Two δ-crystallin polypeptides are derived from a cloned δ1-crystallin cDNA

Eric F. Wawrousek, John M. Nickerson and Joram Piatigorsky

Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

Received 23 July 1986

Previous studies have shown that there are 2 similar δ-crystallin genes (δ1 and δ2) and at least 2 δ-crystallin polypeptides in the chicken eye lens. We show here that both δ-crystallin polypeptides can be synthesized from mRNA transcribed in vitro from a cloned δ1-crystallin cDNA. Both polypeptides co-migrate in SDS-urea-polyacrylamide electrophoresis with their authentic counterparts isolated from 15-day-old embryonic chicken lenses, and both react with sheep anti-chicken δ-crystallin serum. Screening nearly 900 δ-crystallin cDNA clones from a 15-day-old embryonic lens library with an oligonucleotide probe specific for exon 2 of the δ2-crystallin gene failed to detect any δ2 cDNA clones, indicating that the δ2 gene produces little or no mRNA in the lens at this stage of development. Our results suggest that both of the observed δ-crystallin polypeptides are derived from mRNA transcribed from the δ1 gene, with heterogeneity arising at the translational or co-translational level.

δ1-Crystallin cDNA mRNA Polypeptide synthesis

1. INTRODUCTION

Crystallins, the major water-soluble proteins in the transparent eye lens [1,2], compose a heterogeneous population of structural proteins. The four crystallin families (α, β, γ, δ) each consist of several similar polypeptides. Gene duplication [3,4] and alternative RNA splicing [5] have contributed to crystallin heterogeneity at the nucleic acid level, and post-translational modifications further diversify the population of crystallin polypeptides [6–8].

The δ-crystallins, found only in birds and reptiles, are tetrameric proteins consisting of two principal polypeptides with molecular masses near 48 and 50 kDa [9]. Although both δ-crystallin polypeptides contain extremely similar tryptic peptides [9,10], the 50 kDa polypeptide has two additional methionine-containing tryptic peptides that are not present in the 48 kDa polypeptide [11]. Both δ-crystallin polypeptides are synthesized when embryonic chicken lens mRNA is added to reticulocyte or wheat germ extracts [9] or microinjected into monkey kidney cells [12]. Pulse-chase experiments in explanted lenses offer no evidence of precursor product relationship between the 48 and 50 kDa polypeptides [9].

The presence of two linked, extremely similar δ-crystallin genes in the chicken [13–15] is consistent with each encoding one of the δ-crystallin polypeptides. To date, only cDNA clones derived from the δ1 gene (5′ to the δ2 gene) have been identified [16–19]. We show here that screening nearly 900 δ-crystallin cDNA clones from an embryonic chicken lens library with a δ2-specific probe failed to detect any δ2 cDNA clones, suggesting that the δ2 gene is functionally inactive in the embryonic lens. Furthermore, we demonstrate that both δ-crystallin polypeptides can be synthesized in vitro from mRNA derived from a single cloned δ1 cDNA.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2. MATERIALS AND METHODS

2.1. *Screening for δ2 cDNA clones*

Approx. 2500 colonies from a 15-day-old embryonic chicken lens cDNA library [19] were screened with a δ-crystallin probe nick-translated from pgδCr4.1 [13], a fragment from the central portion of the δ2 gene. Strongly hybridizing colonies were transferred to fresh plates, and clones containing the 5′-end of the δ1 gene (pδ1.4) [20], the 5′-end of the δ2 gene (pδ2.2) [20] and a full-length δ1 cDNA (pδCr17) [19] were included on each plate as controls. A 20-nucleotide probe complementary to part of exon 2 in the δ2 gene (shaded portion in fig.1A) was 5′-end-labelled with polynucleotide kinase (P-L Pharmacia) and [γ-^{32}P]ATP (ICN). The nitrocellulose colony blots were prehybridized for 2 h at 37°C in 10 × Denhardt's solution [21], 6 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.2) and 100 μg/ml of *E. coli* DNA.

Hybridization took place at 37°C overnight in 10 × Denhardt's solution, 6 × SSC, and 6.5 × 10^5 cpm/ml of the labelled oligonucleotide probe. The blots were washed three times (20 min each) at room temperature in 6 × SSC then twice (20 min each) at 37°C in 6 × SSC, air-dried, covered with plastic wrap and autoradiographed under Kodak XAR-5 film at −70°C.

