled with [α-^{32}P] dCTP was synthesized according to the procedure described by Chang *et al.* (11) using the purified mRNA as template and avian myeloblastosis virus reverse transcriptase (a gift of J. W. Beard). The reaction products were subjected to gel filtration on a Sephadex G-150 column (0.6 x 20 cm), and the excluded material was ethanol-precipitated and dissolved in 0.1 M NaOH containing 1 mM EDTA. After incubation at 65°C for 30 min, the solution was neutralized and the cDNA was ethanol-precipitated. Double-stranded (ds) cDNA was synthesized on the cDNA template with *E. coli* DNA polymerase I, followed by treatment with S1 nuclease (Sankyo) as described by Chang *et al.* (11). The ds-cDNA was tailed with dCTP by terminal transferase (Bethesda Research Laboratories).

Construction of a Recombinant cDNA·pBR322 DNA. The pBR322 DNA that had been linearized by digestion with *Pst* I was tailed with oligo(dG) by essentially the same procedure described above. The oligo(dG)-tailed plasmid DNA and the oligo(dC)-tailed ds-cDNA were hybridized as described by Chang *et al.* (11). *E. coli* χ1776 (provided by R. Curtis, III) was transformed by the procedure

described by Curtis *et al.* (12). The cells were plated and Tet^r transformants were transferred onto nitrocellulose filters. The hybrid clones were identified by the colony hybridization procedure of Grunstein and Hogness (13).

Hybrid-Mediated Arrest of δ-Crystallin mRNA Translation with cDNA. pBδ2 (10 μg), a cDNA clone that had been linearized by *Hin*dIII digestion was hybridized to cytoplasmic poly(A)-containing RNA (0.25 μg) from lens fibers in a hybridization buffer (0.4 M NaCl, 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid)-NaOH (pH 6.4), 80% formamide) at 51°C for 3 hr. After incubation, the mixture was divided into two portions. One portion was incubated further at 100°C for 1 min and ethanol-precipitated. Another portion was ethanol-precipitated without the heat treatment. The precipitates were then applied individually to the reticulocyte cell-free protein synthesizing system. The [^{3}H] leucine-labeled translation products were electrophoresed on a SDS-polyacrylamide (15%) slab gel according to the procedure described previously (14), and fluorographed.

Cloning of Genomic DNA Fragments. High molecular weight DNA prepared from chick brains was limit-digested with *Eco*RI and fractionated by agarose gel electrophoresis. The fragments containing δ-crystallin gene sequences were identified by Southern blotting (15) followed by hybridization with [^{32}P] labeled δ-crystallin cDNA according to the procedure described previously (16). The fragments corresponding to those hybridized with the probe were recovered from the gel and ligated to the terminal *Eco*RI fragments of λgtWES (obtained from P. Leder). The resulting DNA was packaged into phage particles according to the procedure of B. Hohn (17). The phage was plated on *E. coli* DP50-*supF* and the recombinant clones were identified by the *in situ* screening procedure (18). The partial *Eco*RI digests of the chick brain DNA were cloned in the Charon 4A vector (obtained from F. Blattner). The digests were fractionated by sucrose gradient (10-30%) centrifugation, and the DNA fragments of molecular sizes of 11-22 kilobase pairs (kb) were pooled. The fragments were ligated to the terminal *Eco*RI fragments of the Charon 4A DNA. The rest of the cloning procedure was the same as above.

Electron Microscopy. Recombinant DNA (1 μg) was mixed with mRNA (1 μg), lyophilized, and dissolved in 10 μl of the R-loop buffer (70% deionized formamide, 0.1 N [Tris(hydroxymethyl)methyl]glycine (tricine)-NaOH (pH 8.8), 0.5 M NaCl, 10 mM EDTA). The solution was incubated at 70°C for 10 min, then at 53°C for 2 hr, and subsequently diluted 30 folds with a hyperphase solution containing 55% formamide, 2.6 M urea, 9 mM EDTA and 90 mM tricine-NaOH (pH 8.0). The mixture was incubated at 40°C for 30 sec, then at 0°C, and subse-

quently allowed to equilibrate at room temperature (19). Cytochrome C (100 μg/ml) was added immediately before spreading on a hypophase of water. Samples were transferred onto parlodion-coated grids, stained with uranylacetate, dehydrated in iso-pentane, shadowed with platinum/palladium (80:20), and viewed with a JEM-7A electron microscope. Nucleic acid lengths were measured at a final magnification of 125,000 with a Hewlett-Packard 9820A calculator and a 9864A digitizer using fd RFII DNA as an internal duplex standard and fd viral DNA as an internal single-stranded DNA standard.

