REGULATION OF THE TWO DELTA CRYSTALLIN GENES DURING LENS DEVELOPMENT IN THE CHICKEN EMBRYO

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ABSTRACT

Title of Dissertation: Regulation of the Two Delta Crystallin Genes During Lens Development in the Chicken Embryo

Xuan Li, Doctor of Philosophy, 1991

Dissertation directed by: David C. Beebe, Ph.D.
Chairman and Professor
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Delta-crystallin is the major structural protein in the chicken embryo lens. It is encoded by two very similar genes, δ_1 and δ_2. During lens development, δ_1 mRNA is more abundant than δ_2 mRNA and the δ_1/δ_2 ratio is greater in lens fibers than in lens epithelial cells. Thus, the expression of the two δ-crystallin genes is differentially regulated in different regions of the lens (Thomas, et al., 1990). Although, in vitro transfection studies suggested at regulation might occur at the post-transcriptional level, the mechanism responsible for the different levels of mRNA from these two genes is not known.

In my studies, I first reexamined prior work by others indicating that crystallin mRNA becomes stabilized in lens epithelial cells between days 11 and 13 of embryonic development. My results did not support this conclusion. In contrast, by the tests employed, these mRNAs were already quite stable at these stages of development.

I then developed a new method, which coupled density-

labelling with the polymerase chain reaction (PCR), to study mRNA metabolism in the embryo. Using this technique, I found that the δ_1/δ_2 ratio in newly synthesized mRNA was not significantly different from the ratio in pre-existing mRNA. Using a related method, I showed that there was a similar ratio of δ_1 to δ_2 transcripts in both unprocessed and mature mRNA. These data strongly suggest that the differential regulation of the two δ-crystallin genes was at the level of transcription, with transcription of the δ_1-crystallin gene greatly exceeding transcription of the δ_2-crystallin gene in most parts of the lens.

I also measured the δ_1/δ_2 ratio in the same culture systems used previously by other investigators for <u>in vitro</u> transfection studies. The results showed that these cells expressed equal amounts of the two δ-crystallin mRNAs. Thus, studies in which these cells were used as an assay system may have led to erroneous conclusions about the regulation of δ-crystallin gene expression.

My studies provide a new view of δ-crystallin gene expression. They should lead to additional investigations of this interesting gene family.

REGULATION OF THE TWO DELTA CRYSTALLIN GENES DURING LENS DEVELOPMENT IN THE CHICKEN EMBRYO

By

Xuan Li

Dissertation submitted to the Faculty of the Department of Anatomy Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1991

DEDICATION

To my father and mother

for their love and their dreams.

To my husband Hanlong

for his constantly support and his encouragement.

To my lovely daughter Victoria

for her future understanding.

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This thesis, and the degree it represents, could never have been completed without the help and support of my thesis advisor, Dr. David C. Beebe, who provided the starting point and the motivation for this dissertation, and guided me through the entire line of research. He read and corrected this thesis very carefully and showed me there is a lot to learn in English writing. His comments significantly improved its range, accuracy, and conciseness. I wish to express my heartfelt appreciation towards him.

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Dr. Joram Piatigorsky provided the delta crystallin cDNA clone and Dr. Robert Grainger generously offered the density labelled ribonucleosides. Without such help, the experiments presented in this thesis would not be possible.

I am grateful to my colleagues and good friends Beth Kaplan and Susanne Cerrelli from whom I learned many things about the U.S. and about the laboratory. Beth taught me about basic molecular biology techniques at the beginning of my studies. Susanne helped me to dissect the lenses in my actinomycin D experiments.

I would like to thank Greg Decker for showing me techniques to format this thesis.

Connie Pechura helped me throughout my first two years of studies. She invited me to stay in her house when I first

came to U.S. and helped me with my English. She spent a great deal of time recording all the terms in gross anatomy, to help me correct my pronunciation. I benefitted tremendously during my second year of teaching.

My husband Hanlong Yao, accompanied me many nights to help me run the serial dilution quantitation experiments. Without his support, the thesis data would not be finished so soon.

I sincerely thank my parents and my brother. My father Chujie Li, has always had high standards for research. His scientific attitude motivated me to pursue my scientific career. My mother Daohui Cai, helped me to take care my daughter Vicky during my last half year of studies and provided me extra time to do my experiments and write my thesis. My brother Kai Li, has always been concerned with my studies. His constant moral support and encouragement motivated me to overcome the difficulties that I encountered in my thesis work.

I would like to thank all the people in Department of Anatomy, who helped me every step along the way to make my graduate work successful and my life enjoyable.

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CHAPTER I

Introduction

General Review of Gene Expression and Regulation

Gene expression in eucaryotes can be regulated at many steps in the pathway leading from DNA to protein (Darnell, 1982; see Fig. 1):

1. Transcriptional control, determining when and how often a given gene is transcribed.

2. RNA processing control, altering the rate or extent to which the primary RNA transcript is spliced or processed.

3. RNA transport control, selecting which completed mRNAs in the cell nucleus are exported to the cytoplasm.

4. Translational control, determining which mRNAs in the cytoplasm are translated by ribosomes.

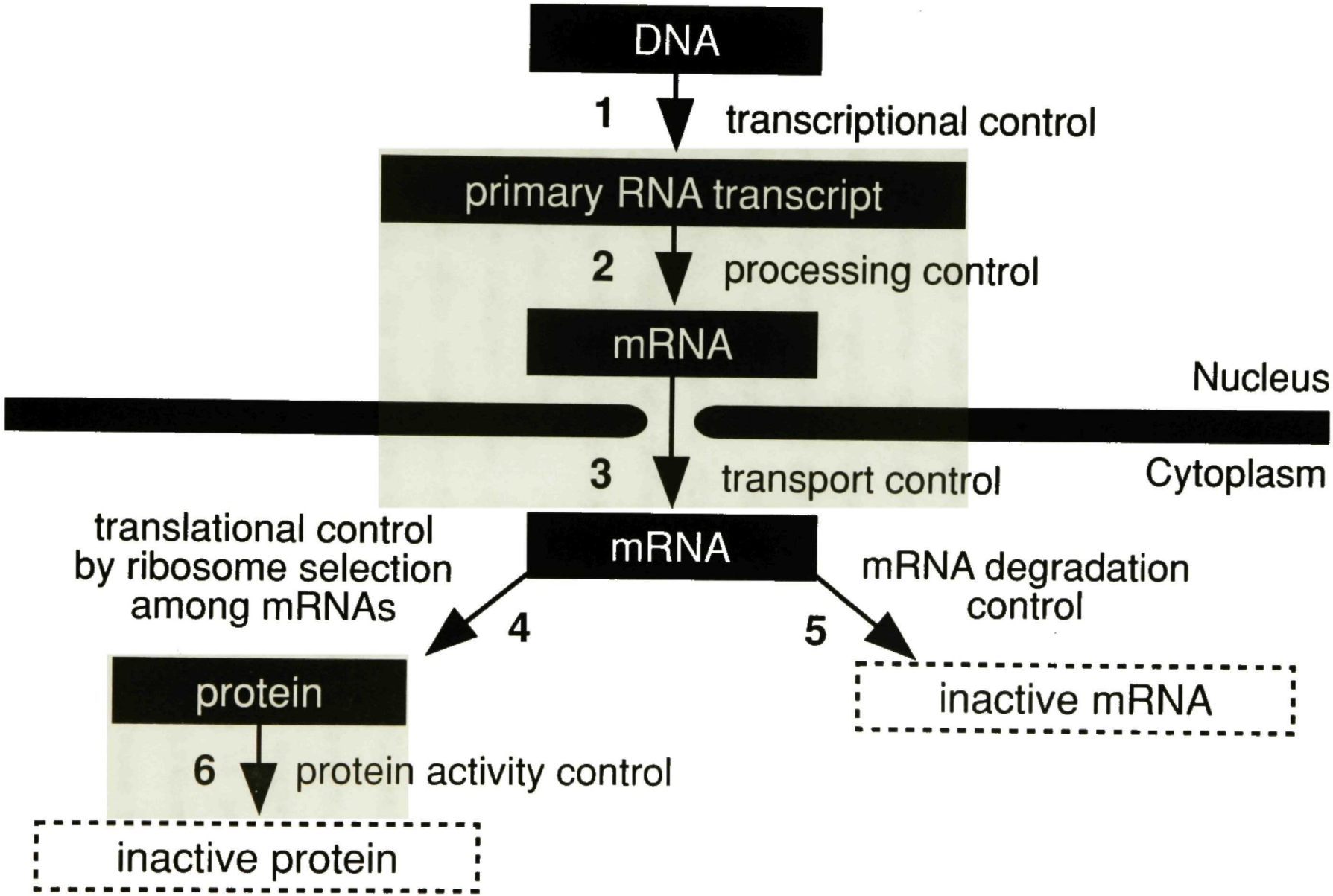
5. mRNA degradation control, selectively destabilizing certain mRNA molecules in the cytoplasm.

6. Protein activity control, selectively activating, inactivating, or compartmentalizing specific protein molecules after they have been made.

1. Transcriptional control:

The expression of most genes is controlled predominantly at the transcriptional level (Derman, et al., 1981). Transcriptional controls depend on gene regulatory proteins

Fig. 1. **Six steps at which gene expression can be controlled in eucaryotes** (from Alberts, et al., 1989).


DNA
1
transcriptional control
primary RNA transcript
2
processing control
mRNA
Nucleus
Cytoplasm
3
transport control
translational control
by ribosome selection
among mRNAs
mRNA
mRNA degradation
control
4
5
protein
inactive mRNA
6
protein activity control
inactive protein

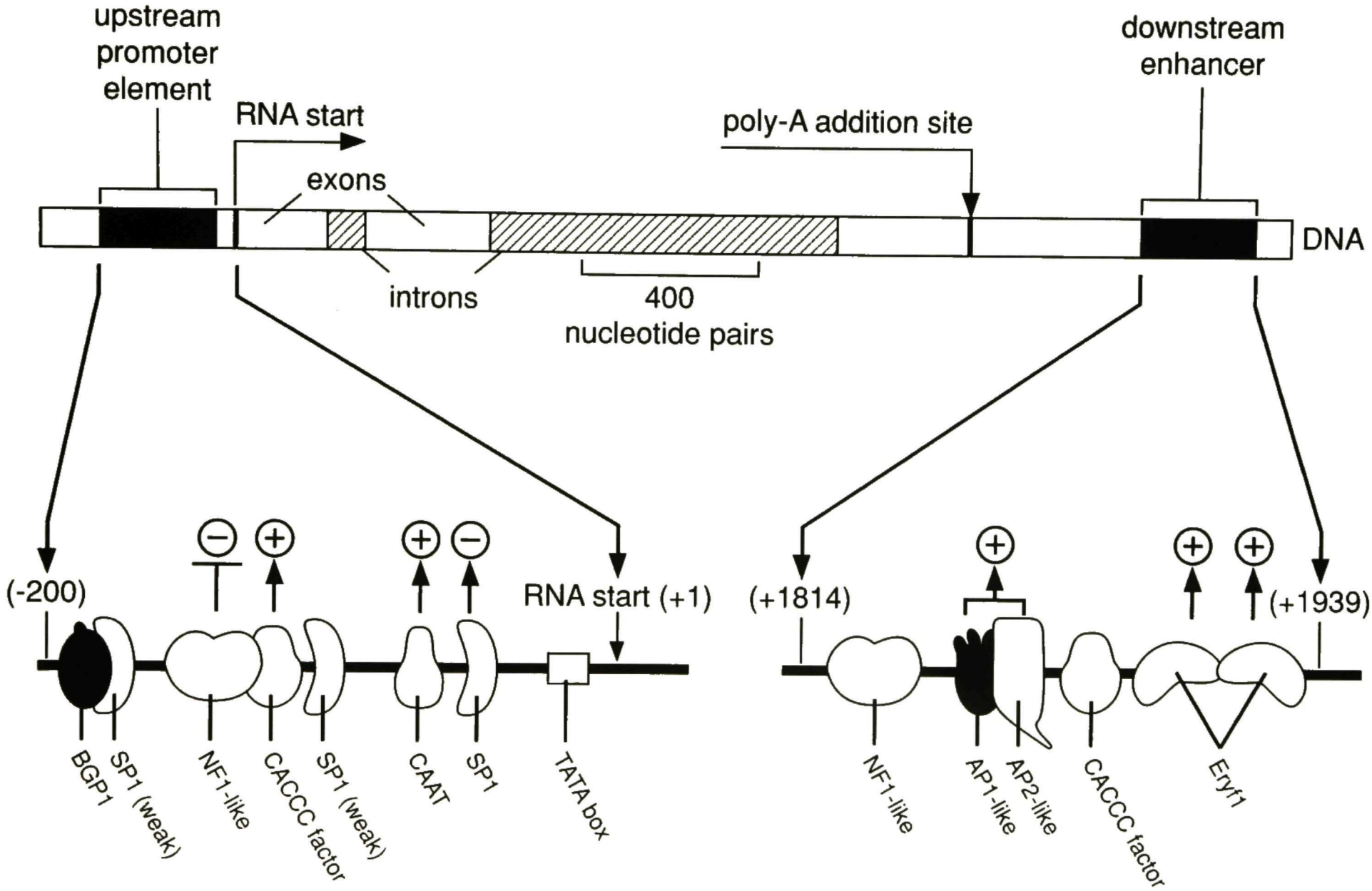
that bind to specific DNA sequences. These proteins can help to turn a gene either on (positive control) or off (negative control) (Maniatis et al., 1987). Genes in higher eucaryotes usually are regulated by the combinatorial effects of several such positive and negative gene regulatory proteins (Sen and Baltimore, 1986; Yamamoto et al., 1988).