2.2. *Construction of pEW7 plasmid*

A 1.8 kb *Hpa*II fragment was excised from pδCr17 [19] and cloned into the *Acc*I site of pSP64 (Promega Biotech). Restriction mapping of 50 of the resulting clones revealed that only one, pEW7, contained the δ1 crystallin cDNA in the correct orientation relative to the SP6 promoter (see fig.2A).

2.3. *Transcription/translation assay*

To prepare template DNA, pEW7 was linearized with *Bam*HI and purified by electrophoresis in a disulfide crosslinked polyacrylamide gel [22]. The transcription reaction was done with the linearized template and SP6 RNA polymerase [23] (Promega Biotech) according to the manufacturer's instructions. The reaction was stopped by addition of diethyl pyrocarbonate, phenol extraction and ethanol precipitation. A small portion of the transcription product was analyzed by electrophoresis in a 1.5% agarose gel containing formaldehyde. The transcription product was treated with guanylyltransferase (BRL) in the presence of GTP and *S*-adenosylmethionine to add a 7-methylguanosyl cap to the 5′-end of the RNA transcripts [23].

Capped in vitro synthesized RNA and poly(A^+) RNA isolated from 15-day-old chicken embryo lens fiber masses were translated in a rabbit reticulocyte lysate (NEN) with [^{35}S]methionine according to the manufacturer's instructions. The RNA concentrations in the translation mixtures were approx. 0.8 and 1.0 ng/μl for the in vitro synthesized RNA and poly(A^+) lens RNA, respectively. Translation products were immunoprecipitated with sheep anti-chicken δ-crystallin serum prior to electrophoresis. To enable visualization of protein bands by Coomassie staining, 2 μg authentic unlabelled δ-crystallin was added to each sample. The samples were subjected to SDS-urea-polyacrylamide gel electrophoresis as described [9] except that the pH of the separating gel was 8.1. The gel was Coomassie stained, dried and autoradiographed.

3. RESULTS

Approx. 880 δ-crystallin cDNA clones which hybridized strongly to a nick-translated probe from pgδCr 4.1 [13] (a fragment of the δ2-crystallin gene spanning exons 5–11) were isolated from a 15-day-old embryonic chicken lens cDNA library [19]. These selected clones were replated along with δ1 and δ2 genomic clones each containing exon 2 and a full-length δ1 cDNA clone [19] in preparation for rescreening with a δ2-specific probe.

To obtain a probe which can discriminate between δ1 and δ2 cDNAs, an alignment was made of sequences available for the two genes (see fig.1) [20,24–26]. The complement of a 20 bp area in the presumptive exon 2 of the δ2 gene (shaded area in fig.1A) was chosen because it has only a 14-nucleotide complementarity to the corresponding region of the δ1 gene and would contain a large loop out. The colony blot hybridization shown in fig.1B clearly demonstrates that under the hybridization and washing conditions used in this study the 20-nucleotide probe binds exclusively to colonies containing exon 2 of the δ2 gene. The