Restriction Endonuclease Mapping. Restriction endonucleases employed and the mapping procedure of their cleavage sites were essentially the same as described previously (16).

Biosafety Precautions. All cloning experiments were carried out in compliance with the guidelines of the Ministry of Education of Japan for recombinant DNA research.

RESULTS

Cloning of δ-Crystallin cDNA. Cytoplasmic poly(A)-containing RNA isolated from the lens fibers of 15-day-old chick embryos and fractionated by oligo(dT)-cellulose chromatography contained δ-crystallin mRNA as the major constituent, as judged by SDS-polyacrylamide gel electrophoresis of the *in vitro* translation products in the reticulocyte system and by immunoprecipitation of the *in vitro* products utilizing antiserum against δ-crystallin (data not shown). When the δ-crystallin mRNA preparation was purified further by sucrose gradient centrifugation, the mRNA was at least 95% pure on the basis of the *in vitro* translation products.

The purified δ-crystallin mRNA preparation was used as template to synthesize double-stranded cDNA. The cDNA molecules were inserted at the *Pst* I site of pBR322 by the dG-dC tailing procedure. Among the Amp^{S}· Tet^{r} transformants, those containing δ-crystallin cDNA sequences were screened by hybridization with [^{32}P]-labeled cDNA. One of the clones thus obtained (designated pBδ2) contained an insert of about 0.4 kb, as judged by electrophoretic mobility on a 1% agarose gel (data not shown). Since our δ-crystallin mRNA preparation was not absolutely pure, it was necessary to examine rigorously whether the insert of pBδ2 represents the δ-crystallin mRNA sequence. This was accomplished by hybrid-mediated arrest of δ-crystallin mRNA translation. When cytoplasmic poly(A)-containing RNA extracted from the lens fibers was hybridized to pBδ2 DNA that had been linearized by digestion with *Hin*dIII and subsequently applied to the *in vitro* protein-synthesizing

system, the synthesis of δ-crystallin was specifically inhibited (Fig. 1b). However, when the RNA-DNA was denatured by heating at 100°C for 1 min, the synthesis of δ-crystallin was restored (Fig. 1a). This indicates that pBδ2 contains the sequence corresponding to δ-crystallin mRNA. Electron microscopic analysis of the mRNA·pBδ2 hybrid shows that the insert of the cDNA clone represents a middle portion of the δ-crystallin mRNA sequence but not the 3' poly(A) sequence (data not shown).

Cloning of δ-Crystallin Gene Sequences. High molecular weight DNA extracted from chick brains was digested to completion with *Eco*RI and fractionated by agarose gel electrophoresis. When the fragments containing δ-crystallin gene sequences were examined by Southern blotting and hybridization to [^{32}P]-labeled δ-crystallin cDNA, the presence of four major fragments of molecular sizes of 11 kb, 7.6 kb, 4.0 kb and 3.0 kb was noted as shown in Fig. 2. This is consistent with the results obtained by Bhat *et al.* (9). When the *Eco*RI digests of the chicken DNA were cloned in the λgtWES vector and subsequently the recombinant phages were screened by hybridization to the labeled δ-crystallin cDNA, many clones containing each of the major *Eco*RI fragments were obtained. For example, λgδ28, λgδ1, λgδ43, and λgδ44 contained the inserts of 11 kb, 7.6 kb, 4.0 kb, and 3.0 kb, respectively (Fig. 3A). These clones may well be the same as those of Bhat *et al.* (9), as judged by