So far, many elements or factors involved in the regulation of transcription have been discovered (see Fig. 2). I have briefly listed some representative factors below:

a) Master gene regulatory proteins: experimentally these proteins can regulate large sets of genes and play a special role in expressing different genes from the same genome (Gehring and Hiromi, 1986). For example, myoD1, a single master gene regulatory protein that normally specifies a "myoblast" phenotype, can convert a fibroblast to a myoblast when it expressed at a high enough concentration in a fibroblast (Davis et al., 1987).

b) TATA factor: This is a sequence-specific DNA-binding protein that binds to the consensus "TATA box" DNA sequence, TATAAA. The activity of the this factor stimulates RNA polymerase II transcription (Workman and Roeder, 1987). It is recognized by RNA polymerase II, the enzyme which synthesizes all precursor mRNAs. The TATA box is generally located at position -25 to -30 relative to the transcription start site. In vitro experiments suggest that once the TATA factor has bound to the DNA at a promoter, which contains the start site for RNA synthesis and signals where RNA synthesis

Fig. 2. **Binding sites for gene regulatory proteins and their effects** (from Alberts, et al., 1989).


upstream
promoter
element
RNA start
exons
poly-A addition site
downstream
enhancer
DNA
introns
400
nucleotide pairs
(-200)
RNA start (+1)
(+1814)
(+1939)
BGP1
SP1 (weak)
NF1-like
CACCC factor
SP1 (weak)
CAAT
SP1
TATA box
NF1-like
AP1-like
AP2-like
CACCC factor
Eryf1

should begin, it tends to remain in a stable transcription complex that can mediate multiple rounds of transcription by RNA polymerase II molecules (Bossy, Hall and Spierer, 1984).

c) Upstream promoter and enhancer elements: Upstream promoter elements are generally located near the promoter region, extend for about 100 nucleotides and include the TATA box. Enhancer elements are generally located further upstream, several thousand nucleotides from the promoter they regulate. Some may be more than 20,000 nucleotides away. Both enhancer and upstream promoter elements contain a series of short nucleotide sequences that bind a corresponding series of gene regulatory proteins. The proteins bound to these two types of regulatory elements cooperate to turn genes on or off (Maniatis et al., 1987; McKnight and Kingsbury, 1982). It has been thought that the rise and fall in transcription of some genes, such as the chicken erythrocyte β-globin gene, is due to a change in the balance of positively and negatively acting gene regulatory proteins (Emerson et al., 1987; Evans et al., 1987).

d) DNA methylation: In general, the DNA of inactive genes is more heavily methylated than that of active genes. In most tissue-specific genes, demethylation is involved in the initiation of transcription. Evidence suggests that DNA methylation accounts for at least a part of the control of gene expression in vertebrates (Cedar, 1988).

2. Post-transcriptional controls:

a) Transcription attenuation causes the transcription of some RNA molecules to be terminated prematurely (Platt, 1986). It regulates the expression of a small proportion of genes in eucaryotes.

b) Alternative splice site selection controls RNA splicing: It can lead to the production of different proteins from a single pre-mRNA or can function as an on-off switch during development (Maniatis, 1991). It is the primary mechanism involved in the regulatory hierarchy of sex determination in *Drosophila*. By regulating the expression of gender-specific genes it controls tissue-specific gene expression (Bingham et al., 1988).

c) Control of translational initiation: Translational control include positive and negative control. A very good example of the negative translational control is regulation of ferritin mRNA translation (Aziz and Munro, 1987). When a protein is bound to the "iron-response element" in a mRNA, the translation of any downstream RNA sequence is repressed. In contrast, if iron dissociates the protein from the mRNA, the rate of translation of the mRNA can be increased about 100-fold. In addition, a special "translation-enhancer" region could be found in certain RNA viruses, the picornaviruses (Pelletier, and Sonenberg, 1988). This translation-enhancer region can cause translation to begin at internal AUG sites.

e) Regulated mRNA degradation: mRNA stability can be differentially controlled (Atwater, et al., 1990). The stability of some mRNAs can be regulated by the extracellular

environment. For instance, hormones or nutrients can alter mRNA half life. Vitellogenin mRNA is stabilized by estrogen treatment, which can cause the amount of the vitellogenin mRNA to increase about 50 fold. This is due to an increase in the half life of the mRNA from 16 hr to about 500 hr (Brock and Shapiro, 1983). Transferrin receptor mRNA is destabilized by iron, which reduces receptor mRNA levels. Conversely, treatment with desferrioxamine, an iron chelator, resulted in a twenty-fold stabilization of transferrin mRNA in the cytoplasm (Muller and Kuhn, 1988).

The stability of some mRNAs can also be regulated by the cells themselves. This can be by translational feedback, cell cycle control, or mRNA structural features.

Lens Structure and Development

The transparent eye lens, which transmits and focuses light onto the retina, has fascinated developmental biologists and ophthalmologists since the early 1890's. In 1891 Colucci discovered lens regeneration (cited in Reyer, 1954). In 1894, Morner described the high concentrations of structural proteins in the lens, called crystallins. Then in 1901, Spemann provided the first studies of lens embryonic induction (Spemann, 1962).

The lens has distinct advantages as an experimental system because of its unusual and very orderly structure. The mature lens is composed of two different cell populations within the lens capsule (Harding, and Crabbe, 1983; Kuwabara,

1983). A monolayer of cuboidal epithelium covers the anterior of the lens, while highly elongated, non-nucleated fiber cells comprise the bulk of the lens. Fiber cells synthesize and accumulate large amounts of crystallins (Bloemendal, 1982).

The fiber cells are responsible for the major functional properties of the lens, such as transparency and high refractive index. These properties are achieved by the regular arrangement of the fiber cells and by the presence of stable, lens-specific proteins, the crystallins. In the transparent lens, light is minimally scattered because fiber cells and their proteins are densely packed in an orderly way (Harding and Dilley, 1976; Bloemendal, 1981). The high concentrations of the crystallin proteins provide the medium of high refractive index that is necessary for lens function (Delaye and Tardieu, 1983; Benedek, 1971).

The epithelium at the anterior of the lens is responsible for maintaining the health of the fibers and for providing new fiber cells throughout life. Epithelial cells transport nutrients into the fiber cells. They also maintain normal levels of intracellular ions in the fiber cells and they produce and transport high levels of glutathione, which is essential to prevent crystallin aggregation and to maintain the health of fiber cell membranes (Goodenough, 1979; Goodenough et al., 1980; Reddy, 1990).

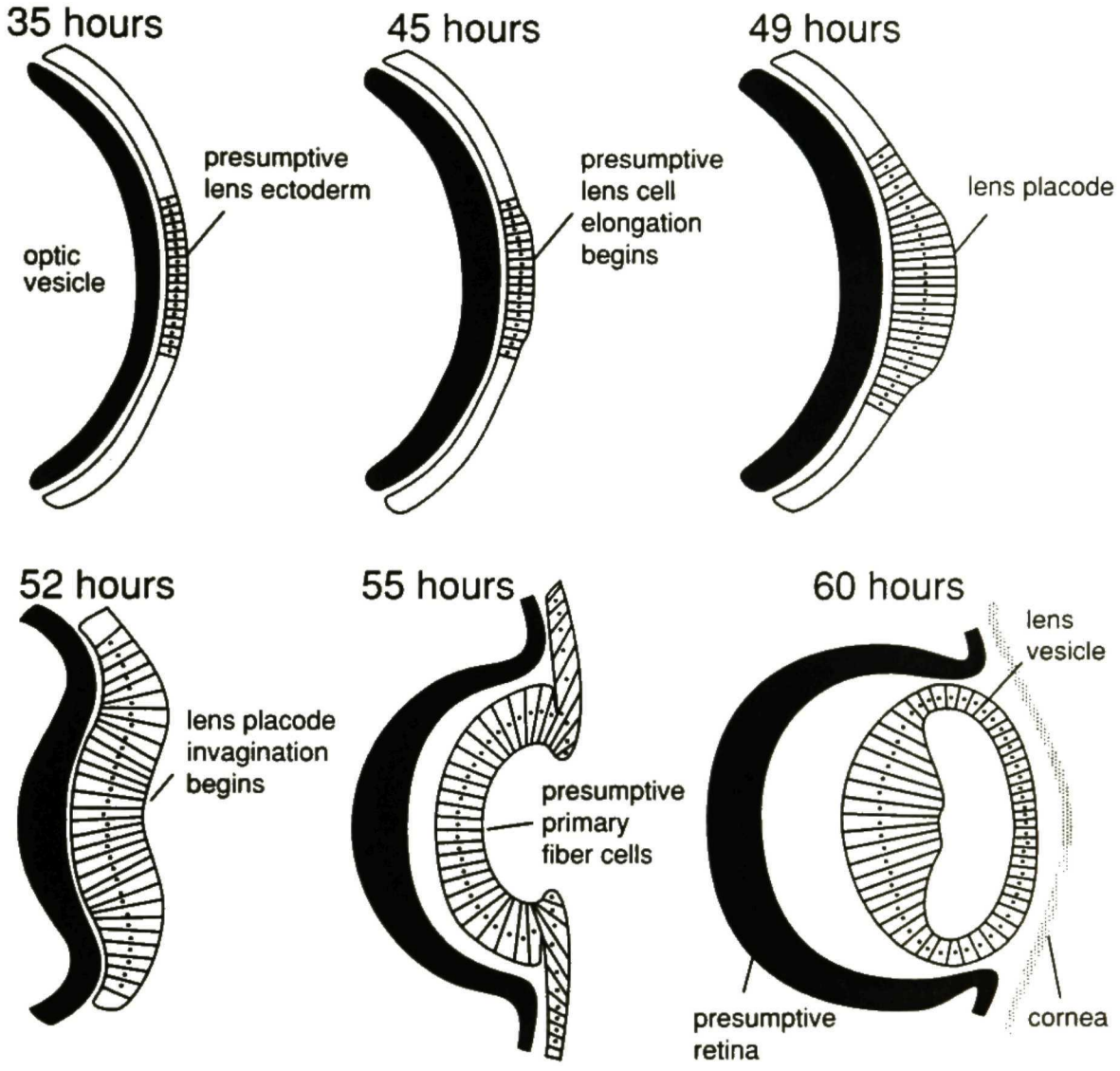
During development and growth, new fiber cells are formed near the lens equator. These new fiber cells are continuously formed at all ages from the progeny of epithelial

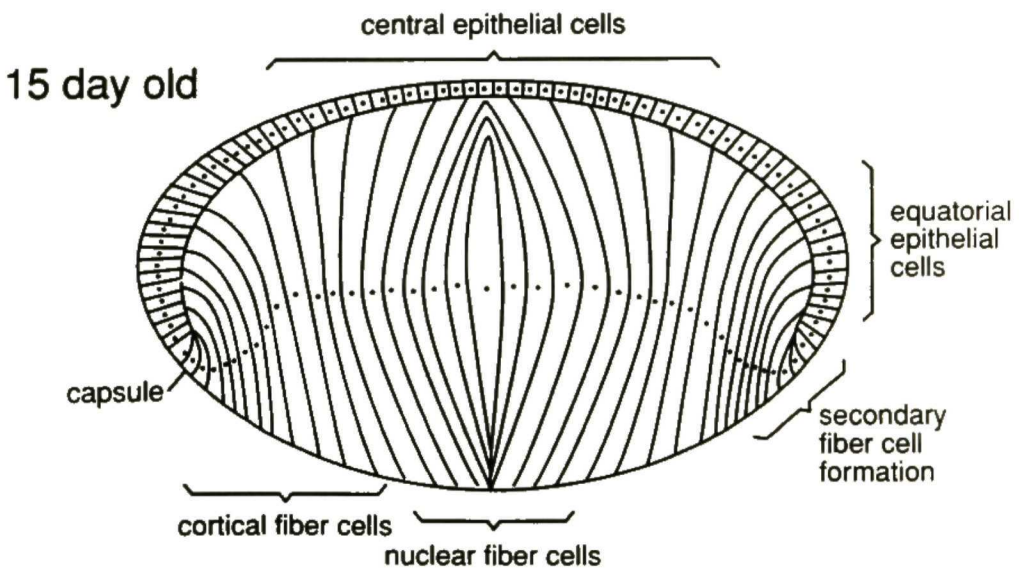
cell divisions (Messier and Leblond,1960; Hanna and O'Brien, 1961; Brolin et al., 1961). As the lens grows, the mitotically active anterior epithelial cells move towards the equator of the lens from the fringe of the epithelium. As they pass through this equatorial region they are induced to differentiate (Hayden and Rothstein, 1979). These equatorial epithelial cells are added to the posterior compartment forming the peripheral, or cortical lens fiber cells. Fiber cell differentiation is characterized by cell elongation, the synthesis and accumulation of large concentrations of lens-specific crystallins, the cessation of DNA synthesis and cell division, and the eventual degradation of membrane bound organelles (see review; Piatigorsky, 1981).

The eye develops in a similar manner in all vertebrates (Duke-Elder and Cook 1963; Coulombre, 1965; Pei and Rhodin, 1970; Aguirre, Rubin and Bistner 1972; Bistner, Rubin, and Aguirre, 1973; O'Rahilly, 1975). The major differences are in the timing of the events. The lens develops from the embryonic ectoderm following exposure to inductive influences of the embryonic optic vesicle. During the early stages of vertebrate development the diencephalon bulges out laterally from the neural tube, producing the optic vesicles. When these vesicles come into close proximity with the surface ectoderm on each side of the head fold (Fig. 3, 35 hr), cells of the ectoderm elongate (Fig. 3, 45 hr) and undergo a series of morphological and biochemical changes that results in the formation of a thickened lens placode (Fig. 3, 49 hr). The

Fig. 3. **Stages of lens development in the chicken.** (Piatigorsky, 1981) Upper Panel: Diagrammatic representation (axial section in the naso-temporal plane) of lens induction and early development in the chicken embryo. Lens induction begins near the 35th hr of development. The number of presumptive lens cells approximately doubles within the next 9-10 hr; the presumptive lens cells then begin elongating to form the lens placode. The lens placode invaginates along with the optic vesicle at around 52 hr of development and eventually loses contact with the optic vesicle (which becomes the neural retina). The invaginating placode separates completely from the ectoderm to form the lens; the peripheral ectoderm fuses over the newly formed lens and develops into the cornea. Lower Panel: Diagrammatic representation of a cross-section of a 15-day-old embryonic chicken lens. The nuclei in the center of the fiber mass (nuclear fiber cells) are pycnotic and will eventually disintegrate.