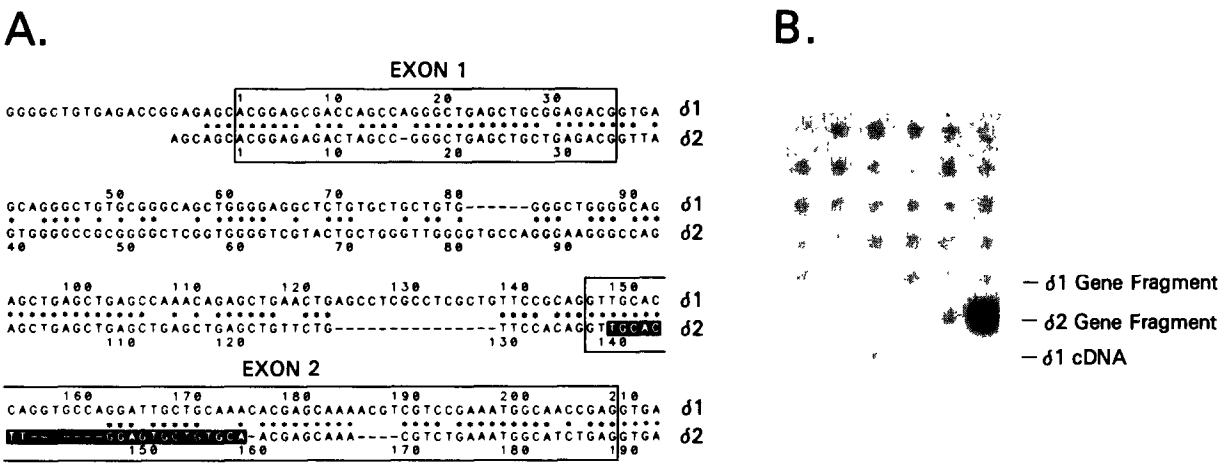

Fig.1. (A) Computer-generated alignment [38] of sequences at the 5′-ends of the $\delta 1$ and $\delta 2$ genes. Exons 1 and 2 are boxed and the portion of the $\delta 2$ exon 2 whose complement was used as a $\delta 2$-specific probe is blackened. (B) Autoradiogram of a colony filter blot hybridized with the $\delta 2$-specific probe. δ-Crystallin cDNA clones selected from a 15-day-old embryonic chicken lens cDNA library were replica plated along with three previously characterized clones and rescreened with a $\delta 2$-specific oligonucleotide probe as described in section 2. The positive control colony contains a $\delta 2$ gene fragment spanning exon 2 (pδ2.2 [20]); the negative control colonies contain a $\delta 1$ gene fragment spanning exon 2 (pδ1.4 [20]) and a full-length $\delta 1$ cDNA (pδCr17 [19]). The 44 δ-crystallin cDNA clones exhibit the same background levels of hybridization as the $\delta 1$ negative control colonies.

880 cDNA clones isolated from the library exhibited only background levels of hybridization as did the known $\delta 1$ cDNA and the genomic clone containing exon 2 from the $\delta 1$ gene. Thus, none of the cDNA clones appear to contain the $\delta 2$ exon 2.

A full-length $\delta 1$ cDNA [19] was subcloned into an SP6 promoter vector [23] to enable synthesis of a pure $\delta 1$ mRNA. The resulting plasmid, pEW7, is schematized in fig.2A, and the sequence of the RNA synthesized from the *Bam*HI linearized plasma by SP6 RNA polymerase is shown in fig.2B. The RNA has, in addition to the $\delta 1$ mRNA, some pSP64 and Okayama-Berg vector [27] sequences at its 5′- and 3′-ends, respectively, but the additional 5′-sequences contain neither AUG codons nor translation-termination codons which might interfere with translation studies [28–30]. The in vitro synthesized RNA migrated in formaldehyde-agarose gel electrophoresis as a single band of approx. 1700 nucleotides (not shown), indicating that a homogeneous population of $\delta 1$-crystallin RNA was produced. Translation of the in vitro synthesized $\delta 1$ mRNA (7-methylguanosyl capped with vaccinia virus guanylyltransferase) and poly(A^+) RNA (isolated from 15-day-old chicken embryo lenses) in a rabbit reticulocyte lysate yielded both the 48 and 50 kDa δ-crystallin polypeptides, as shown in fig.3, lanes C and D, respectively. The polypeptides co-migrate with native δ-crystallin (fig.3, lane B) in SDS-urea-polyacrylamide gel electrophoresis and are immunologically similar to native δ-crystallin as judged by immunoprecipitation with anti-chicken δ-crystallin serum. The ratio of 48 kDa/50 kDa polypeptide synthesis is 3.4:1 for the in vitro synthesized mRNA and 2.2:1 for the poly(A^+) mRNA, in reasonably close agreement with the 3:1 ratio found for native δ-crystallin [9], for δ-crystallin produced in explanted lenses with vitreous body attached [31] (see fig.3, lane B), and in previous in vitro translation experiments using lens poly(A^+) RNA [32] under similar conditions. These data clearly demonstrate that both of the observed δ-crystallin polypeptides can be synthesized in approximately the correct ratio from mRNA derived from a single cloned $\delta 1$ cDNA.

