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Accumulation of Putative mRNA Precursors of Crystallin Genes in Neural Retina of Chick Embryos

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Abstract. Crystallins are proteins specific to lens tissue. However, it has been demonstrated that gene coding for δ-crystallin is also transcribed in several non-lens tissues (neural retina, brain, limb bud) of early chick embryos by Northern blots (Agata et al., 1983). The present paper reports two separate analyses to determine whether gene expression is similar in both lens and non-lens tissue for crystallin genes. First, the quantity of transcripts of the δ-crystallin gene in embryonic neural retina was estimated by liquid hybridization using a single-stranded probe corresponding to the mRNA complementary strand. Results showed that 0.0028% of poly(A)+RNAs of neural retina in both 3.5-day and 8.5-day chick embryos were δ-crystallin transcripts. This suggests that (1) the larger RNA transcripts detected in Northern blots in the previous study (Agata et al., 1983) are derived from the coding strand of the δ-crystallin gene and maybe precursors of mature mRNA; and (2) these precursors are major components of δ-crystallin transcripts in embryonic neural retina. Second, expression of αA- and δ-crystallins were compared using Northern blotting. No mature-sized transcripts in contrast to larger RNA species were detected in neural retina of 3.5-day-old chick embryos using the αA-crystallin probe. However, both mature-sized transcripts and larger RNA species were detected with the δ-crystallin probe. These observations suggest that lens cell specific genes are transcribed in non-lens tissues of early chick embryos. However, the maturation process for precursor RNAs in heterologous tissues are different from those in the lens.

Introduction

Embryonic cells have the potential for several pathways of differentiation. Some of these possibillties are revealed only by manipulative procedures; for example, transplantation or *in vitro* cell culture. As differentiation proceeds, cell lose their potential for expression of alternative pathways and become restricted to a single phenotype. To investigate the molecular basis of this phenomenon, transcription of lens specific genes in the neural retina of early chick embryos was analyzed. It is well-known that the neural retina (NR) cells of early chick embryos may transdifferentiate into lens under *in vitro* conditions (Okada, 1980, 1983; Clayton, 1982).

Crystallins are proteins specifically located in lens. However, in a previous paper (Agata

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et al., 1983), it was demonstrated that gene coding for δ-crystallin is transcribed in several non-lens tissues of early chick embryos. This was shown by Northern blot analyses using cloned δ-crystallin cDNA. The Northern blot of poly(A)$^+$RNA of 3.5-day-old embryonic NR showed that the ratio of mature δ-crystallin mRNA to total poly(A)$^+$RNAs in non-lens tissues was 0.001% based on the intensity of the band in the Northern blot. Furthermore, we have observed abundant larger RNA species hybridizing with δ-crystallin cDNA in non-lens tissues, in addition to mature mRNA. These larger RNA species may be precursors of δ-crystallin transcripts, since no other DNA sequence hybridizing with the same probe is detected besides the δ-crystallin gene in the chicken genome (Yasuda et al., 1982). In the present study, Rot analysis was conducted to investigate whether these larger RNA transcripts were actually derived from the coding strand of the δ-crystallin gene, using a single-stranded probe corresponding to the mRNA complementary strand. In addition, Northern blot analysis was used to determine whether the αA-crystallin gene is also transcribed in NR.

Materials and Methods

Preparation of poly(A)$^+$RNAs. NRs were isolated from 1,700 3.5-day and 140 8.5-day chick embryos. Lenses and livers were collected from 100 one-day chickens. These tissues were homogenized in a solution containing 0.1 M Tris-HCl (pH 9.0), 0.5% Nonidet P–40 and 10 mM vanadyl-ribonucleoside complexes (Berger and Birkenmeier, 1979). The homogenate was centrifuged at 4°C for 5 min at 10.000×*g*. The cytoplasmic fraction in the supernatant was extracted twice with an equal volume of phenol saturated with 0.1 M Tris-HCl (pH 9.0) containing 10 mM EDTA. Cytoplasmic RNA was precipitated with ethanol at −20°C and suspended in water. Cytoplasmic RNA was passed through an oligo (dT) cellulose column (Collaborative Research) to collect bound RNA as poly(A)$^+$RNA (Avin and Leder, 1972). From 3,400 3.5-day NR and 280 8.5-day NR, 127 μg and 220 μg, respectively, of poly(A)$^+$RNA were obtained. The percentages of the bound RNA were 1.9% and 1.7%, respectively.