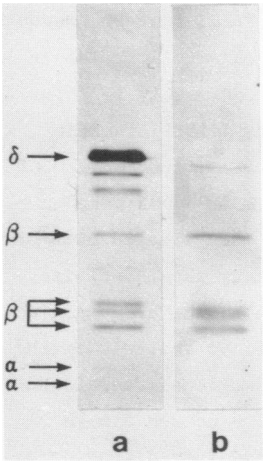

Figure 1. Hybrid-mediated arrest of δ-crystallin mRNA translation by pBδ2 DNA. Cytoplasmic poly(A)-containing RNA (0.25 μg) from the lens fibers was hybridized to the linearized pBδ2 DNA (10 μg) as described in MATERIALS AND METHODS. The mixture was then divided into two portions; one portion was incubated further at 100°C for 1 min and subsequently ethanol-precipitated (lane a), whereas the other portion was ethanol-precipitated without the heat treatment (lane b). The precipitates were then applied individually to the *in vitro* protein synthesizing system. The [^{3}H]-leucine-labeled products were electrophoresed (from top to bottom) on a SDS-polyacrylamide gel and fluorographed. The slowest migrating band (δ) of lane a is a mixture of two proteins of about 50,000 and 48,000 daltons and is indistinguishable from the band of δ-crystallin purified from the chicken lens fibers. The two protein bands immediately below the δ bands were also precipitable with antiserum against δ-crystallin and detectable only in the *in vitro* translation products but not in δ-crystallin from the lens cells. The nature of these protein bands is not known. α and β in the figure denote α- and β-crystallins, respectively.

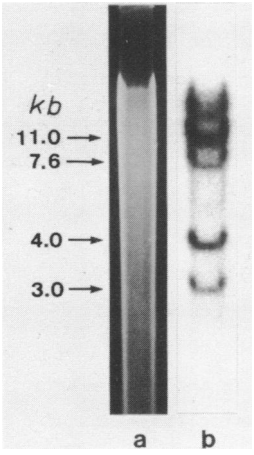

Figure 2. Identification of *Eco*RI fragments containing δ-crystallin gene sequences. DNA (20 μg) from chick brains was digested with *Eco*RI and the digests were electrophoresed on a 0.8% agarose gel. DNA bands were stained with ethidium bromide and photographed as described previously (16)(lane a). DNA bands of the same gel were transferred onto nitrocellulose filter and hybridized to [^{32}P] labeled cDNA of nearly full length (2 x 10^6 dpm input, approximately 4 x 10^7 dpm per μg specific activity). The labeled cDNA was prepared by reverse transcriptase reaction using the purified δ-crystallin mRNA preparation as template and [α-^{32}P] dCTP as the radioactive substrate. After hybridization, the filter was autoradiographed (lane b).

the sizes and the restriction maps of the inserts. The genomic clones isolated by the previous workers were shown to contain portions of δ-crystallin gene sequences. Accordingly, attempts were made to clone larger genomic DNA fragments. From the partial *Eco*RI digests of the chicken DNA, the fragments larger than 11 kb were cloned in the Charon 4A vector and the clones were screened by hybridization to [^{32}P]-labeled nick-translated cDNA from the plasmid clone pBδ2.

One of the clones thus obtained (designated λCδ106) contained an insert of about 15 kb, which was split, by digestion with *Eco*RI, into four fragments of 7.6 kb, 4.0 kb, 2.6 kb, and 0.8 kb in length. Among the partial *Eco*RI digests of the chicken DNA, we detected a fragment of approximately 15 kb, that was hybridizable to the [^{32}P]-labeled cDNA probe and indistiguishable from the insert of λCδ106 as judged by the electrophoretic mobility on a 0.8% agarose gel (data not shown). When the *Eco*RI digests of λCδ106 DNA were electrophoresed on a 0.8% agarose gel and hybridized to [^{32}P]-labeled cDNA of nearly full length, the 7.6-kb, 4.0-kb, and 0.8-kb fragments were hybridizable to the cDNA but the 2.6-kb fragment was not (Fig. 3A). The alignment of these *Eco*RI fragments in the chicken genome was determined by analysis of the partial *Eco*RI digests of λCδ106 DNA. Thus, a fragment that consisted of the 7.6-kb and 0.8-kb fragments and another fragment of the 7.6-kb and 2.6-kb fragments were detected in the partial digests. An intermediary digestion product in which the 0.8-kb and 4.0-kb fragments were linked in tandem was also observed. In addition, a Charon 4A clone carrying the *Eco*RI fragments of 8.0 kb, 2.6 kb, and 7.6 kb has been isolated. Therefore, the alignment of the four *Eco*RI fragments in λCδ106 DNA has been concluded to be 2.6 kb - 7.6