Stages of Lens Development in the Chicken





lens placode subsequently invaginates (Fig. 3, 52 hr), and the outer margins of the lens cup gradually close, forming the lens vesicle. As the lens vesicle breaks away from the adjacent ectoderm, cells in the posterior region of the vesicle elongate thereby filling the cavity of the lens vesicle. These elongating cells form the primary lens fibers (Fig. 3, 55 hr). Although the anterior epithelial cells of the lens vesicle continue to divide, the elongating posterior fibers cease cell division.

Lens induction does not appear to be an exclusive event in which the inductive influences of the optic vesicle initiates a set of developmental responses in a competent ectoderm. Instead, lens differentiation results from a myriad of tissue interactions which are thought to begin before the optic vesicle induces the presumptive lens ectoderm (Saha et. al., 1989).

Lens Crystallins

By virtue of their abundance and their short-range interactions (Delaye and Tardieu, 1981), the crystallins contribute to the transparency and ability of the lens to refract light without aberrations (Sivak, 1985). They are, therefore, an essential element in lens function. Delta crystallin was the first crystallin to be cloned (Bhat and Piatigorsky, 1979). Since this time, ophthalmology has been propelled into the field of molecular biology. I will, therefore, briefly review the molecular biology studies of

crystallin gene regulation.

Lens Crystallins and Their Gene Families

Classically, crystallins have been divided into three major families, α, β, and γ. These proteins are represented in the lenses of nearly all species of vertebrates. In contrast to these ancestral crystallins, a new major protein, δ-crystallin was discovered in the chicken lens (Rabaey, 1962). Its expression appears to be confined to the lenses of birds and reptiles (Clayton, 1974). This kind of crystallin protein, found only in certain species, is known as a taxon-specific crystallin (Piatigorsky and Wistow, 1989). These four crystallin families are synthesized at different times and in different regions of the developing lens (Papaconstantinou, 1965; Zwaan and Hendrix, 1968; Piatigorsky, 1981). Each family consists of several polypeptides with related amino acid sequences (Piatigorsky, 1984a). The polypeptides of each family aggregate to form higher molecular weight complexes, except for the γ-crystallins and β_s-crystallin, which remain monomeric. Experiments have also shown that the various members within each crystallin family are independently regulated and differentially expressed during development (Hejtmancik et al., 1985; van Leen et al., 1987).

The α- and β-crystallins are present in lenses of all vertebrate classes; δ-crystallin, however, replaces γ-crystallin in avian and reptilian lenses (Clayton, 1974). Interestingly, α-crystallin is the first to appear during

development in rats and mice, δ-crystallin is the first to appear in chickens, and β-crystallin is the first to appear in newts (Piatigorsky, 1981).

α-crystallins

The α-crystallins are encoded by two similar genes, αA and αB (Wistow and Piatigorsky, 1988). They are partially homologous to the small heat shock proteins of Drosophila (Ingolia and Craig, 1982). The αA-crystallin gene is lens-specific. In contrast, the αB-crystallin gene, in addition to being expressed at high levels in the lens, is expressed at low levels in a number of tissues including heart, brain, kidney and retina (Overbeek, et al., 1985; Bhat and Nagineni, 1989; Dubin et al., 1989; Piatigorsky and Wistow, 1989).

β-crystallins

Beta-crystallins are encoded by multiple genes (estimated 8) scattered among several chromosomes (Piatigorsky and Zelenka, 1991; Wistow and Piatigorsky, 1988; Lubsen et al., 1988). The β-crystallin polypeptides are 50%-60% homologous (Piatigorsky, 1984a). They are lens-specific, present in the lens epithelia and the fibers of the newt and chicken, but only found in lens fibers of the rat (Piatigorsky and Zelenka, 1991; Piatigorsky, 1981; McAvoy, 1978). βA3/A1 and βB1 crystallin genes of the chicken have been examined, and both appear lens-specific by Northern blot analysis (Piatigorsky and Zelenka, 1991). In adult chicken lens, the β-crystallins

are the principal proteins, probably due to increased mRNA accumulation after hatching (Piatigorsky, 1987).

γ-crystallins

Gamma-crystallins also are encoded by a family of genes (Piatigorsky, 1984a), which are clustered on the same chromosome (Hejtmancik and Piatigorsky, 1991). Their polypeptides are 70%-85% homologous. Gamma-crystallins are used as lens-specific markers of fiber cell differentiation in bovine (Papaconstantinou, 1965), rat (McAvoy, 1978; Shubert et al., 1970), and amphibian (McDevitt et al., 1969) lenses. They are poorly represented or absent in birds and reptiles (Hejtmancik and Piatigorsky, 1991).

δ-crystallins

Delta-crystallins were discovered in 1962 (Rabaey, 1962). Delta-crystallins, which are only found in lenses of birds and reptiles (Piatigorsky, 1984b), are tetrameric proteins consisting of two principal polypeptides with molecular masses near 48 and 50 kDa (Reszelbach et al., 1977). Delta-crystallins, like α-crystallins, are expressed at high level in lens and at lower levels in the embryonic heart, brain (Thomas et al., 1990) and in the developing retina (Clayton et al., 1979). There are a number of special features of δ-crystallin which make this protein attractive to investigators. Delta-crystallin is a prominent marker for gene expression during development (Piatigorsky et al., 1972).

Delta-crystallin mRNA is first detected a few hours after lens induction, accumulates to very high levels during embryonic development and gradually disappears 2-3 months after hatching. However, δ-crystallin remains the major protein (40%) in the central region of the mature lens (Genis-Galvez et al., 1968; Thomas et al., 1990).

Regulation of δ-crystallin gene expression

Early experiments indicated that the amount of δ-crystallin synthesized in the chicken lens is related to the level of δ-crystallin mRNA present in the cytoplasm (Zelenka and Piatigorsky, 1974; Milstone et al., 1976). Synthesis of δ-crystallin is also partially regulated at the translational level (Beebe and Piatigorsky, 1977). It is still not clear whether the accumulation of δ-crystallin mRNA during fiber cell differentiation involves regulation at the level of transcription, RNA processing, or differential mRNA stability (Piatigorsky, 1981). Thus, the study of δ-crystallin synthesis offers the opportunity to investigate several mechanisms involved in regulation the synthesis of a gene product during normal development (Piatigorsky, 1984b).

In chickens, there are two extremely similar δ-crystallin genes, δ_1 and δ_2, which are 4 kilobases (kb) apart in the genome (Bhat et al., 1980; Yasuda et al., 1982; Hawkins et al., 1984). Each gene contains 16 introns and 17 exons (Ohno et al., 1985; Nickerson, et al., 1985; Nickerson et al., 1986), with coding regions having 91% sequence identity

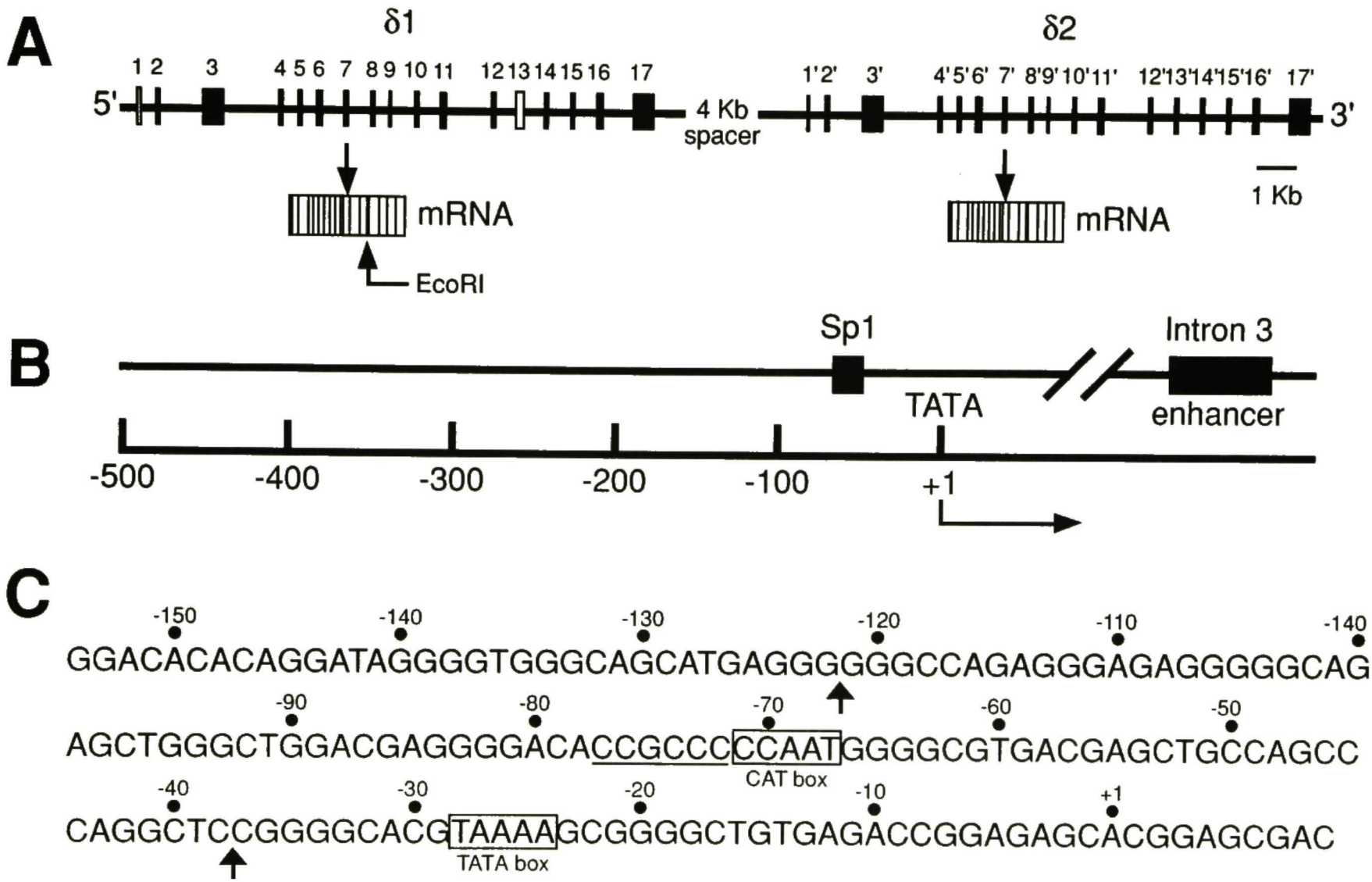
(Wistow and Piatigorsky, 1988). Both δ genes are oriented with the same transcriptional polarity (5′ δ_1-δ_2 3′)(Piatigorsky, 1984a; Borras et al. 1985. See Fig. 4A). The 5′ flanking regions of both the δ_1- and δ_2-crystallin genes contains a TATA box, a CCAAT box, and an Sp1 binding site (Fig. 4B,C).

The lens structural protein δ-crystallin and the metabolic enzyme argininosuccinate lyase (ASL) have striking sequence similarity (Wistow and Piatigorsky, 1987; Yeh et al. 1988). It has been demonstrated that duck δ-crystallin has enormously high ASL activity, while chicken δ-crystallin has lower but significant activity (Piatigorsky et al., 1988).

When nearly 900 δ-crystallin cDNA clones from a 15-day-old embryonic lens library were screened with an oligonucleotide probe specific for exon 2 of the δ_2-crystallin gene, no δ_2-crystallin cDNA clones were detected (Wawrousek et al., 1986). This experiment established that δ_1 is the principal δ-crystallin mRNA found in the embryonic chicken lens (Piatigorsky and Zelenka, 1991). It appears that δ_2-crystallin is identical to the urea cycle enzyme, argininosuccinate lyase, while δ_1-crystallin is a related form that predominates in the lens.

Primer extension experiments using gene-specific oligonucleotides showed that most of the δ-crystallin mRNA in the 14-day-old embryonic lens contained transcripts derived from the δ_1 gene, while only about 1-2% of the extended products were derived from δ_2 mRNA (Parker et al. 1988).

Fig. 4. (A) **Structure and expression of the chicken δ-crystallin gene locus** (Piatigorsky, 1987). Exon 13 is represented as an open box in the δ_1 gene to indicate that it contains an EcoR1 site that is lacking in the corresponding exon of the δ_2 gene. (B) **Diagrammatic representation of regulatory sequences associated with the chicken δ_1 crystallin gene** (Piatigorsky, 1991). Sp1, a GC box binding the trans-acting factor Sp1; enhancer, present in third intron. A similar enhancer is present in the third intron of the δ_2 gene despite the fact that this gene is not expressed to the same extent as the δ_1 gene in the lens. (C) **DNA sequence showing significant features of a portion of the 5′ flanking region of the δ_1-crystallin gene** (Das and Piatigorsky, 1986). The RNA initiation site (+1), CAAT box, TATA box, and CCGCCC sequences are marked.


A
δ1
δ2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
1'2' 3' 4'5'6' 7' 8'9'10'11' 12'13'14'15'16' 17'
5'
3'
4 Kb spacer
1 Kb
mRNA
mRNA
EcoRI
B
Sp1
Intron 3
TATA
enhancer
-500 -400 -300 -200 -100 +1
C
-150 -140 -130 -120 -110 -140
GGACACACAGGATAGGGGTGGGCAGCATGAGGGGGGCCAGAGGGAGAGGGGGGCAG
-90 -80 -70 -60 -50
AGCTGGGCTGGACGAGGGGACACCGCCCCCAATGGGGCGTGACGAGCTGCCAGCC
CAT box
-40 -30 -20 -10 +1
CAGGCTCCGGGGCACGTAAAAGCGGGGCTGTGAGACCGGAGAGCACGGAGCGAC
TATA box

These experiments demonstrated that the δ_1/δ_2 mRNA ratio may differ in the lens fiber cells and suggested that the two δ-crystallin genes are differentially regulated during lens development (Parker et al., 1988).

Recently, polymerase chain reaction experiments confirmed the differential expression of the two δ-crystallin genes during lens development. δ_1 mRNA accumulates to a higher level than δ_2 in lens epithelial and fiber cells. The δ_1/δ_2 ratio was greater in fibers than in epithelial cells. The data showed that δ_1 mRNA increases from approximately 95% to over 99% of the δ-crystallin mRNA in the fibers, while it decreases from approximately 95% to about 80% of the δ-crystallin mRNA in the epithelium between 6 and 14 days of development of the chicken embryo.