Preparation of ^{3}H-labeled δ-crystallin cDNA (δ-cDNA). δ-crystallin mRNA (δ-mRNA) was purified by essentially the same procedure as used by Yasuda *et al* (1982). Lens poly(A)$^+$RNA was fractionated by sucrose density gradient (5%–20%) centrifugation. Each RNA fraction was translated *in vitro* by a reticulocyte lysate (Amersham), and the products analyzed by SDS gel electrophoresis (Laemmli, 1970) (Fig. 1A). The fractions with high δ-mRNA and low non-δ-mRNA were combined and further purified by one additional cycle of density gradient centrifugation (Fig. 1B). δ-cDNA labeled with ^{3}H-dCTP (50 Ci/mmol, Amersham) was synthesized by reverse transcription using the purified δ-mRNA as the template (Chang et al., 1978). δ-cDNA was denatured in a mixture consisting of 1 M glyoxal, 50% dimethylsulfoxide (DMSO) and 10 mM sodium phosphate (pH 7.0) for 60 min at 50°C (McMaster and Carmichael, 1977), electrophoresed on a 1% agarose gel, and fluorographed. Most cDNA migrated to a position similar to that of δ-mRNA (Fig. 2), indicating that same-size cDNA were synthesized. The specific activity of the δ-cDNA obtained by this procedure was 1.2×10^6 cpm/μg. This δ-cDNA was used as the probe in liquid hybridization reactions.

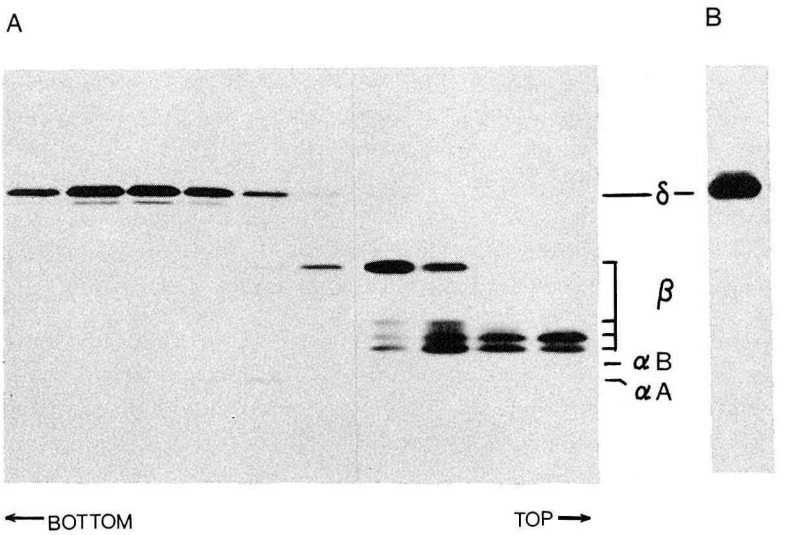

Fig. 1. SDS-polyacrylamide gel electrophoresis of *in vitro* translation products of fractionated lens poly(A)$^+$RNAs. Panel A shows products of lens poly(A)$^+$RNAs fractionated by first step centrifugation. Each crystallin class (α-, β-,δ -) is indicated on the right side of the panel. Panel B shows that the product of the fraction of the second step centrifugation is only δ-crystallin. This purified fraction was used as a template for synthesis of ^{3}H-δ-cDNA.

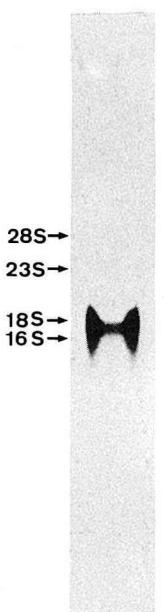

Fig. 2. Molecular size analysis of synthesized ^{3}H-δ-cDNA. Denatured ^{3}H-δ-cDNA was electrophoresed on a vertical agarose gel and fluorographed. Ribosomal RNAs of chicken and *E. coli* were used as the size marker.