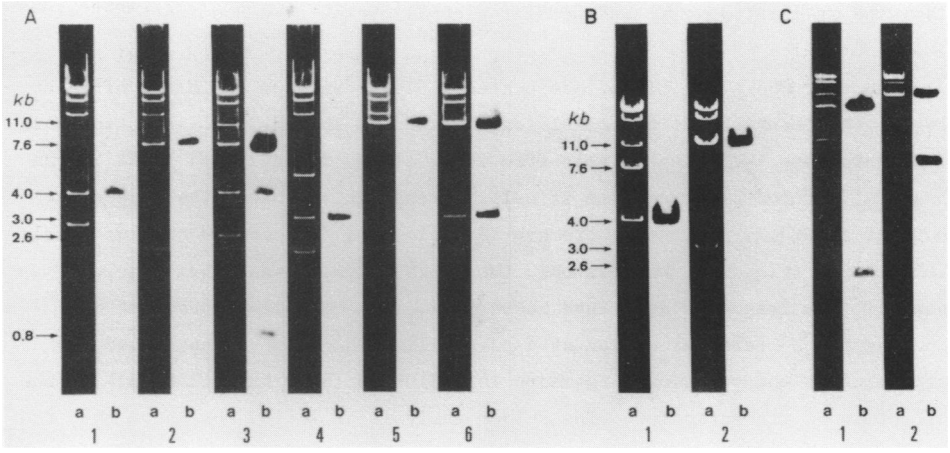

Figure 3. Hybridization of the *Eco*RI digests of genomic clones with cDNA probes. DNA (0.5 μg) of each genomic clone was limit-digested with *Eco*RI and electrophoresed on a 0.8% agarose gel. DNA bands were stained with ethidium bromide and photographed as in Fig. 2 (lane a). DNA bands of the same gel were subjected to Southern hybridization with various [^{32}P]-cDNA probes and autoradiographed (lane b).
Panel A. Single-stranded [^{32}P]-cDNA of nearly full length was used as the hybridization probe. Lanes: 1, λgδ43; 2, λgδ1; 3, λCδ106; 4, λgδ44; 5, λgδ28; 6, λCδ109.
Panel B. Single-stranded [^{32}P]-cDNA fragments of about 500 nucleotides long representing the 3' terminal side of δ-crystallin mRNA were used as the hybridization probe. The cDNA fragments were prepared as described in the text. Lanes: 1, λCδ106; 2, λCδ109.
Panel C. The large *Eco*RI fragment of the pBδ2 insert was nick-translated and used as the hybridization probe. Lanes: 1, λCδ106; 2, λCδ109.

kb - 0.8 kb - 4.0 kb. The polarity of these sequences relative to δ-crystallin mRNA was determined by hybridization to [^{32}P]-labeled cDNA fragments of about 500 nucleotides long representing the 3' terminal side of the mRNA sequence. The cDNA fragments were obtained from the partial reaction products of cDNA synthesis by electrophoretic fractionation on a 1% agarose gel. When the *Eco*RI digests of λCδ106 DNA were hybridized to the partial cDNA fragments, only the 4.0-kb fragment was hybridizable (Fig. 3B). Thus, the 4.0-kb fragment must represent the 3' terminal region of δ-crystallin mRNA. Conversely, the 7.6-kb fragment includes the 5' terminal region of the mRNA sequence. It is worth noting that the 7.6-kb and 4.0-kb fragments of λCδ106 are identical with those cloned in λgδ1 and λgδ43, as judged by restriction endonuclease mapping and by electron microscopy of heteroduplexes formed between λCδ106 DNA and λgδ1 or λgδ43 DNA (Agata *et al.*, manuscript in preparation). We have

isolated at least several independent genomic clones that are identical with λCδ106.