4. DISCUSSION

Before the present study, the simplest explana-

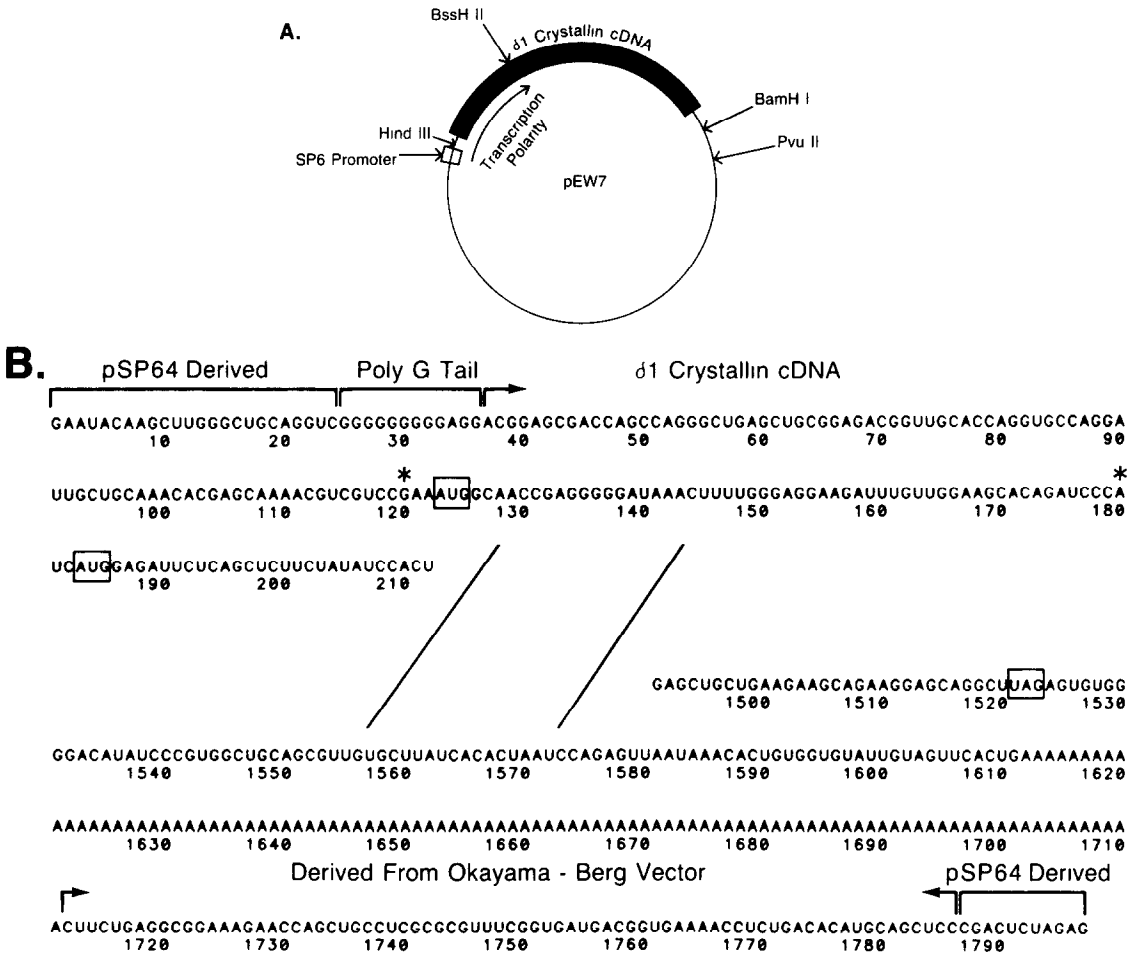

Fig.2. (A) Schematic representation of the pEW7 plasmid. The cDNA insert from pδCr17 [19] was subcloned into the SP6 promoter vector pSP64. The insert is oriented such that transcription with SP6 RNA polymerase will produce δ-crystallin mRNA. (B) Composite sequence of the RNA synthesized by SP6 RNA polymerase using *Bam*HI-linearized pEW7 as template. The RNA contains sequences derived from the Okayama-Berg vector [27] and pSP64 at its 5′- and 3′-termini as indicated. The central portion of the δ-crystallin cDNA insert has been omitted since it has been reported [18,19] and amended [24,26] elsewhere. The first two in-frame AUG codons are boxed as is the first in-frame translation-termination codon.

tion for the origin of the two very similar δ-crystallin polypeptides was that each was derived from one of the two closely linked δ-crystallin genes. The predicted amino acid sequences encoded in the δ1 and δ2 genes are 91% homologous [25] and would yield the tryptic peptides which have been observed for native δ-crystallin [9,11]. Moreover, transfection experiments using the pSVO-CAT vector and explanted lens epithelia have shown that the δ1 gene promoter is more efficient than that of the δ2 gene [20], consistent with the fact that there is more of one δ-crystallin polypeptide (48 kDa) than of the other (50 kDa).