Liquid hybridization reactions. Hybridization was performed in 20–50 μl of the reaction mixture containing 0.6 M NaCl, 0.2 mM EDTA, 20 mM Tris-HCl (pH 7.4), 0.1 ng of ^{3}H-cDNA and 0.1 μg/ml–5.0 mg/ml of RNA (Yasuda et al., 1983). The mixture was overlaid with buffer-

saturated liquid paraffin in an 1.5 ml polyethylene tube and the reaction was allowed to proceed at 75°C for 6 min–28 hr, to give appropriate Rot values. Hybridization was stopped by diluting the mixture in 1 ml of cold 50 mM sodium acetate (pH 4.5), 2 mM $ZnSO_4$, 0.1 M NaCl and 10 μg/ml of heat-denatured, sonicated, salmon sperm DNA. Each sample was divided into two aliquots. These were incubated for 1 hr at 37°C either in the presence of absence of 20 U of S_1 nuclease (Sigma, Type III). The ^{3}H-cDNA precipitated with 10% TCA was collected on Whatman GF/C glass-fiber filters and the radioactivity counted. The percentage of ^{3}H-cDNA hybridized was calculated from the ratio of ^{3}H-cDNA precipitated in a tube containing S_1 nuclease compared to a tube without enzyme.

Probes for Northern blots. A cloned δ-cDNA, designated pBδ 5, was obtained from Dr. K. Yasuda (Yasuda et al., 1982). By *Pst* I digestion, 0.8 kb of δ-cDNA fragment inserted in this plasmid was prepared and then was radiolabeled with ^{32}P by nick translation (Amersham nick translation kit, PB. 5100). A cloned αA-crystallin cDNA (αA-cDNA) was obtained from K. Okazaki (Okazaki et al., 1984).

Northern blot analyses. RNAs were denatured in a mixture of 1 M deionized glyoxal, 50% DMSO and 10 mM phosphate buffer (pH 7.0) at 50°C for 1 hr (McMaster and Carmichael, 1977). Samples were electrophoresed on a horizontal 1.0% agarose gel and transferred to a nitrocellulose filter by a technique developed by Thomas (1980). Each blot was baked in a vacuum oven for 4 hr at 80°C and prehybridized in a mixture of 50% (v/v) formamide, 5×SSC (1×SSC contains 0.15 M NaCl and 0.015 M trisodium citrate), 5×Denhardt's solution (1×Denhardt's solution contains 0.02% bovine serum aíbumin, 0.002% Ficoll, and 0.002% polyvinylpyrrolidone), 50 mM sodium phosphate (pH 6.5), 1% glycine, and denatured salmon sperm DNA at 0.25 mg/ml for 15 hr at 42°C. Nick-translated probes were denatured at 100°C for 3 min, cooled and added to a hybridization buffer containing 50% (v/v) formamide, 5× SSC, 1×Denhaldt's solution, 50 mM sodium phosphate (pH 6.5), and 10% (w/v) dextran sulfate. Each RNA blot was hybridized for 20 hr at 42°C, washed at the stringent condition, with four changes of 2×SSC containing 0.1% SDS for 5 min at room temperature, and then washed with two changes of 0.1×SSC containing 0.1% SDS for 15 min at 50°C. Blots were exposed to X-ray film at −70°C. Each RNA blot hybridized with ^{32}P-labeled δ-cDNA was washed in buffer containing 5 mM Tris-HCl at pH 8.0, 0.2 mM EDTA, 0.05% sodium pyrophosphate and 0.002% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone for 2 hr at 65°C (Thomas, 1978). RNA blots were then hybridized with nick-translated αA-cDNA.