Another Charon 4A clone, designated λCδ109, contains an insert of about 14 kb, that consists of two *Eco*RI fragments of 11 kb and 3 kb. The two *Eco*RI fragments were indistinguishable from those cloned in λgδ28 and λgδ44 on the basis of heteroduplex analyses as well as restriction endonuclease mapping. Both of the *Eco*RI fragments were hybridizable to [^{32}P]-labeled cDNA of nearly full length (Fig. 3A), whereas only the 11-kb fragment was hybridizable to the partial cDNA fragments described above (Fig. 3B). The 11-kb fragment must include the 3' terminal region of δ-crystallin mRNA gene sequences and the alignment of the two *Eco*RI fragments in λCδ109 is (5')3 kb - 11 kb(3') in the direction of transcription. The R-loop analysis of λCδ109 DNA shows that the genomic insert of the clone covers approximately the 3' terminal two-thirds of δ-crystallin mRNA sequence (Agata *et al.*, manuscript in preparation). This δ-crystallin gene appears to be different from that of λCδ106 on the basis of restriction endonuclease mapping (Fig. 4). It is most likely that the two gene sequences represent the two non-allelic genes reported by Bhat *et al.*(9).

It has been suggested by Jones *et al.* (10) that the δ-crystallin gene sequences of their genomic clones containing the 7.5-kb and 4-kb fragments (gδCr1 and gδCr3) are transcribed on the basis of *Eco*RI mapping of these clones as well as their cDNA clone. Consistent with this notion are our

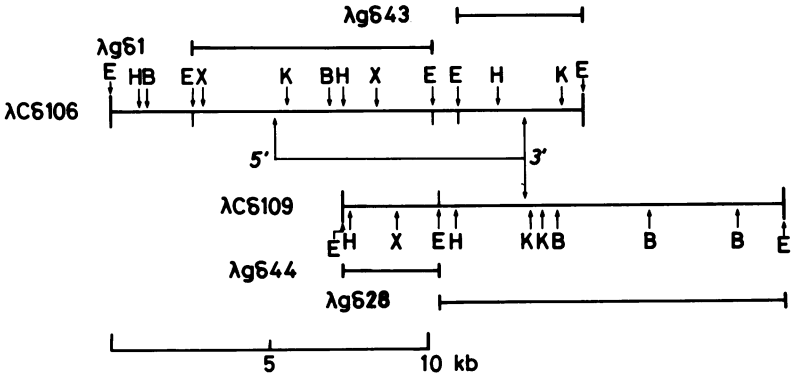

Figure 4. Restriction maps of genomic DNA fragments of λCδ106 and λCδ109. The following restriction endonuclease cleavage sites are shown: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; X, *Xba* I; K, *Kpn* I. The four *Eco*RI fragments cloned in λgδ1 (7.6 kb), λgδ43 (4.0 kb), λgδ44 (3.0 kb), and λgδ28 (11 kb) are positioned along the λCδ106 and λCδ109 fragments. The arrows marked 5' and 3' indicate the locations of the 5' and 3' termini of δ-crystallin mRNA gene sequences revealed by electron microscopy of the mRNA-DNA hybrids.

results suggesting that the δ-crystallin gene in λCδ106 is transcribed in chick embryos. The cDNA sequence of pBδ2 has a single *Eco*RI site located closely to a terminus of the sequence. Thus, digestion of the pBδ2 insert with *Eco*RI results in two cDNA fragments. When the larger *Eco*RI fragment was nick-translated and hybridized to the *Eco*RI digests of λCδ106 DNA, the 7.6-kb and 0.8-kb fragments were hybridized but the 4.0-kb fragment was not (Fig. 3C). On the other hand, when the cDNA fragment was hybridized to the *Eco*RI digests of λCδ109 DNA, both of the 3-kb and 11-kb fragments were hybridized (Fig. 3C). These results suggest that the *Eco*RI site of pBδ2 insert is identical with that between the 0.8-kb and 4.0-kb fragments of λCδ106 DNA but not with that between the 3-kb and 11-kb fragments of λCδ109 DNA. If the latter were the case, the *Eco*RI site of the cDNA insert would be created as a result of post-transcriptional processing of the transcript of the λCδ109 gene. This seems unlikely, considering the consensus sequence of splicing sites of many eukaryotic genes and the *Eco*RI recognition sequence.