However, the apparent absence of δ2 cDNA clones in a 15-day-old embryonic chicken lens cDNA library in our present experiments indicates that the δ2 gene is not expressed in tiny amounts or not at all in the lens at this stage of development. We cannot rule out the possibility that δ2 cDNAs lacking exon 2 are represented in the cDNA

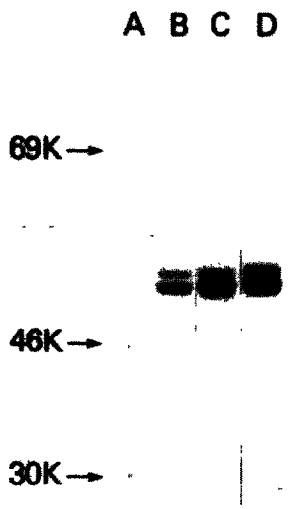

Fig.3. Labelling pattern of δ-crystallin polypeptides synthesized in the cultured lens and in a reticulocyte lysate supplemented with the indicated RNA. Lanes: (A) ^{14}C-labelled protein size markers. (B) δ-Crystallin isolated from 15-day-old embryonic chicken lenses after a 3 h incubation (with vitreous bodies attached) in the presence of [^{35}S]methionine. (C) In vitro synthesized δ1 mRNA was translated in a reticulocyte lysate in the presence of [^{35}S]methionine. The translation products were immunoprecipitated with sheep anti-chicken δ-crystallin serum; 2 μg unlabelled native δ-crystallin was added prior to electrophoresis. (D) Poly(A$^+$) RNA isolated from 15-day-old embryonic chicken lenses was treated as in lane C. In lanes B–D the labelled bands co-migrated with δ-crystallin polypeptides visualized by Coomassie staining of the gel (not shown).

library. This could occur by truncation during cDNA synthesis or by elimination of exon 2 from the δ2 mRNA by alternative RNA splicing. Since the Okayama-Berg cDNA synthesis protocol [27] yields a high percentage of full-length cDNAs and our library contains a significant number of full-length δ1 cDNA clones [19], δ2 clones containing sequences from exon 2 of the δ2 gene should have been identified if a δ2 mRNA produces even the minor (50 kDa) δ-crystallin polypeptide.

Since the δ2 gene generates either trace amounts or no mRNA, both of the observed δ-crystallin polypeptides should be derived from the δ1 gene. Translation studies showed that it is possible for mRNA derived from a cloned δ1 cDNA to produce both δ-crystallin polypeptides in a pattern similar to that observed for translation of lens poly(A$^+$) RNA and for in vivo synthesis of δ-crystallin [9,31]. Although we still cannot rule out minor contributions from the δ2 gene, our data show that mRNA derived from the δ1 gene produces both of the δ-crystallin polypeptides in the embryonic chicken lens.

The mechanism for the synthesis of both δ-crystallin polypeptides from the δ1 mRNA remains unknown. Pulse-chase experiments in explanted lenses argue against post-translational modifications [9] but do not exclude the possibility that protein modifications occur co-translationally (while the protein is being formed on the ribosome). Alternative translation initiation at two in-frame methionine codons (boxed in fig.2B) has been suggested earlier for generation of the 2 δ-crystallin polypeptides [24], as appears to occur during synthesis of the 2 βA3/A1 crystallin polypeptides in several species [33–35]. Both of these AUG codons are flanked by sequences favorable for translation initiation as defined by Kozak [36,37]. However, current experiments indicate that this mechanism is not operational in δ-crystallin synthesis (in preparation).

Whatever the mechanism, our data establish that both δ-crystallin polypeptides can be produced from δ1 mRNA and that the δ2 gene is either not expressed or expressed at very low levels in the embryonic chicken lens.

ACKNOWLEDGEMENTS

We thank Dawn Chicchirichi for expert secretarial assistance.

REFERENCES

[1] Harding, J.J. and Dilley, K.J. (1976) Exp. Eye Res. 22, 1–73.
[2] Bloemendal, H.B. (1982) CRC Crit. Rev. Biochem. 12, 1–38.
[3] Piatigorsky, J. (1984) Cell 38, 620–621.
[4] De Jong, W.W. (1981) in: Molecular and Cellular Biology of the Eye Lens (Bloemendal, H. ed.) pp.221–278, Wiley, New York.
[5] King, C.R. and Piatigorsky, J. (1983) Cell 32, 707–712.
[6] Stauffer, J., Rothschild, C., Wandel, T. and Spector, A. (1974) Invest. Ophthalmol. 13, 135–146.