It is not known whether is differential control of the amounts of δ_1 and δ_2 mRNA during development are regulated at the level of transcription, post-transcriptional processing, mRNA stability, or a combination of these mechanisms. Borras et al., (1985) sequenced the 5′ flanking regions of the δ_1- and δ_2-crystallin genes and tested their ability to promote chloramphenicol acetyltransferase (CAT) activity using the pSV0-CAT expression vector in transfected embryonic lens epithelia. Their sequence data showed that both promoters contain a TATA box and are GC rich, with a CCAAT box being present and continuous with an Sp1 site in the δ_1 gene. Their transfection experiments showed that the δ_1 promoter was only five times stronger than the δ_2 promoter, with neither

promoter being very effective in lens-specific expression (Piatigorsky and Zelenka, 1991).

In vitro transcription of the two δ genes was generally similar in a Hela cell extract, showing that the δ_1 promoter was only several-fold stronger than that of δ_2 promoter under these conditions (Das and Piatigorsky, 1988).

Additional experiments have shown that the third intron of the δ_1-crystallin gene has an enhancer that serves as a major determinant for lens specificity (Hayashi et al. 1987). This enhancer contains several elements that appear to be able to direct expression of a thymidine kinase (tk) promoter/reporter gene construct to the lens (Goto et al., 1990). Thomas et al. have shown, in their transfection experiments, that the third intron of the δ_2 gene has an enhancer that is comparable to that of the δ_1 gene. The enhancers and promoters of the two δ-crystallin genes were functionally interchangeable in these transfection studies.

All the evidence regarding δ-crystallin gene expression comes from studies under in vitro conditions. These results suggest that the differential expression of the two δ-crystallin genes cannot be explained by transcriptional regulation by previously-identified regulatory sequences (Piatigorsky and Zelenka, 1991). Either additional cis-acting sequences remain to be identified, or the differential expression of these genes may be due to differential mRNA processing, transport, or stability. This issue has not yet

been conclusively answered.

My present work focuses on this problem. I developed a new density-labeling method to quantitate the ratio of δ_1- to δ_2-crystallin sequences in newly synthesized and pre-existing mRNA from 14-day-old lens fiber cells. Then I used PCR with either gene-specific exon primers, or with an intron-specific primer to quantitate the ratio of processed and unprocessed δ_1 and δ_2 messages from the central epithelium, annular pad, central fiber or cortical fiber cells. Finally, I tested the culture systems used for previous transfection studies. These studies showed, contrary to previous belief, that these cultured cells produced similar amounts of the two δ-crystallin messages. Taken together, my studies suggested that the differential expression of the two δ-crystallin genes is primarily regulated at the level of transcription.

CHAPTER II

Material and Methods

Lens Preparation

Fertilized eggs were obtained from Truslow Farms, Inc., Chestertown, Maryland, kept refrigerated at 10°C for up one week, then incubated in a Humidaire model 55 forced draft incubator at 38°C.

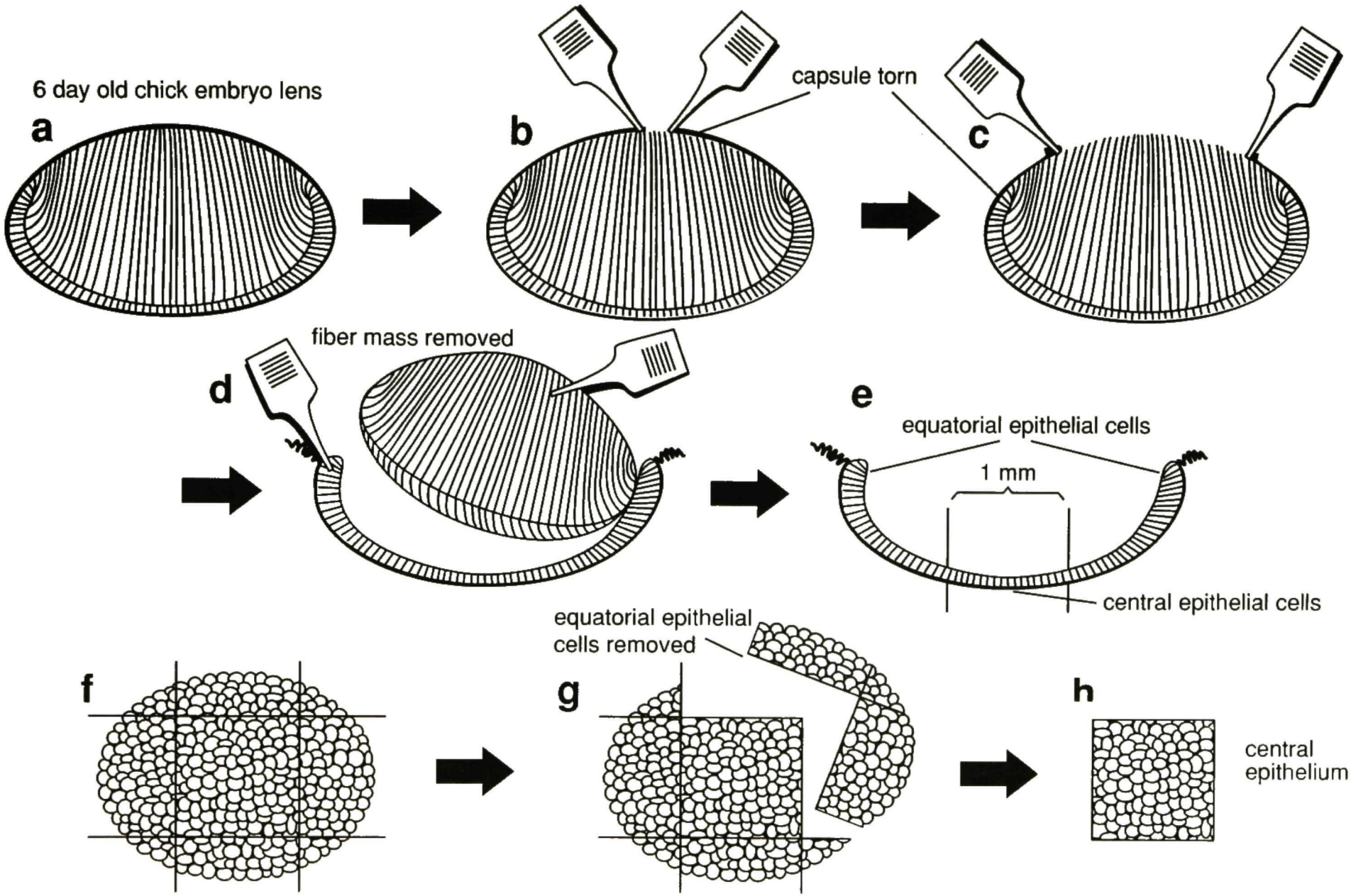
Embryos were obtained aseptically by carefully breaking the egg, lifting the embryo from the yolk and extraembryonic membranes with watchmakers forceps previously sterilized in 70% ethanol and transferring the embryo to a sterile 10 mm x 60 mm culture dish. The embryonic cornea was pierced at a point overlying the eyecup/lens boundary with sharpened watchmakers forceps and the eyes opened by separating the tips of the forceps. Each lens was lifted off the vitreous body by cradling it between the tips of the forceps and immediately transferred to Ham's F-10 medium. For different experiments, lenses from chicken embryos of different ages were incubated for the indicated time period in basal medium (Ham's F-10) which had been supplemented with different reagents (described below) and maintained at 37°C in the presence of 95% air/5% CO_2. At the end of the treatment period, epithelial and fiber cells were separated under a binocular microscope and processed as described. In some

experiments, samples were frozen on dry ice and stored at -70°C before processing.

Lens Explantation and Culture

Each lens was transferred to 2 ml of Hams F-10 culture medium (GIBCO Labs, Grand Island, N.Y.) in a 10 x 35 mm plastic culture dish (for most experiments, except actinomycin D and density labeling). Lens epithelia were removed by positioning the lens with the epithelium against the surface of the culture dish, puncturing the posterior lens capsule with sharpened forceps and, while grasping the capsule with one pair of forceps, peeling portions of the capsule off of the posterior half of the lens until the entire posterior surface of the lens fiber mass was uncovered (Fig. 5). An edge of the lens epithelium was then held against the surface of the dish while the mass of fiber cells were carefully lifted off and placed in a fresh ice cold tube containing Solution D (see section on RNA extraction), or, in some cases the fiber mass was discarded. The remaining circular epithelium and attached lens capsule was then inverted so that the capsule faced upward. A square explant containing approximately 2 x 104 cells, from the central region of the lens epithelium, was simultaneously cut (Fig. 5) and attached to the bottom of a 35 mm Petri dish with a sterile #15 scalpel blade. The peripheral epithelial cells were discarded, leaving approximately 0.7 mm square monolayer of centralepithelial cells lying beneath the capsule at the

Fig. 5. **Diagrammatic representation of the explantation of a lens epithelium** (from Piatigorsky, 1975): axial sections (a-e) and top views (f-h). The lens is removed from the eye of a 6-day-old chick embryo, placed into sterile medium contained in a plastic Petri dish, cleaned of vitreous and other adhering debris, and rotated so that the epithelium faces the surface of the dish (a). The capsule (dark line) is torn on the posterior side of the lens with sharpened jeweler's forceps and the fiber mass (F) is removed (b-d), leaving the central (C) and equatorial (E) epithelial cells still attached to the lens capsule. A square piece with sides of approximately 1 mm is cut out from the central region of the epithelium by pushing the peripheral regions of the explant into the surface of the plastic culture dish with a scalpel (e, f). Next, the equatorial epithelial cells are carefully removed with forceps (g). This yields a piece of isolated central epithelium with the cells adhering to the lens capsule, which is anchored to the dish (h).


6 day old chick embryo lens
a
b
capsule torn
c
fiber mass removed
d
e
equatorial epithelial cells
1 mm
central epithelial cells
equatorial epithelial
cells removed
f
g
h
central
epithelium

surface of the dish (Fig. 5). For each independent experiment, lens explants were dissected from eight to ten embryos.

Vitreous humor was prepared from day-15 embryonic chickens according to methods previously described (Beebe et al., 1980). Vitreous bodies were removed from decapitated day-15 chicken embryos. Prior to the removal of the vitreous body, the heads were thoroughly washed with 0.01 M PBS (pH 7.4) to minimize contamination of the vitreous substance with embryonic blood. A 90° lateral incision was made at the corneal limbus, and the vitreous body was expressed with gentle pressure from the outside of the eye. Lenses that remained attached to the vitreous bodies were carefully removed. Vitreous bodies were pooled in chilled tubes in ice, then centrifuged for 10 min at 4°C at 26,000 x g in a Sorvall SS-34 rotor. The supernatant was collected, then stored at -80°. Vitreous humor was tested for its ability to stimulate cell elongation in cultured central lens epithelial cells. Vitreous humor that did not stimulate fiber formation was not utilized in these experimental protocols.

Explanted lens epithelia were incubated in 2 ml of basal (Hams F-10) medium with or without 50% vitreous humor for 3 days at 37°C, in 95% air/5% CO_2.

Primary Lens Cell Culture Methods

Lens explants were obtained from 14-day-old embryonic chicken lenses as described above and incubated for 3 days at

37°C in 5% CO_2 in unsupplemented Ham's F-10 medium (GIBCO), with 20% fetal calf serum, or with 50% vitreous humor (from 15-day-old chicken embryos).

Some lens explants were obtained from 14-day-old embryonic chicken lenses following the methods of Chepelinsky et al. (1985), and incubated for 3 days at 37°C in a humidified-air environment (at 10% CO_2) in Coon's modified F-12 medium (gift from Dr. Anna Chepelinsky). This medium contained insulin (10 µg/ml), 10 nM hydrocortisone, transferrin (5 µg/ml), glycyl-L-hystidyl-L-lysine acetate (10 ng/ml), somatostatin (10 ng/ml), thyrotropin (10 mU/ml), 0.5% calf serum, and gentamicin (50 µg/ml).

Cultures of dissociated cells (primary patched epithelial cells) were prepared from 11- or 14-day-old chicken embryo by following the method of Borras et al. (1985), and incubated for 3 days at 37°C in the presence of 10% CO_2. We also used freshly-dissected lens central epithelia and lens peripheral fibers from 14-day-old chicken embryos for controls in our studies.

Tritiated Uridine Incorporation

Lenses at 10 and 13 days of embryonic development were prelabeled with [3H]-uridine (ICN, 100 µCi/ml, 45 Ci/mmole) for 1 hr then incubated in the label for an additional 5 hr in the presence of actinomycin D (0.5 or 30 µg/ml).

Protein Labeling Experiments

Protein labeling experiments were performed in multiwell tissue culture clusters (24 wells, 16 mm diameter) in a total of 1 ml of Ham's F-10 medium alone or with 0.5 or 30 µg/ml actinomycin D (Dactinomycin; Merck Sharp & Dohme). Lenses from 11-day-old embryos were labeled in the presence or absence of the drug with [^{35}S]-methionine (New England Nuclear, 100 µCi/ml, >1000 Ci/mmole) for 5 hr. In separate experiments, lenses from 10- and 13-day-old embryos were incubated with actinomycin D for 5 hr, then pulse-labeled with [^{35}S]-methionine for 2 hr. Incubations were terminated by washing the tissues twice with warm Ham's F-10 medium.

Glucosamine Treatments

Explanted lens epithelia from 13-day-old chicken embryos were incubated in Ham's F-10 medium containing [^{3}H]uridine (10 µCi/ml) with or without 20 mM glucosamine, pH 7.4, at 37°C in 95% air/5% CO_2. At the beginning, or after 30 or 60 min, 75 µM 4-thiouridine was added and the samples were incubated for an additional 4 hr under the same conditions. At the end of the incubation period lens epithelia were washed four times with warm Ham's F-10 medium, and immediately lysed in solution D (see RNA extraction).