Results

Kinetics of hybridization of poly(A)$^+$RNAs of embryonic NR to ^{3}H-labeled-cDNA

^{3}H-labeled cDNA synthesized using highly purified δ-mRNA as template was used as the probe in lqiuid hybridizations. Only transcripts derived from the coding strand of the δ-crystallin gene were detected in this experiment. Results of liquid hybridizations between δ-cDNA and poly(A)$^+$RNAs extracted from embryonic NR are given in Fig. 3. Assays were also done with purified δ-mRNA, and cytoplasmic RNAs of lens and liver. The percentage

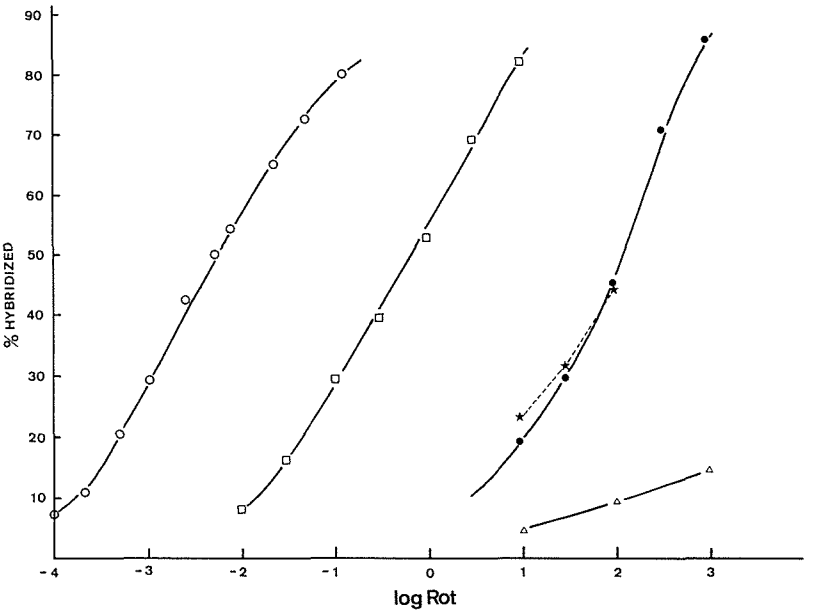

Fig. 3. Liquid hybridization reactions of ^{4}H-δ-cDNA to poly(A)$^{+}$RNAs of embryonic NR. (★——★), 3.5-day embryonic NR; (●——●), 8.5-day embryonic NR. Kinetics of hybridization with purified δ-mRNA (○——○), cytoplasmic RNA of lens (□——□) and cytoplasmic RNA of liver (△——△) from chickens one day after hatching are also given.

Table 1. Relative abundance of δ-crystallin transcripts

Sourse of material	RNA sample	$Rot_{1/2}$ (M · sec)	Relative abundance
1-day chicken lens	purified δ-mRNA	2.5×10^{-3}	1.0
1-day chicken lens	total cytoplasmic RNA	3.0×10^{-1}	0.0083
3.5-day embryo NR	poly(A)$^{+}$RNA	9.0×10^{1}*	0.000028*
8.5-day embryo NR	poly(A)$^{+}$RNA	9.0×10^{1}	0.000028

* These values are estimated on the assumption that $Rot_{1/2}$ is the same as for the 8.5-day sample.

of cDNA hybridized with δ-mRNA, whole cytoplasmic RNA of lens and poly(A)$^{+}$RNA of 8.5-day embryonic NR, increased up to 81%, 84% and 91%, respectively. The $Rot_{1/2}$ values (M · sec) are given in Table 1. Poly(A)$^{+}$RNA prepared from 8.5-day-old embryonic NR reacted 36,000 times more slowly ($Rot_{1/2}$ of 9.0×10^{1} M · sec) than purified δ-mRNA ($Rot_{1/2}$ of 2.5×10^{-3} M · sec). By calculation, 0.0028% of the poly(A)$^{+}$RNA in 8.5-day embryonic NR hybridized with the single-stranded probe of δ-cDNA (Table 1). Using cytoplasmic RNA of liver, 15% of cDNA hybridized at a Rot value of 1.0×10^{3} M · sec.

Hybridization with poly(A)$^{+}$RNA of 3.5-day embryonic NR did not reach saturation levels at the highest Rot value tested. However, the Rot curve of 3.5-day embryonic NR (from a Rot value of 1.0×10^{1} M · sec to 1.0×10^{2} M · sec) was very close to that obtained with poly(A)$^{+}$RNA of 8.5-day embryonic NR. The $Rot_{1/2}$ value of 3.5-day embryonic NR was estimated as 9.0×10^{1} M · sec on the assumption that the $Rot_{1/2}$ vlaue was the same as the 8.5-day sample (Table 1).