Electron Microscopy of λCδ106 DNA. We have used electron microscopy to characterize the δ-crystallin gene of λCδ106. The electron micrograph presented in Fig. 5 shows the R-loops made by hybridizing δ-crystallin mRNA to λCδ106 DNA. The mRNA·DNA hybrid is interrupted by thirteen loops of single-stranded DNA (letters A to M), indicating discontinuity of the gene sequences

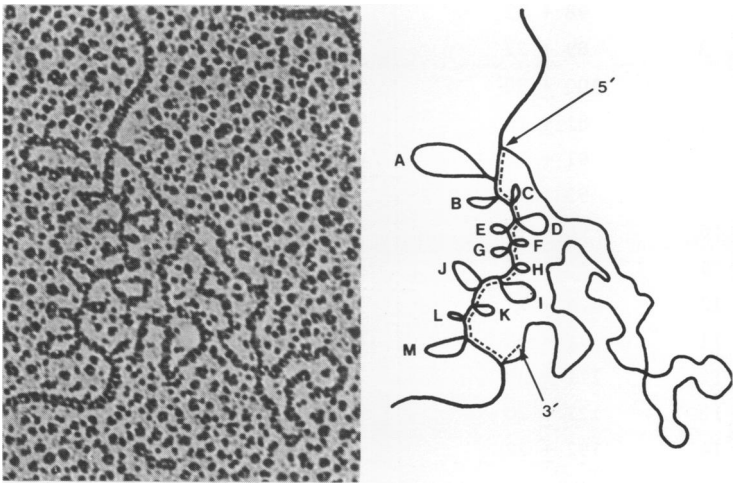

Figure 5. Electron micrograph and schematic interpretation of a δ-crystallin mRNA·λCδ106 DNA hybrid. In the line drawing, the solid line is DNA and the dotted line is mRNA. The letters denote intervening sequence regions.

complementary to δ-crystallin mRNA. The RNA tail to the right of intervening sequence M in the figure represents, in all likelihood, the 3' terminal poly-(A) sequence of the mRNA. The other terminus of the mRNA molecule is in duplex form with the DNA. In view of the fact that our mRNA preparation directs the synthesis of δ-crystallin *in vitro* and yet cannot be phosphorylated at the 5' terminus, after alkaline phosphatase treatment, in the presence of [γ-^{32}P] ATP and polynucleotide kinase, it is almost certain that the majority of mRNA molecules are capped at the 5' termini, thereby indicating that the RNA molecules are largely intact. Thus, the R-loop analyses of the δ-crystallin mRNA·λCδ106 DNA hybrids led us to conclude that the cloned DNA contains the entire mRNA gene sequences and their flanking regions and that the δ-crystallin gene contains at least thirteen intervening sequences. The mean lengths (±SD) of the hybridized regions (exons) and the loops (introns) are listed in Table 1. The mean cumulative length of the exons is 1.6 ± 0.3 kb and the mean total length of the introns is 6.04 ± 1.1 kb. On the basis

Table 1. Chain lengths of exons and introns of the δ-crystallin gene of λCδ106

Exons		Introns	
1	188 ± 35	A	1151 ± 127
2	98 ± 22	B	391 ± 62
3	89 ± 22	C	326 ± 62
4	100 ± 20	D	400 ± 74
5	81 ± 16	E	323 ± 48
6	91 ± 16	F	279 ± 71
7	93 ± 33	G	350 ± 59
8	90 ± 18	H	312 ± 65
9	113 ± 25	I	603 ± 116
10	124 ± 22	J	464 ± 145
11	111 ± 18	K	359 ± 76
12	111 ± 20	L	441 ± 110
13	122 ± 20	M	641 ± 108
14	192 ± 22		

The lengths are given in nucleotides and the mean values (± SD) of 15 measurements. Exons are numbered in order from the 5' proximal side of mRNA sequence. The letters of introns correspond to those in Fig. 5.

of these results, the δ-crystallin gene of λCδ106 is schematically illustrated as shown in Fig. 6.