DNA Extraction from Chicken Embryo Blood Cells

Embryonic chicken blood was obtained by puncturing one of the chorioallantoic vessels with a drawn glass capillary attached to a polyethylene tube by which suction could be

applied by mouth. Whole blood was collected and DNA was isolated as in Miller (1988). The red blood cells were centrifuged at 4°C for 30 min at 13,000 X g in a microcentrifuge. Cells were pelleted, the plasma was removed and packed red blood cells were resuspended in 3 ml of lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, pH 8.2) in a 15 ml polypropylene tube. The nuclei were pelleted and remaining cellular and nucleoprotein were digested by adding 0.2 ml 10% SDS and 0.5 ml proteinase K (2 mg/ml, 1% SDS and 2 mM Na_2EDTA) to the suspension of nuclei with gentle shaking in a shaker bath for 2 hr at 50°C. Proteins were precipitated after digestion by adding 1 ml of 5 M NaCl, vortexing, and then pelleting at 3000 g for 15 min. The supernatant containing the DNA was then transferred to another tube. Following the same procedure, the supernatant was deproteinized with two NaCl extractions. The clear supernatant was then transferred to a new tube and the DNA precipitated with equal volume of isopropanol. The DNA was removed by winding it around a heat-sealed Pasteur pipette, washed with 70% ethanol, air dried and redissolved in 2 ml of Tris-EDTA buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.5). The DNA was placed in a 95°C water bath for 10 min to denature DNase activity.

Plasmid (pGem) Mini Lysate

Plasmid lysates were performed using the alkaline lysis method (Birnboim and Doty, 1979). Five ml of LB containing 50 µg/ml ampicillin were inoculated with a 50 µl of

plasmid stock and incubated overnight. The next morning 1.5 ml of the culture was transferred into an Eppendorf tube and centrifuged at 2000 X g for 15 min at 4°C. The medium was removed and the pellet was resuspended by vortexing in 350 µl of ice-cold STET solution (8% sucrose, 5% Triton X-100, 50 mM Na_2EDTA and 50 mM Tris-HCl, pH 8.0) for 15 min at 4°C. Then the sample was transferred to another fresh Eppendorf tube. After 25 µl of lysozyme (10 mg/ml in 50 mM Tris, pH 8.0) was added, 4 ml of 50 mM glucose, 10mM EDTA, 25 mM Tris-HCl, pH 8.0, 5 mg/ml lysosome was added and incubated for 5 min on ice. A freshly-prepared solution of 200 µl of 0.2 N NaOH, 1% SDS was then added. The sample was mixed and incubated on ice for 5 min. To precipitate bacterial proteins, 150 µl of an ice-cold solution of potassium acetate (pH 4.8) was added (60 ml of 5 M potassium acetate, 1.5 ml of glacial acetic acid and 28.5 ml of H_2O). The sample was mixed by vortexing gently and centrifuged for 5 min at 4°C. The supernatant was transferred to a fresh tube, extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and DNA was precipitated from the aqueous phase with 2.5 volumes of 100% ethanol at room temperature. The precipitate was centrifuged at 10,000 X g for 30 min and the DNA pellet dried under vacuum and resuspended in 50 µl of TE containing DNase-free pancreatic RNase (20 µg/ml).

Large Scale Preparation of Plasmid DNA

Based on the methods of Maniatis et al. (1982), *E. coli*

strain DH5α, which had been transfected with the appropriate plasmid, were grown in 1 liter of LB broth with ampicillin (50 µg/ml) overnight at 37°C. The bacteria were harvested by centrifugation at 7000 rpm for 7 min at 4°C, and the pellet was resuspended in 50 ml cold TE, pH 8.0, and again spun at 7000 rpm for 7 min at 4°C. The cells were resuspended in 9 ml of Sucrose-EDTA-Tris (SET; 25% sucrose, 0.05 M Tris-HCl and 40 mM EDTA). One ml of lysozyme solution was added (10 mg/ml of lysozyme in SET). Incubation was carried out for 5 min and 3.7 ml of cold 0.25 M EDTA, pH 8.0 was then added. After 5 min on ice, 14.5 ml of Triton solution (40% Triton X-100, 62.5 mM EDTA and 50 mM Tris-HCl, pH 8.0) was added and the sample was incubated at room temperature for 10 min. The sample was transferred to 1 x 3-1/2" polyallomer centrifuge tubes and spun at 25,000 rpm in an SW28 rotor for 2 hr at 4°C. The supernatant was transferred to 50 ml tubes and the plasmid DNA was purified by buoyant density centrifugation in cesium chloride ethidium bromide gradients. The CsCl gradients were prepared according to the following formula: (1) X ml supernatant; (2) 1/20 X ml ethidium bromide (10 mg/ml); (3) X g + 1/10 X g of CsCl. The gradients were centrifuged in sealed polypropylene tubes (Beckman Quick Seal) at 47,000 rpm in a VTi-50 rotor for 18 hr at 18°C. Two bands were visible after centrifugation when illuminated with 302 nm light from a hand held UV illuminator. The lower band, corresponding to supercoiled covalently closed circular plasmid DNA was removed by piercing the side of the centrifuge tube with a wide-bore

syringe needle. Ethidium bromide was removed from the CsCl solution containing the plasmid by extracting 4-6 times with isopropanol saturated with NaCl-saturated distilled water. To precipitate the plasmid DNA, one vol of TE (10 mM Tris-HCl, pH 8; 1 mM EDTA) and two vol of ice-cold absolute alcohol were added. The plasmid DNA was pelleted after incubation at -70°C for 1 hr and centrifuged at 3.4k rpm for 15 min at 4°C. The pellet was washed with 70% alcohol, dried under vacuum and resuspended in TE.

Agarose Gel Electrophoresis

Agarose (BRL) was boiled in 1 X TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) cooled to 50°C, and ethidium bromide (1 µg/ml) was added. Then the gel solution was cast in a gel casting platform. The concentration of agarose in the gel dependended on the experiment. After the gel was solidified, 1 X TBE buffer was added up to the level of the gel surface and samples of DNA were mixed with 6X gel-loading buffer [0.25% (w/v) bromophenol blue, 40% glycerol in H_2O], and vortexed briefly and centrifuged before loading onto the gel. The gel was electrophoresed at 100 V in 1X MOPS running buffer until the bromophenol blue migrated to within 1 cm of the bottom of the gel, typically requiring 45 min to 2 hr depending the size and purpose.

Polyacrylamide Gel Electrophoresis and Autoradiography

Labeled proteins were extracted from lens epithelial cells and fibers and denatured at 100°C for 5 min in electrophoresis sample buffer (62.5 mM Tris, pH 6.8, 4% SDS, 10% (v/v) glycerol, 5% 2-Mercaptoethanol). Denatured, labeled proteins were then electrophoresed on a 10% polyacrylamide gel according to the method of Laemmli (1970). After electrophoresis the gels were fixed with 10% acetic acid, 40% methanol, stained, destained, dried and exposed to X-ray film (Kodak XAR-5).

RNA Extraction

Total RNA extraction was modified from the method of Chomczynski and Sacchi (1986). Lens tissue was lysed or thawed in 100 µl or 200 µl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% Sarcosyl and 0.1 M 2-mercaptoethanol), vortexed well, and one tenth vol of 3 M sodium acetate (pH 4.0) was added. The mixture was extracted with water-saturated phenol:chloroform:isoamyl alcohol (25:24:1). The RNA was precipitated from the aqueous phase with 2.7 vol of 100% ethanol and 10 µg of tRNA as carrier at -20°C overnight. Precipitates were centrifuged at 10,000 x g for 30 min at 4°C, washed with 70% ethanol, dried and redissolved in sterile, DEPC treated water.

RNA Blots

RNA samples were denatured by heating at 65°C for 15 min in 3 vol of a solution of 6.15 M formaldehyde, and loaded

onto a nitrocellulose filter in 10 x SSC using a BRL slot-blot manifold. Before the samples were loaded, the nitrocellulose filters were wet with H_2O first, then in 10 X SSC. After loading, the sample wells were washed with 10 X SSC. The RNA was bound to the nitrocellulose filter by drying at 80°C for 2 hr under vacuum.

mRNA Translation

Translation of total RNA samples extracted from lens epithelia was performed using a New England Nuclear (Dupont-/NEN, Boston, MA) reticulocyte lysate kit, employing ^{35}S-methionine as the radiolabel. The newly-synthesized protein products were analyzed on 10% SDS-polyacrylamide gels, which were fixed, dried, and exposed to Kodak XAR-5 film.

cDNA Hybridization

Specific δ-crystallin mRNA was quantitated using cDNA probes. The cDNA insert of pδCR17 (gift from Dr. Joram Piatigorsky) was excised with HpaII. The cDNA was nick-translated using a nick-translation kit from BRL to a specific activity of > 10^8 cpm/μg DNA using ^{32}P-dCTP. Nitrocellulose filters were prehybridized for 3 hr at 42°C with constant agitation. Hybridization was performed at 42°C for 16 hr in the prehybridization solution without glycine. The final concentration of the probe was 2 X 10^5 cpm/ml. Filters were washed for 30 min at room temperature in 2 X SSC; two times at 65°C in 2 X SSC, 1% SDS for 30 min; 2 times at 65°C in 0.1 X

SSC, 0.1% SDS for 30 min and finally at 65°C in 0.1 X SSC for 20 min. All the wash procedures were with constant agitation. The dried filters were subjected to autoradiography at -70°C using Kodak XAR-5 film.

Quantitation of Autoradiograms

Autoradiograms were scanned with a Quick-Scan (Helena Labs) densitometer. The integrated area under specific bands or the entire lane was used to estimate changes in protein incorporation or the percentage of total incorporation represented by a single polypeptide band.

Northern Blots

Total lens RNA was isolated using the method as described above. Then following Rosen's method (1990), 1% (w/v) agarose/6.6% (w/v) formaldehyde gel was prepared as follows: 1 g of agarose was melted in 72 ml of distilled water and allowed to cool to approximately 55°C. Ten ml of 10 X running buffer [0.2 M MOPS, 10 mM EDTA, 10 mM sodium acetate, (pH 7.0)] were mixed with 18 ml of deionized 37% (w/v) formaldehyde and added to the cooled agarose. The gel was cast and allowed to solidify for 1 hr at room temperature in a fume hood. Each RNA sample was dissolved in 2 µl of sterile, DEPC treated, distilled water. Formamide (5 µl), formaldehyde (2 µl), 10 X running buffer (1 µl), and 400 µg/ml ethidium bromide (1 µl) were added to each sample. The samples were then vortexed and heated for 10 min at 65°C. After heating,

the samples were placed on ice. Following a brief centrifugation, 2 µl of 6X dye mix [0.2% (w/v) xylene cyanol, 0.2% (w/v) bromophenol blue, 10 mM EDTA, 50% (w/v) glycerol] was added. The samples were vortexed briefly and centrifuged before loading onto the gel. The gel was electrophoresed at 100 V in 1X MOPS running buffer until the bromophenol blue migrated to within 1 cm of the bottom of the gel.

After electrophoresis was complete, the gel was soaked briefly (5 min) in distilled water to remove excess formaldehyde, then photographed under UV illumination. RNA was transferred to Nytran filters (0.45µm) overnight using 10 X SSC as the transfer buffer. Following transfer, the Nytran membrane was baked at 80°C for 2 hr under vacuum.

Density Labeling of RNA

Density-labeled ribonucleosides with 84% substitution of ^{13}C for ^{12}C and 94% substitution of ^{15}N for ^{14}N were a generous gift from Dr. Rob Grainger of the University of Virginia (Grainger, 1976). Ham's F-10 culture medium was purchased from GIBCO, Grand Island, N.Y. RNase-free DNase I was obtained from Boehringer Mannheim (Indianapolis, IN) and Moloney murine leukemia virus (M-MLV) reverse transcriptase, yeast tRNA, T4 polynucleotide kinase and agarose were purchased from BRL/Life Technologies (Gaithersburg, MD). RNasin, Taq DNA polymerase and 10 x PCR reaction buffer were obtained from Promega (Madison, WI). Deoxynucleoside triphosphates (dNTPs) were obtained from Perkin Elmer Cetus (Norwalk, CT).

White mineral oil was obtained from Thomas Scientific (Swedesboro, NJ), ^{32}P-deoxycytidine-5-triphosphate from Dupont/NEN (Boston, MA), ^{3}H-uridine from ICN Biomedicals, Inc. (Costa Mesa, CA) and potassium and sodium iodide from Aldrich Chemical Company, Inc. (Milwaukee, WI). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Lenses from 6-, 10- or 14-day-old chicken embryos were removed and incubated for 4 hr in 200 µl drop of medium at the center of a 35 mm plastic culture dish. The medium was Ham's F-10 supplemented with ^{3}H-uridine (100 µCi/ml) and a total of 10^{-4}M of the four density-labeled ribonucleosides (25 µM each). Lenses used for controls were incubated in the same medium lacking the density-labeled nucleosides. In preliminary studies we determined that this concentration of nucleosides maximally labeled the lens nucleoside pools. These tests were performed as described by Bowman and Emerson (1980). The ^{3}H-uridine was included to identify newly-synthesized RNA. At the end of the labeling period lenses were removed from the medium and washed twice in warm Ham's F-10 medium. Lens fibers were separated from the epithelia by dissection and lysed in cold solution D (see RNA extraction), and the central portion of the lens epithelia were separated from the annular pad and also immediately lysed in solution D.

Separation of Density-labeled and Light RNA by Equilibrium Density Gradient Centrifugation

Following the method of Winkles and Grainger (1985),

RNA samples in sterile DEPC-treated water were added to 0.305 ml NaI stock (saturated, density = 1.896 gm/ml) prepared in 0.8 mM Na_2SO_3 to prevent oxidation, 0.984 ml KI stock (saturated, density = 1.718 gm/ml) in 0.8 mM Na_2SO_3, 0.05 ml deionized formamide, 2 µl of 2-mercaptoethanol and enough additional sterile distilled H_2O to bring the final volume to 1.50 ml. The gradient mixtures were prepared in Beckman Quick Seal Tubes (11 x 32 mm), overlaid with white mineral oil and centrifuged at 48,000 rpm for 48 hr at 20°C in a Beckman SW60 Ti swinging bucket rotor (Beckman Instruments Inc., Palo Alto, CA) in a Dupont/Sorvall OTD-65 ultracentrifuge. Four drop fractions (~50 µl each) were collected from the bottom of the tube by lowering a micropipette through the gradient. The density of selected fractions was determined by weighing aliquots. The distribution of radioactive RNA in the gradient was determined after mixing 15 µl aliquots of each fraction with 0.5 ml 0.1% (v/v) 2-mercaptoethanol and counting in a Beckman LS-9000 scintillation counter. Data were plotted using Fig P (BIOSOFT, Milltown, NJ).