These results suggest that the transcripts including RNA species larger than δ-mRNA detected in Northern blots (Agata et al., 1983) (see also Fig. 4A) are derived from the coding strand of the δ-crystallin gene and may be precursors of mature δ-mRNA. Northern blots in the previous study (Agata et al., 1983) have shown that the ratio of mature δ-mRNA in poly(A)$^+$RNA of embryonic NR decreased with development. Very little mature δ-mRNA was detected in 8.5-day embryonic NR, and the ratio of mature δ-mRNA was not more than 0.001% even in 3.5-day embryonic NR. These results indicate that precursor RNAs are major components of δ-crystallin transcripts in embryonic NR.

Northern blot analyses using αA- and δ-crystallin cDNAs as probes

The poly(A)$^+$RNA of 3.5-day-old embryonic NR was analyzed by Northern blot using cloned δ-cDNA and αA-cDNA as probes to compare the transcription mode among lens specific genes in embryonic NR. 10 μg of poly(A)$^+$RNA of 3.5-day-old embryonic NR was electrophoresed and compared with 100 ng of lens cytoplasmic RNA of 1-day-old chicken electrophoresed in parallel. Using the δ-cDNA probe, an RNA species of the same molecular size as δ-mRNA, in addition to larger RNA species hybridized with δ-cDNA were clearly seen in poly(A)$^+$RNA of 3.5-day embryonic NR (Fig. 4A). This comfirms the report in the previous paper (Agata et al., 1983).

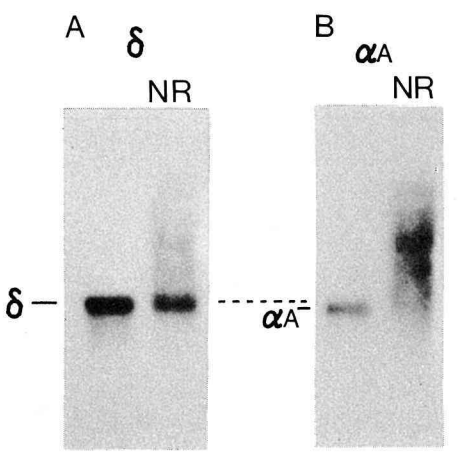

Fig. 4. Northern blot analysis of poly(A)$^+$RNA of 3.5-day embryonic NR. Lens cytoplasmic RNA of 1-day (hatched) chickens (100 ng) and poly(A)$^+$RNA of 3.5-day embryonic NR (10 μg) were electrophoresed. A, hybridized with δ-cDNA. B, the same filter hybridized with αA-cDNA.

The filter was washed to remove the δ-cDNA probe and then hybridized with nick-translated αA-cDNA (Fig. 4B). The band corresponding to αA-crystallin mRNA was not seen in poly(A)$^+$RNA of 3.5-day embryonic NR, although such a band was clearly seen in lens cytoplasmic RNA. Instead, larger RNA species hybridizing with αA-cDNA were detected in NR. These results are confirmed in three separate experiments. Hybridization of re-washed filters with ^{32}P-labeled pBR322 probe did not show any hybridizing RNA species. It has been shown that there is no DNA sequence hybridizing with the same probe other than the αA-crystallin gene in the chicken genome (Okazaki et al., 1984). This suggests that αA-crystallin transcripts are present in 3.5-day embryonic NR. However, these transcripts are not processed