DISCUSSION

The δ-crystallin mRNA preparation from the lens fibers of chick embryos was sufficiently pure, so that the majority of cDNA synthesized on the RNA template represented δ-crystallin mRNA sequence. Using single-stranded δ-crystallin cDNA as well as the cloned duplex cDNA as probes, we have isolated many genomic DNA clones containing δ-crystallin gene sequences from chick brain DNA. Among the clones were those carrying the four *Eco*RI fragments of 11 kb, 7.6 kb, 4.0 kb, and 3.0 kb. They are similar, in many respects, to and probably the same as those isolated by Bhat *et al.* (9).

The organization of δ-crystallin genes has been investigated by Jones *et al.*, using their genomic clones of the four *Eco*RI fragments and a library clone containing the 3-kb *Eco*RI fragment and its 5' flanking as well as short 3' flanking sequences (10). The short 3' flanking sequence of the library clone appears to coincide with a small 5' terminal region of the 11-kb *Eco*RI fragment. The library clone, however, does not cover the whole δ-crystallin mRNA gene sequences. Jones *et al.* have concluded that the genomic fragment of the library clone and the 3-kb and 11-kb *Eco*RI fragments represent a δ-crystallin gene and that the gene contains at least 15 intervening sequences on the basis of the R-loop analyses of the indifidual clones (10). They have also indicated that the 7.5-kb and 4.0-kb fragments represent another δ-crystallin gene. Although they have suggested the presence of a small region connecting the two *Eco*RI fragments, there has been no direct evidence to prove it. They have proposed that this δ-crystallin gene contains at least 14

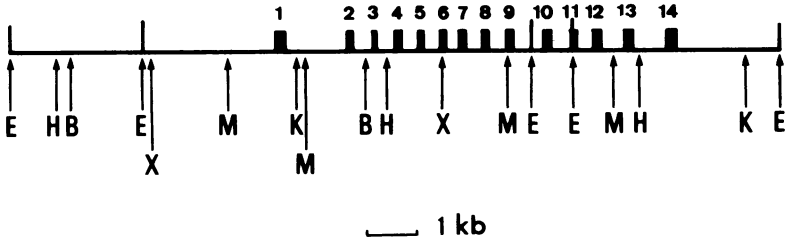

Figure 6. Schematic representation of the δ-crystallin gene in λCδ106. ——, Flanking and intervening sequences; ■, mRNA gene sequences. The arrows indicate restriction endonuclease cleavage sites: E, *Eco*RI; H, *Hin*dIII; X, *Xba* I; M, *Msp* I; K, *Kpn* I; B, *Bam*HI.

intervening sequences.

One of our genomic clones, λCδ106, appears to contain the entire δ-crystallin mRNA gene sequences; the mRNA gene sequences are included in the three *Eco*RI fragments of (5')7.6 kb - 0.8 kb - 4.0 kb(3') of the clone. The R-loop analyses of the λCδ106 DNA·mRNA hybrids have revealed that this gene is interrupted by at least 13 intervening sequences. This is different from the number of intervening sequences reported by Jones *et al.* (10) on the comparable δ-crystallin gene. Comparison of the introns and exons of λCδ106 with those of gδCr1 and gδCr3 of Bhat *et al.* (9) and Jones *et al.* (10) suggests that possibly the 5' exon of gδCr1 is missing in the R-loop of λCδ106. This would also mean that intervening sequence A of gδCr1 described by Bhat *et al.* (9) was not visualized in the R-loop analysis of λCδ106. The reason underlying the different observations of the two groups on the occurrence of intervening sequences is not known. It is possible that the difference is due to the conditions of DNA-RNA hybridization. It is possible to assume that the 5' proximal exon and its flanking intervening sequence of gδCr1 were not visualized in λCδ106, because their mRNA-DNA hybridization in the R-loop was somehow eliminated by branch migration of the DNA-DNA hybridization 5' to it. Alternatively, the possibility that the gene in λCδ106 is a polymorphic allele of the gene analyzed by Bhat *et al.* and Jones *et al.* may not be ruled out. There is still another possibility that the gene in λCδ106 is an entirely different δ-crystallin gene.

The δ-crystallin gene sequence in λCδ109 is in the two *Eco*RI fragments of 3 kb and 11 kb that are linked in tandem and possibly represents another non-allelic gene. Thus, our results obtained with this clone support those of Jones *et al.* (10) obtained with their gδCr4, gδCr2, and the library clone. The organization of this δ-crystallin gene has not been clarified in the present studies, since a portion of the mRNA gene sequences is still missing in the genomic fragment of λCδ109. Molecular cloning of a genomic fragment that covers the entire sequences of this δ-crystallin gene is in progress.