Amplification of Density-labeled mRNA by PCR

Typically, 15 µl portions of three consecutive fractions were pooled and precipitated at -20°C. after the addition of 2 vol of 0.2 M potassium acetate, 10 µg of tRNA as carrier, and 6 vol of cold 100% ethanol. The RNA was pelleted, washed in cold 70% ethanol and dissolved in 10 µl DEPC-treated H_2O. In some cases, before reverse

transcription, RNA samples were pre-treated with 27 units of RNase-free DNase I in the presence of 40 units of RNAsin and 2 mM $MgCl_2$ for 1 h at 37°C in a total volume of 10 µl (Grillo and Margolis 1990). Reverse transcription was performed in a total volume of 25 µl by adding concentrated stock solutions to the RNA samples to achieve final concentrations of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3.0 mM $MgCl_2$, 500 µM each of dNTP and 2 µM of the 3′ PCR primer (#139, see below and Fig. 18) Samples were briefly vortexed, centrifuged and heated at 70°C for 5 min. Five hundred units of M-MLV reverse transcriptase were added, the mixture was incubated for 20 min at 37°C., heated for 10 min at 70°C and immediately cooled on ice.

PCR was performed in a 50 µl reaction mix containing 2 µl of cDNA template from the reverse transcription reaction, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 250 µM each dNTP, 2.5 µCi of ^{32}P-dCTP (for serial dilution experiments) and 1 µM each primer. Prior to the addition of 2.25 units of *Taq* DNA polymerase, reaction mixes were overlaid with 50 µl of mineral oil and heated at 94°C for 5 min. Negative controls, containing all reagents except template, were run with each primer set. The primers used in these experiments are diagrammed in Fig. 18. PCR was carried out in a Programmable Thermal Controller (MJ Research, Inc.) for 28 cycles. Each cycle consisted of denaturing at 94°C for 40 seconds, annealing at 55°C for 1 min and extending at 72°C for 1 min. At the end of the 28th cycle, the heat denatur-

ation step was omitted and extension was allowed to proceed at 72°C for 7 min.

The oligonucleotide primers for PCR were synthesized using β-cyanoethyl phosphoramidate chemistry on a #391 PCR-Mate oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). We used the following primers: #999, TAAAG AAAGA AGAGG GGCAG G, an intron-specific, sense-strand primer identical to sequences in intron L of the δ_1- and δ_2-crystallin genes; #137, CACTG GCAGC AGCCT GTTGC, a sense-strand primer identical to sequences in exon 12 of both δ-crystallin genes; #139, CTGGA GGGTA GAAAT CACTC an antisense primer complementary to identical sequences in exon 14 of both δ-crystallin genes; #513, CTCAT GGTTC TCAAA GGAAT TCCAA GCACC TTCAG, a sense-strand 35-mer specific for a sequence in exon 13 of the δ_1-crystallin gene; #492, GGACT TCCAA GCACC TACAA, a 20-mer specific for exon 13 of the δ_2-crystallin gene. As can be seen, primers 513 and 492 have similar 3′ sequences. In spite of this similarity, oligo #513 was over 1,000-fold more effective than #492 in amplifying cloned δ_1-crystallin cDNA and both primers specifically amplified only their cognate gene from total genomic DNA (see below).

For the experiments described, cDNA was amplified using primers 137 and 139 to generate a 229 bp product from mature δ-crystallin mRNA. Any unprocessed δ-crystallin mRNA in this mixture would also be amplified, yielding products of 581 and 627 bp for δ_1- and δ_2-crystallin transcripts, respectively. In some experiments a 2 μl aliquot of these PCR-

amplified, unprocessed δ-crystallin sequences was used as a template for a second PCR amplification. This used intron primer #999 and exon primer #139 to selectively amplify the unprocessed sequences.

Quantitation of PCR Products by Serial Dilution

This was done in a manner similar to that described by Thomas, et al., 1990. After cDNA synthesis, an aliquot of the reverse transcription reaction was serially diluted in 5-fold steps, the PCR reaction mixture, including ^{32}P-dCTP, was added and the template was amplified as described above. PCR products were analyzed by agarose electrophoresis, stained with ethidium bromide and photographed. The bands containing amplified DNA were cut out and counted in a Beckman LS-9000 scintillation counter.

Southern Blot Hybridization of PCR products

Southern blots were performed according to the method of Leonard, Dibner and Battey (1986). Once electrophoresis was completed, the gel was soaked in 1.5 M NaCl/0.5 M NaOH for 30 min at room temperature with agitation, then neutralized by being shaken in 1 M ammonium acetate/0.2 M NaOH for 30 min. The DNA was transferred to Nytran membranes (Schleicher & Schuell, Inc. Keene, NH) in the neutralization buffer, the membrane baked at 80°C for 2 hr under vacuum and prehybridized for 5 hr at 42°C with constant agitation, following the manufacturer's instruction (Dupont/NEN). The oligodeoxynucle-

otide #385 (AGCAG TCAGA GTGTC CACAA), which binds to a sequence present in exon 14 of both δ-crystallin genes, was ^{32}P end-labeled by T4 polynucleotide kinase. Hybridization was performed at 40°C for 16 hr in the prehybridization solution without glycine. The final concentration of the probe was 5 x 10^6 cpm/ml. Filters were washed for 30 min at room temperature and twice at 49°C in 2 x SSC/0.1% SDS for 45 min. The dried filter was subject to autoradiography at -70°C using Kodak XAR-5 film.

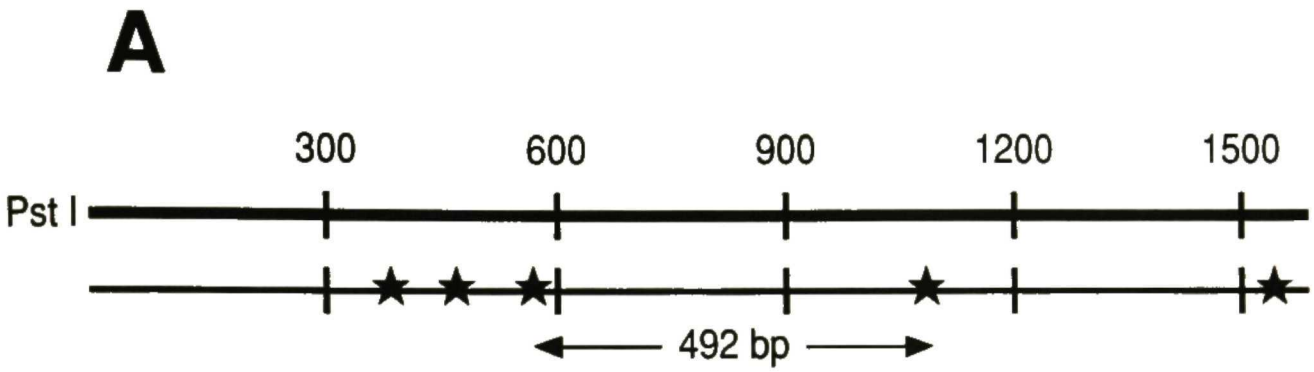
Riboprobe Construction

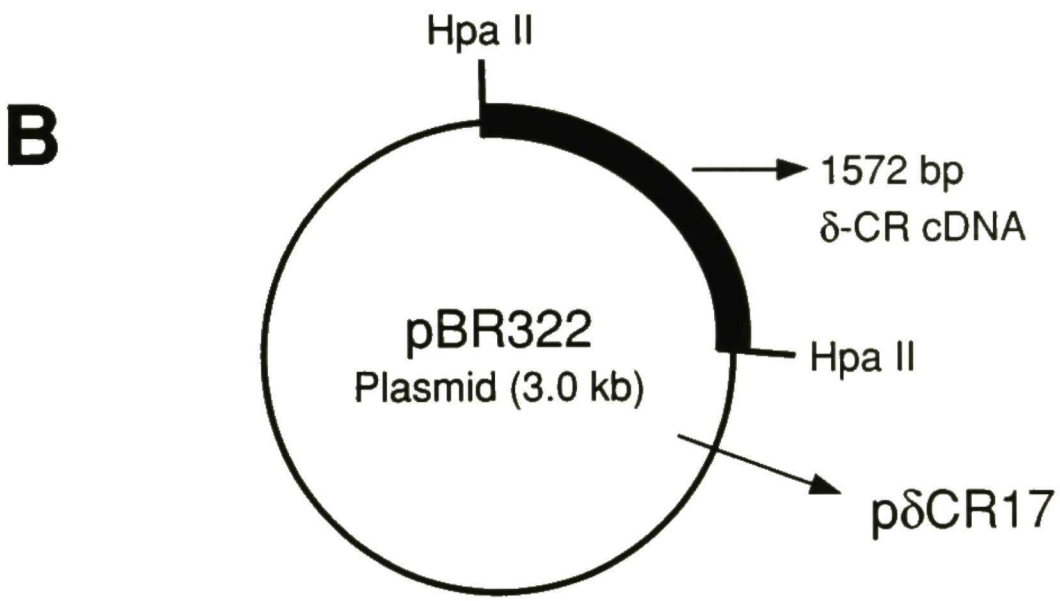
A 492 bp δ_1-crystallin cDNA insert (base 572 to base 1063) was cut from the full length δ-crystallin cDNA with the restriction enzyme, *Pst* I (Fig. 6A). The full length of δ-crystallin cDNA insert was isolated from clone pδCR17 (a gift from Dr. Piatigorsky) with the restriction enzyme *Hpa* II (Fig. 6B). The *Pst* I fragment was purified by agarose gel electrophoresis and subcloned (Fig. 6C) into the PstI cloning site of pGem blue vector (Promega Biotech). For generation of antisense riboprobes, this construct was linearized with SmaI and was gel purified (see DNA isolation from gels) by NA-45 DEAE membrane. Synthesis of antisense RNA with Sp6 RNA polymerase (as described below) generated a 548 base transcript containing 56 bases of pGem plasmid sequence and 492 bases complementary to δ-crystallin mRNA (δCR492).

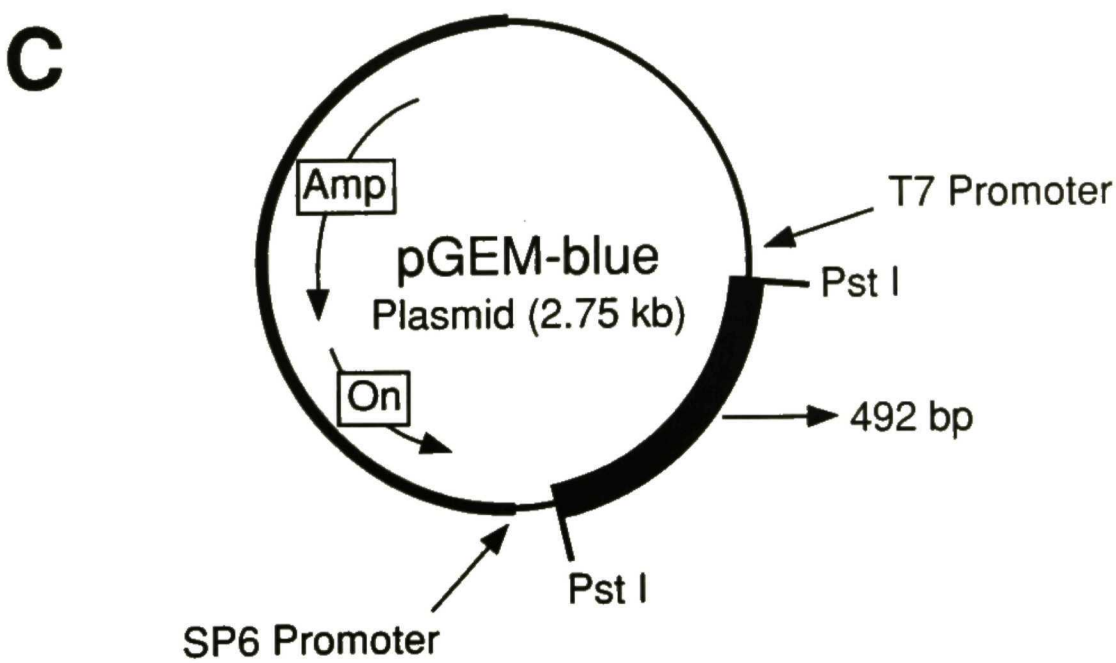
RNase Protection Assay

Protection assays were performed according the methods

Fig. 6. **Construction of δ-crystallin <u>in</u> <u>vitro</u> transcription vector.** (A). *Pst* I restriction enzyme. The dark line represents the full length of δ-crystallin cDNA (1572 bp). The star represents the restriction sites of *Pst* I. The 492 bp fragment was cut by *Pst* I (base 572 to base 1063). (B). Structure of clone pδCR17. (C). Structure of subcloned 492 bp of δ-crystallin cDNA in a pGEM Blue vector.


A
300
600
900
1200
1500
Pst I
492 bp


B
Hpa II
1572 bp
δ-CR cDNA
pBR322
Plasmid (3.0 kb)
Hpa II
pδCR17


C
Amp
pGEM-blue
Plasmid (2.75 kb)
On
T7 Promoter
Pst I
492 bp
Pst I
SP6 Promoter

of Lowe, et al. (1988). The δ-crystallin riboprobe was synthesized and labeled using ^{32}P-UTP and Sp6 polymerase according to the manufacturer's instructions (Promega Biotech) except that the specific activity of the labeled riboprobe was reduced by doubling the concentration of unlabeled UTP.