[7] Zigler, J.S. and Goosey, J. (1981) Trends Biochem. Sci. 6, 133–136.
[8] Harding, J.J. and Crabbe, M.J.C. (1984) in: The Eye, 3rd edn (Davson, H. ed.) pp.207–492, Academic Press, New York.
[9] Reszelbach, R., Shinohara, T. and Piatigorsky, J. (1977) Exp. Eye Res. 25, 583–593.
[10] Piatigorsky, J. (1976) J. Biol. Chem. 251, 4416–4420.
[11] Shinohara, T., Reszelbach, R. and Piatigorsky, J. (1980) Exp. Eye Res. 30, 361–370.
[12] Piatigorsky, J. (1984) J. Mol. Cell Biochem. 59, 33–56.
[13] Jones, R.E., Bhat, S.P., Sullivan, M.A. and Piatigorsky, J. (1980) Proc. Natl. Acad. Sci. USA 77, 5879–5883.
[14] Yasuda, K. and Okada, T.S. (1983) Dev. Growth Differ. 25, 422–427.
[15] Hawkins, J.W., Nickerson, J.M., Sullivan, M.A. and Piatigorsky, J. (1984) J. Biol. Chem. 259, 9821–9825.
[16] Bhat, S.P. and Piatigorsky, J. (1979) Proc. Natl. Acad. Sci. USA 76, 3299–3303.
[17] Bower, D.J., Errington, L.H., Wainwright, N.R., Sime, C., Morris, S. and Clayton, R.M. (1982) Biochem. J. 201, 339–344.
[18] Yasuda, K., Nakajima, N., Isobe, T., Okada, T.S. and Shimura, Y. (1984) EMBO J. 3, 1397–1402.
[19] Nickerson, J.M. and Piatigorsky, J. (1984) Proc. Natl. Acad. Sci. USA 81, 2611–2615.
[20] Borras, T., Nickerson, J.M., Chepelinsky, A.B. and Piatigorsky, J. (1985) EMBO J. 4, 445–452.
[21] Denhardt, D.T. (1966) Biochem. Biophys. Res. Commun. 23, 641–646.
[22] Hansen, J.N. (1981) Anal. Biochem. 116, 146–151.
[23] Krieg, P.A. and Melton, D.A. (1984) Nucleic Acids Res. 12, 7057–7070.
[24] Nickerson, J.M., Wawrousek, E.F., Hawkins, J.W., Wakil, A.S., Wistow, G.J., Thomas, G., Norman, B.L. and Piatigorsky, J. (1985) J. Biol. Chem. 260, 9100–9106.
[25] Nickerson, J.M., Wawrousek, E.F., Borras, T., Hawkins, J.W., Norman, B.L., Filpula, D.R., Nagle, J.W., Ally, A.H. and Piatigorsky, J. (1986) J. Biol. Chem. 261, 552–557.
[26] Ohno, M., Sakamoto, H., Yasuda, K., Okada, T.S. and Shimuar, Y. (1985) Nucleic Acids Res. 13, 1593–1606.
[27] Okayama, H. and Berg, P. (1982) Mol. Cell. Biol. 2, 161–170.
[28] Johansen, H., Schumperli, D. and Rosenberg, M. (1984) Proc. Natl. Acad. Sci. USA 81, 7698–7782.
[29] Kozak, M. (1984) Nucleic Acids Res. 12, 3873–3893.
[30] Kozak, M. (1983) Cell 34, 971–978.
[31] Piatigorsky, J. and Shinohara, T. (1977) Science 196, 1345–1347.
[32] Shinohara, T. and Piatigorsky, J. (1980) Exp. Eye Res. 30, 351–360.
[33] Gorin, M.B. and Horwitz, J. (1984) Curr. Eye Res. 3, 939–948.
[34] Hejtmancik, J.F. and Piatigorsky, J. (1983) J. Biol. Chem. 258, 3382–3387.
[35] Inana, G., Shinohara, T., Maizel, J.V. and Piatigorsky, J. (1982) J. Biol. Chem. 257, 9064–9071.
[36] Kozak, M. (1984) Nucleic Acids Res. 12, 857–872.
[37] Kozak, M. (1984) Nature 308, 241–246.
[38] Wilbur, W.J. and Lipman, D.J. (1983) Proc. Natl. Acad. Sci. USA 80, 726–730.