The hybridization profiles of the *Eco*RI digests of λCδ106 and λCδ109 to the larger *Eco*RI fragment of the pBδ2 insert suggest that the cDNA sequence was derived from the transcript of the δ-crystallin gene of λCδ106. Thus, it is likely that the gene is transcribed in chick embryos. More direct evidence on the gene expression will be obtained when nucleotide sequences of the δ-crystallin cDNA and the genomic DNA of λCδ106 are determined. The sequence analyses of these DNAs are currently in progress and will be published elsewhere. It still remains to be solved whether the other δ-crystallin gene that

has been partially cloned in λCδ109 is actually expressed. So far, no cDNA sequence consistent with this gene sequence has been detected. Although Jones *et al.* (20) have recently shown that the two δ-crystallin genes studied in their laboratory are transcribed, it remains to be shown that both transcripts are processed to functional mRNAs.

ACKNOWLEDGEMENTS

We thank Dr. J. W. Beard for the gift of AMV reverse transcriptase and Drs. P. Leder, F. Blattner, B. Hohn, and R. Curtis, III for providing the phage and bacterial strains. We also thank Dr. H. Yamagishi for advices on electron microscopy. This work was supported in part by grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Clayton, R. M. (1974) in *The Eye,* Davson, H. and Graham, L. T. Eds., Vol. 5, pp. 399-494, Academic Press, London.
2. Williams, L. A. and Piatigorsky, J. (1979) *Eur. J. Biochem. 100,* 349-357.
3. Okada, T. S. (1980) in *Current Topics in Developmental Biology,* Moscona, A. A., Monroy, A., and Hunt, H. K. Eds., Vol. 16, pp. 349-380, Academic Press, New York.
4. Piatigorsky, J. *Differentiation* (in press).
5. Zwaan, J. and Ikeda, A. (1968) *Exp. Eye Res. 7,* 301-311.
6. Genis-Galvez, J. M., Maisel, H., and Castro, J. (1968) *Exp. Eye Res. 7,* 593-602.
7. Rezelbach, R., Shinohara, T., and Piatigorsky, J. (1977) *Exp. Eye Res. 25,* 583-593.
8. Bhat, S. P. and Piatigorsky, J. (1979) *Proc. Natl. Acad. Sci. USA. 76,* 3299-3303.
9. Bhat, S. P., Jones, R. E., Sullivan, M. A., and Piatigorsky, J. (1980) *Nature (London) 284,* 234-238.
10. Jones, R. E., Bhat, S. P., Sullivan, M. A., and Piatigorsky, J. (1980) *Proc. Natl. Acad. Sci. USA. 77,* 5879-5833.
11. Chang, A. C. Y., Nunberg, J. H., Kaufman, R. J., Erlich, H. A., Schimke, R. T., and Cohen, S. N. (1978) *Nature (London) 275,* 617-624.
12. Curtis, R., III, Inoue, M., Pereira, D., Hsu, J. C., Alexander, L., and Rock, L. (1977) in *Molceular Cloning of Recombinant DNA,* Scott, W. A. and Werner, R. Eds., pp. 99-114, Academic Press, New York.
13. Grunstein, M. and Hogness, D. S. (1975) *Proc. Natl Acad. Sci. USA. 72,* 3961-3965.
14. Laemmli, U. K. (1970) *Nature (London) 227,* 680-685.
15. Southern, E. M. (1975) *J. Mol. Biol. 98,* 503-517.
16. Nakajima, N., Ozeki, H., and Shimura, Y. (1981) *Cell 23,* 239-249.
17. Hohn, B. (1979) *Methods Enzymol. 68,* 299-309.
18. Benton, W. D. and Davis, R. W. (1977) *Science 196,* 180-182.
19. Schfer, M. P., Boyed, C. D., Tolstoshev, P., and Crystal, R. G. (1980) *Nucleic Acids Res. 8,* 2241-2253.
20. Jones, R. E., DeFeo, D., and Piatigorsky, J. (1981) *J. Biol. Chem. 256,* 8172-8176.