After transcription, 1µg of DNase I (Cooper Biomedicals, Malvern, PA) 8 µg of tRNA and 1µl of 200 mM vanadyl-ribonucleoside complex (New England Biolabs) were added and mixture was incubated for 15 min at 37°C. The mixture was then extracted with 0.5 vol of phenol and 1 Vol of chloroform:IAA (24:1 v/v). The labeled riboprobe was recovered by addition of 0.1 Vol of 3 M sodium acetate and precipitated with 2 Vol of ethanol. The labeled riboprobe was dried under vacuum and resuspended in H_2O.

RNA used in the assay was precipitated from 0.5 M NaCl with 2 Vol of ethanol, recovered by centrifugation, and dried under vacuum. Total RNA from each sample was resuspended in 29.5 µl of hybridization buffer (20 mM Tris.HCl, PH 7.6/1 mM EDTA/0.4 M NaCl /0.1% SDS/ 75% deionized formamide) to which 0.5 µl of H_2O containing 20,000 dpm of labeled riboprobe was added. The mixture was heated to 85°C for 5 min and incubated at 45°C for 16 hours. After hybridization, 270 µl of RNase digestion buffer (10 mM Tris.HCl, PH 7.6/5 mM EDTA/300 mM NaCl/40 µg of RNase A per ml (Sigma)/2 µg of RNase T1 per ml (Pharmacia)] was added and the mixture was incubated for 1 hr at 30°C. $NaDodSO_4$ was then added to a final concentration of 0.6% followed by addition of 50 µg of proteinase K and

incubation at 37°C for 15 min. The mixture was extracted with 0.5 vol of phenol and 1 vol of $CHCH_3$. Twenty micrograms of tRNA and 2 vol of ethanol were added to the aqueous layer. After 1 hr at -20°C, the precipitate was collected by centrifugation, dried in vacuum, and resuspended in formamide sample buffer (80% deionized formamide and 0.2% each of bromophenol blue and xylene cyanol). The sample was heated to 95°C for 3 min, chilled on ice, and loaded on an 8% polyacrylamide/8 M urea denaturing gel. The gel was run at 50°C in 0.5 X TBE (1 X TBE is 89 mM Tris borate/89 mM boric acid/2 mM EDTA), dried and exposed to Kodak X-Omat for the indicated period of time.

DNA Isolation from Agarose Gels

Quick gel purification:

The DNA samples, including uncut plasmid, cut plasmid, or linearized inserts, were run in 1 or 1.2% agarose gels. The bands were visualized under the UV light and cut out as closely to bands as possible. Then the bands were weighed (no more than 0.3 g) and cut in small pieces, transferred to microcentrifuge tubes and 0.6 ml of phenol was added. The tubes were vortexed and the samples were incubated on dry ice for 5 min. and centrifuged at 10,000 X g for 10 min at room temperature. The supernatant containing the DNA was adjusted by adding TE (10 mM Tris, pH 8.0, 1 mM EDTA) to final volume of 0.4 ml, and one tenth vol of 3 M sodium acetate (pH 5.2) was added. The mixture was extracted with Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Then DNA was

precipitated from the aqueous phase with 2.5 vol of 100% ethanol at -20°C for 30 min. Precipitates were centrifuged for 30 min at 10,000 X g at 4°C, washed with 70% ethanol, dried and redissolved in 10 mM Tris, pH 8.0, 1 mM EDTA (TE) or sterile water.

Use of NA-45 DEAE membrane for the isolation of DNA fragments and fractions from agarose gels:

NA-45 DEAE membrane was obtained from S & S (Schleicher & Schuell, Inc. Keene, N.H.). Sheets of membrane were cut into a number of conveniently-sized rectangles (0.5 x 5 cm), activated by soaking in 10 mM EDTA, pH 7.4, for 10 min, then in 0.5 N NaOH for 5 min, followed by rinsing several times in distilled H_2O. DNA was run in appropriately-sized wells on the outer edges of a horizontal agarose gel, with molecular-weight standards in a center well. After electrophoresis sufficient to resolve the DNA fragments of interest from other size species, gels were stained in ethidium bromide (1 µg/ml in electrophoresis buffer) and destained in electrophoresis buffer. Gels were placed on short-wavelength UV transilluminator, and incised immediately in front of the band of interest. The incision was extended from the edge of the gel to slightly inside the lane containing the DNA to be isolated. A piece of DEAE membrane was inserted into the slit so that it was positioned to intercept the DNA, the gel was returned to the gel apparatus and electrophoresis was continued at twice the original running voltage until the DNA

migrated into the membrane. Band position was monitored by periodically transferring the gel in a UV-transparent tray to the transilluminator and visualizing the progression of the DNA band of interest. After sufficient electrophoresis, the membrane was removed and excess membrane was cut off. The membrane was immediately immersed into 5 ml of low salt concentration buffer containing 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA in a screw-cap test tube and the tube was placed on a shaker for 30 min at room temperature to remove any residual agarose. Then the membrane was transferred to 0.3 ml a high salt concentration buffer containing 2.5 M NH_4 acetate, 20 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA in a microfuge tube, and incubated at 60°C for 30 min, checking periodically to ensure that membrane remained submerged. After 30 min, the membrane was rinsed with 0.15 ml of high salt buffer for 5 min. The high salt buffer washes were combined and extracted with 3 volumes of H_2O-saturated n-butanol, then with one half volume of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated from the aqueous phase with 5 µg carrier tRNA and 2.5 volumes of 100% ethanol at -20°C overnight. The precipitates were centrifuged at 10,000 X g in microfuge at 4°C for 20 min, dried under the vacuum and resuspended in small volume of 10 mM Tris, pH 8.0, 1 mM EDTA (TE).

Labeling and Purification of 4-Thiouridine- or 6-Thioguanosine-substituted RNA

Explanted lens epithelia from 10- or 13-day-old chicken embryos were incubated in 35-mm dishes containing 2 ml of basal (Hams F-10) medium with 20 µCi of [^{3}H]-uridine (ICN, 45 Ci/mmole) and 75 µM 4-thiouridine (Sigma) or 3 µM 6-thioguanosine (Sigma) for 4, 6, or 24 hr at 37°C in 95% air/5% CO_2. At the end of the labeling period epithelia were removed from the medium and washed four times in warm Ham's F-10 medium, then immediately lysed in solution D (see RNA extraction).

The separation procedure followed the method of Woodford et al.(1988). Total RNA was redissolved in 50 mM sodium acetate, pH 5.5, 0.1% $NaDodSO_4$, 0.15 M NaCl, and 4 mM EDTA (buffer A), heated at 65°C for 5 min, cooled rapidly on ice, and batch-adsorbed to phenylmercury agarose (Affi-Gel 501, Bio-Rad) for 2 h at 4°C. The matrix was packed into a sterile RNase free column (2.5 ml, 5 Prime -> 3 Prime, Inc. West Chester, PA), and washed with 2.5X the column volume with buffer A at a flow rate of 5 ml/hr. Nonspecifically- bound RNA was eluted with 10 ml of buffer A containing 0.5 M NaCl. Thiol-substituted RNA (4-thiouridine or 6-thioguanosine labeled) was eluted with buffer A containing 10 mM 2-mercapto-ethanol, at a flow rate of 5 ml/hr. Fractions were collected and the radioactivity present was determined by liquid scintillation counting. RNA was recovered by precipitation with 2.5 volume of 100% ethanol at -20°C overnight and centrifuged at 4°C for 30 min. The pellet was washed with 70% ethanol and dried for slot blot or Northern blot analysis.

CHAPTER III

A Reexamination of Lens mRNA Stability

Introduction

Crystallin mRNAs accumulate to high levels during lens fiber formation (Piatigorsky, 1981). Messenger RNA stabilization is likely to be one mechanism contributing to crystallin mRNA accumulation. Evidence for stable mRNAs in the lens fibers originated from studies on calf (Stewart and Papaconstantinou 1967) and chicken embryo lenses (Reeder and Bell 1965, 1967; Yoshida and Katoh 1971). In all these studies, mRNA stability was estimated by measuring the decrease in protein synthesis in cells treated with actinomycin D, an inhibitor of transcription. So far, only one direct test of lens mRNA stability has been reported. In this study it was not possible to compare mRNA stability in lens epithelia and fibers (Treton, Shinohara and Piatigorsky, 1982).

The experiments of Yoshida and Katoh (1972) suggested that the sensitivity of lens epithelial cells to actinomycin D changed during development. Protein synthesis in epithelial cells of 10- or 11⅝-day-old embryonic lenses was almost completely arrested by actinomycin D. However, lenses from 12⅝-day-old embryos were relatively insensitive to the drug. Yoshida and Katoh concluded that mRNA stabilization occurred

between 11½ and 12½ days of development. In my studies I repeated this previous work and, in addition, directly measured the effects of treatment with actinomycin on lens mRNAs.

Results

Effects of actinomycin D on uridine incorporation

The effects of actinomycin D on RNA synthesis in embryonic lens epithelia were determined by measuring [^{3}H]-uridine incorporation into total RNA. Whole lenses from 10- or 13-day-old chicken embryos were preincubated for one hr in [^{3}H]-uridine, then incubated for an additional 3 or 5 hr in the presence or absence of the actinomycin D. After incubation, lens epithelia were separated from fibers, RNA was extracted and incorporation of uridine into RNA was measured by liquid scintillation counting. Figure 7 shows that, in lens epithelia of both ages, actinomycin D at either 0.5 or 30 µg/ml, blocked [^{3}H]-uridine incorporation by >90%.

Effects of actinomycin D on lens protein synthesis

Lenses from 11-day-old chicken embryos were labeled for 5 hr with [^{35}S]-methionine in the presence or absence of 0.5 or 30 µg/ml of actinomycin D. Treatment with the drug caused a 34 or 40% decrease in methionine incorporation in lens epithelia at the two doses employed (Fig. 8). This result was surprising, because Yoshida and Katoh (1971, 1972) showed complete inhibition of protein labeling by 30 µg/ml

Fig. 7 (A and B). **Effect of actinomycin D on [^{3}H]-uridine incorporation in 10- (A) and 13-day-old (B) chicken embryo lens epithelia.** Lenses were labeled with [^{3}H]-uridine for 1 hr, then actinomycin D was added to a final concentration of 30 or 0.5 µg/ml and lenses cultured for an additional 3 or 5 hr. At the end of the incubation lenses were washed and the epithelial cells were separated from the fibers. Total RNA was extracted and the labeled RNA from single lens epithelia was quantitated. Data points represent the means of counts from twenty lens epithelia. Error bars are ±SEM. Symbols: control lens tissue, (square), 0.5 µg/ml actinomycin D, (inverted triangle), 30 µg/ml actinomycin D (triangle).

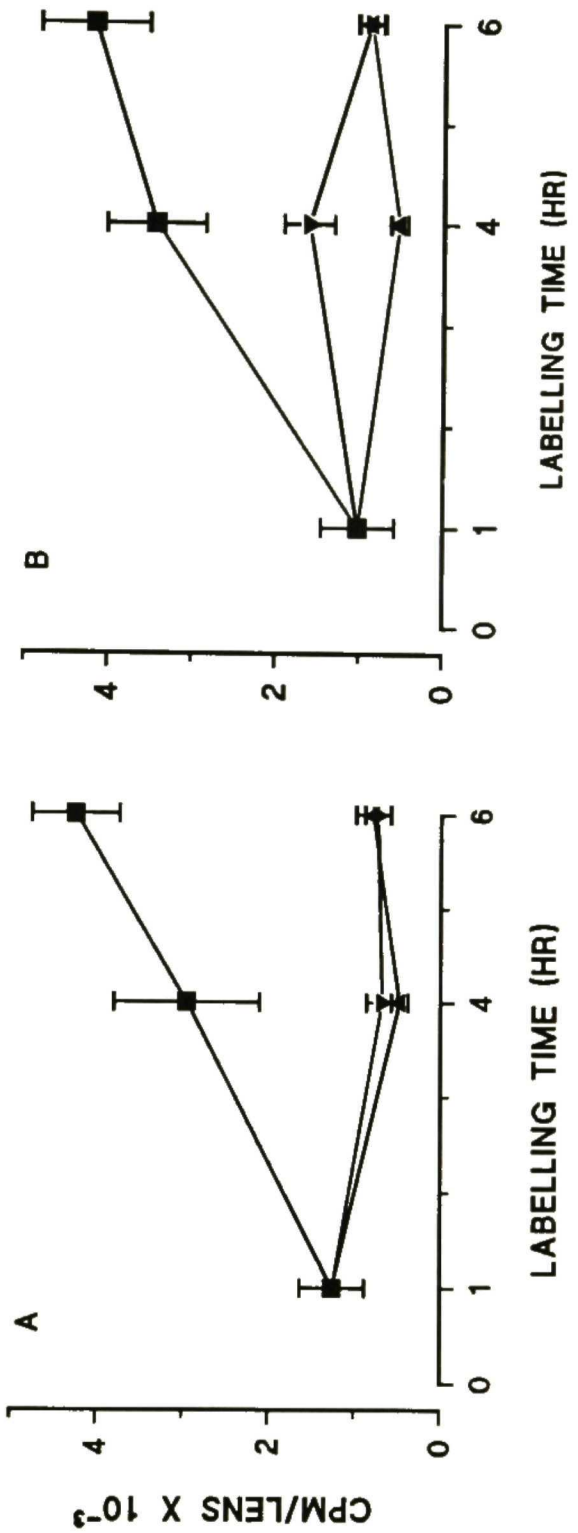
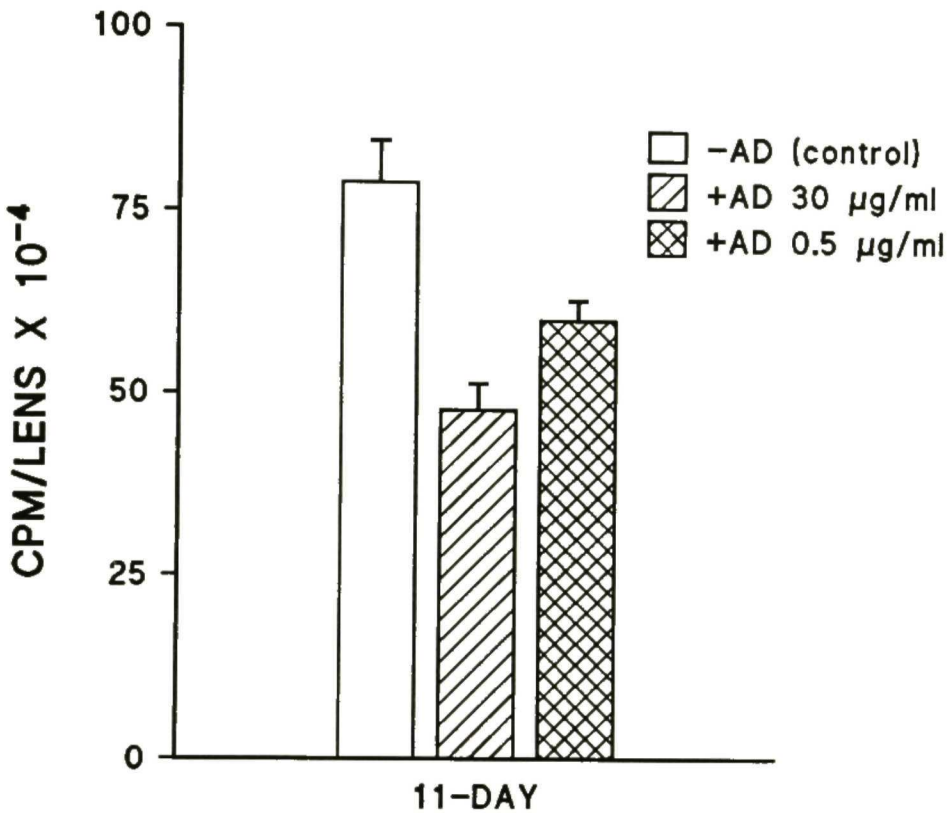