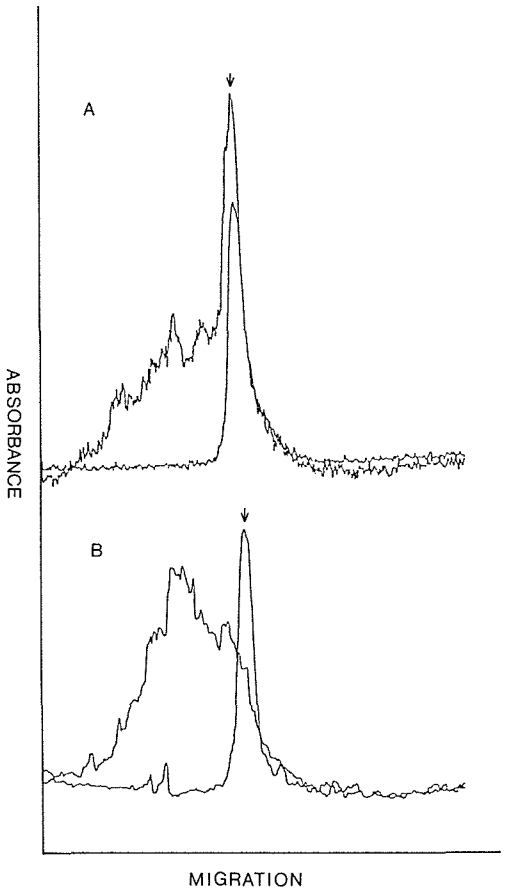

Fig. 5. Densitometry of autoradiographs of Northern blots given in Fig. 4. Migration of RNA was from left to right. A, Scans of blots hybridized with δ-cDNA. Arrow indicates the peak of the mature size δ-mRNA. B, Scans of blots hybridized with αA-cDNA. Arrow indicates the peak of the mature size αA-mRNA.

to mature mRNA in non-lens tissues. These results were further confirmed by desnsitometric analysis (Fig. 5).

Discussion

In this paper, the previous observation (Agata et al., 1983) of transcription of the lens-specific δ-crystallin gene in NR of early chick embryos was confirmed and quantitated more accurately using the technique of liquid hybridization. The proportion of δ-crystallin transcripts in total poly(A)$^+$RNAs in NR was much lower than in lens cells. Only 0.0028% of poly(A)$^+$RNA as δ-crystallin transcripts in embryonic NR was calculated from $Rot_{1/2}$ values in contrast to 70%–80% of poly(A)$^+$RNA as δ-mRNA in lens (Piatigorsky, 1984). In addition, it was possible to estimate the copy numbers of δ-crystallin transcripts per cell of 3.5-day NR from $Rot_{1/2}$ values. If the δ-crystallin gene is transcribed equally in every NR cell of 3.5-day embryos, δ-crystallin transcripts accumulate to approximately 10 copies per cell.

In the present study, the $Rot_{1/2}$ value of 8.5-day embryonic NR is almost the same as that of 3.5-day embryonic NR. However, it has been shown in the previous paper (Agata et al.,

1983) that the amount of mature δ-mRNA decreases with development and disappears in 1-day-old chicken NR. This apparent discrepancy is accounted for by the presence of larger RNA species hybridizing with δ-cDNA. These larger RNA species reassociate with the single-stranded probe corresponding to the mRNA complementary strand in liquid hybridization. Thus, larger RNA species derived from the coding strand of the δ-crystallin gene may be precursors of the mature δ-mRNA. It has been shown that the amount of larger RNA speices reached a peak in 5.5–8.5-day embryos in Northern blots (Agata et al., 1983). Most δ-crystallin transcripts are the putative precursors in embryonic NR at these stages.

There are three major classes of crystallins (α-, β- and δ-crystallin) in the chicken lens (Fig. 1A). These crystallins are expressed coordinately with differentiation of lens *in vivo* (Zwaan and Ikeda, 1968) and with transdifferentiation of NR into lens *in vitro* (Thomson et al., 1978; Araki et al., 1979). The Northern blot suing αA-cDNA shows that the αA-crystallin gene is also transcribed in NR but the putative precursor RNAs are not processed.

These observations suggest that lens specific genes are transcribed in embryonic NR but that the RNA processing machinery of heterologous tissue is different from that of lens tissue. It remains to be examined whether mature-sized δ-mRNAs in NR can be translated *in vivo* with the same efficiency as in lens cells. In this regard, Barabanov (1977) reported that δ-crystallin is transiently expressed in adenohypophysis of early chick embryos. The presence of mature δ-mRNA in non-lens tissues may reflect similar transient expression of δ-crystallin in early chick embryos. I conclude that lens cell specific genes are transcribed in some non-lens tissues of early chick embryos, however, the maturation process for precursor RNAs in heterologous tissues may be different from those in the lens.

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