A
B
CPM/LENS X 10⁻³
0
2
4
0
1
4
6
LABELLING TIME (HR)

Fig. 8. **Effect of actinomycin D on protein synthesis in lens epithelia from 11-day-old chicken embryos.** Lenses were labeled with [^{35}S]-methionine for 5 hr in the absence or presence of actinomycin D (0.5 or 30 µg/ml). Values are the means ±SEM for ten groups of three lens epithelia.

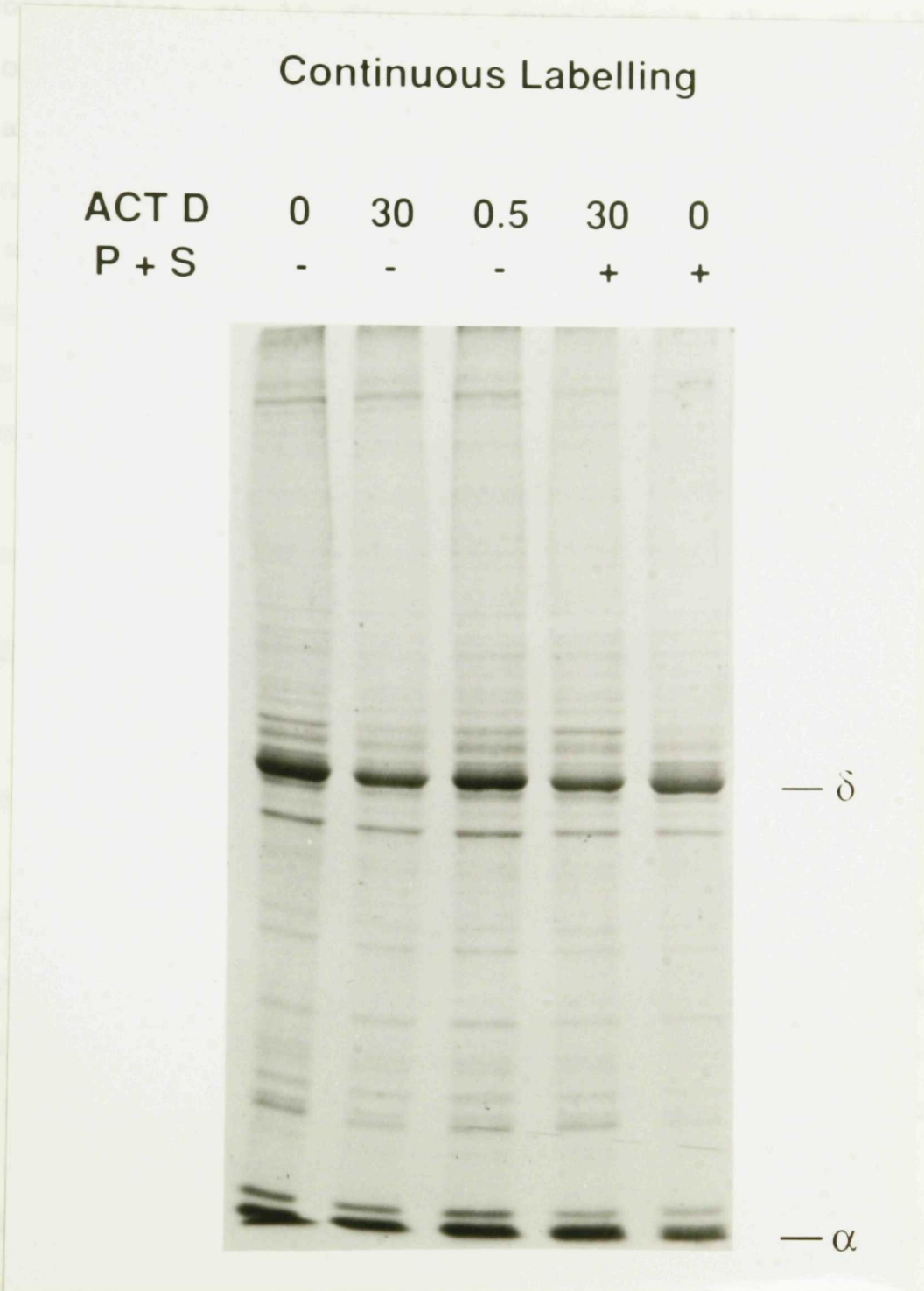

100
75
50
25
0
CPM/LENS X 10^{-4}
11-DAY
−AD (control)
+AD 30 µg/ml
+AD 0.5 µg/ml

actinomycin D in a similar experiment. Fig. 9 is an autoradiogram of the polypeptides labeled in this experiment after they were separated by polyacrylamide gel electrophoresis. The gels confirmed the inhibition of incorporation shown by scintillation counting. They also suggested that the drug inhibited incorporation into most polypeptides to a similar degree, since no major changes in the relative abundance of the major bands were obvious by inspection. Scanning the autoradiograms with a densitometer confirmed these impressions. For example, δ-crystallin accounted for between 8 and 11% of the total incorporation, whether actinomycin was present or not (data not shown).

In the experiments of Yoshida and Katoh (1971, 1972), penicillin and streptomycin were present during incubation. To test whether these antibiotics altered the effects of actinomycin, the experiment was repeated in their presence. No additional inhibition of labeling by actinomycin D was seen in this experiment (Fig. 9).

Lenses were then preincubated with actinomycin D prior to labeling to further test its effects on lens epithelial protein synthesis. The lenses of 10- and 13-day-old were chosen for this study because Yoshida and Katoh concluded that mRNA stabilization occurred between 11⅔ and 12⅔ days of embryonic development. Lenses were pretreated for 5 hr with actinomycin D, [^{35}S]-methionine was added and incubation was continued for an additional 2 hr. Incorporation of labeled methionine into lenses treated with 30 µg/ml of actinomycin D

Fig. 9. **Autoradiogram of proteins from three 11-day-old embryonic lens epithelia labeled with [^{35}S]-methionine and separated by polyacrylamide gel electrophoresis.** When actinomycin was used, it was present at 0.5 or 30 µg/ml. Although incorporation was decreased by treatment with actinomycin D, the kinds and relative amounts of polypeptides synthesized in control and drug-treated lens epithelia were similar. The presence of penicillin and streptomycin in the medium did not appreciably alter the effects of the drug. Autoradiogram was exposed for 23 hr.


Continuous Labelling
ACT D 0 30 0.5 30 0
P + S - - - + +
— δ
— α

was decreased by more than 60% of controls at both ages (Fig. 10) The lower concentration of actinomycin D (0.5 μg/ml) was approximately twice as effective in inhibiting methionine incorporation at 10 days of development than at 13 days. Autoradiograms of SDS gels again reflected the decrease in total protein labeling (Fig. 11). When these gels were quantitated by densitometry, the percentage of label associated with the δ-crystallin band increased nearly two-fold in drug-treated cells (data not shown). This suggested either that δ-crystallin mRNA was degraded more slowly than most messages in the epithelial cells, or that δ-crystallin mRNA was translated more efficiently than most messages in the presence of the drug.

Effects of actinomycin D on δ-crystallin mRNA levels

The effects of actinomycin D on δ-crystallin mRNA levels were measured by nucleic acid hybridization. Total RNA was extracted, blotted onto nitrocellulose membranes and hybridized to a [^{32}P]-labeled δ-crystallin cDNA probe. A representative autoradiogram (Fig. 12) shows that δ-crystallin message levels in 10- and 13-day-old chicken lens epithelia and fibers were essentially unchanged when lenses were treated for 6 hr with either 0.5 or 30 μg/ml of actinomycin. Levels of δ-crystallin mRNA were similar in 10- and 13-day-old lens epithelia.

The results of the previous experiment showed thattreatment with actinomycin D did not cause the loss of δ-

Fig. 10. **Effect of actinomycin D on protein synthesis in lens epithelia from 10- and 13-day-old chicken embryos.** Lenses were preincubated for 5 hr in the presence or absence of actinomycin, then pulse labeled for 2 hr with [^{35}S]-methionine in the same medium. The results are the means ±SEM of ten determinations on groups of three lens epithelia.

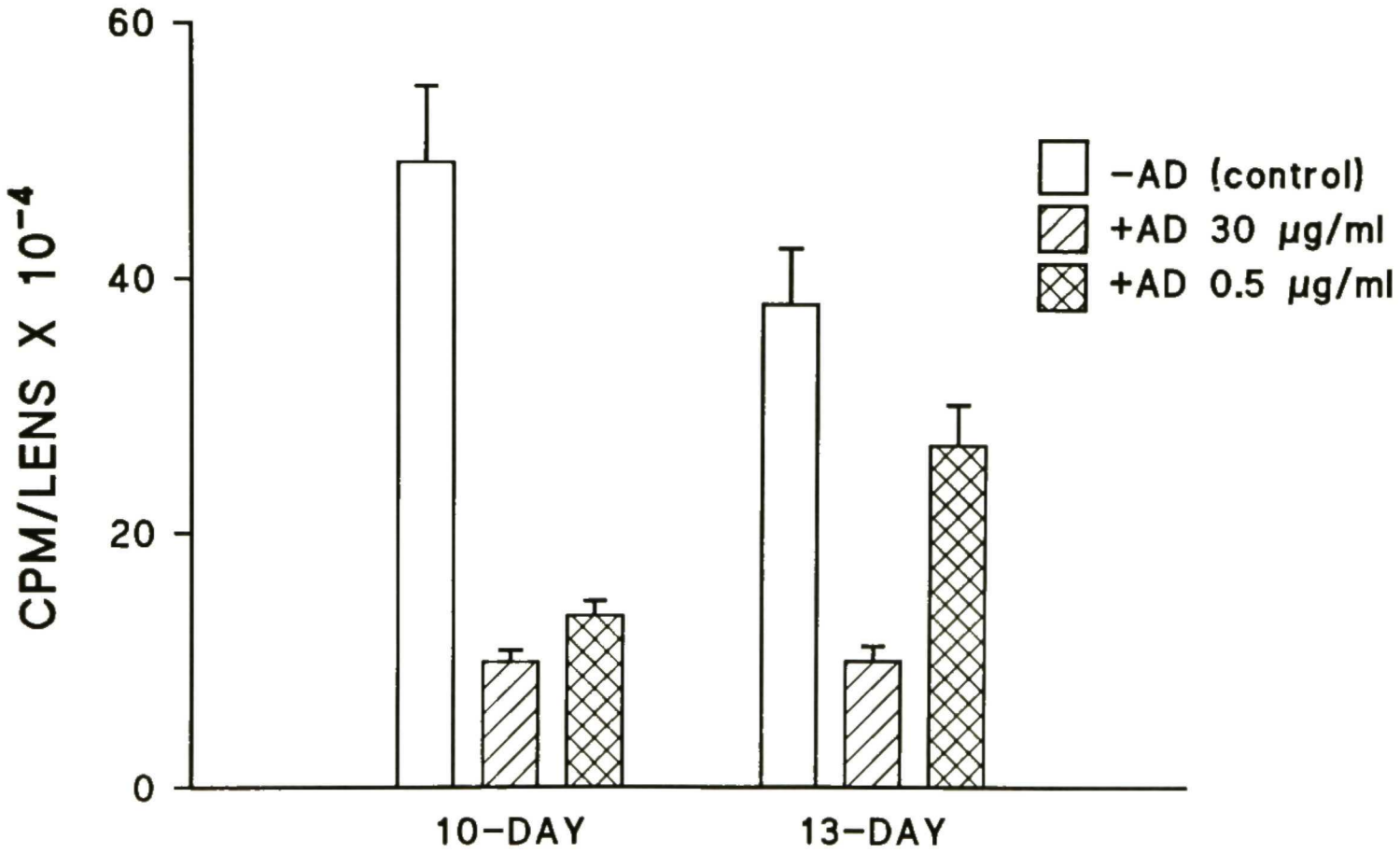

60
40
20
0
CPM/LENS X 10^{-4}
10-DAY
13-DAY
-AD (control)
+AD 30 µg/ml
+AD 0.5 µg/ml

Fig. 11. **Autoradiograms of proteins extracted from 10- and 13-day-old embryonic lens epithelia labeled with [^{35}S]-methionine and separated by polyacrylamide gel electrophoresis.** Groups of three lenses were cultured and labeled as described. Densitometer scans of the gels indicated that the δ-crystallin band accounted for approximately twice as much of the total protein labeling in actinomycin-treated lens epithelia, when compared to controls. Autoradiograms were exposed for 4 days.

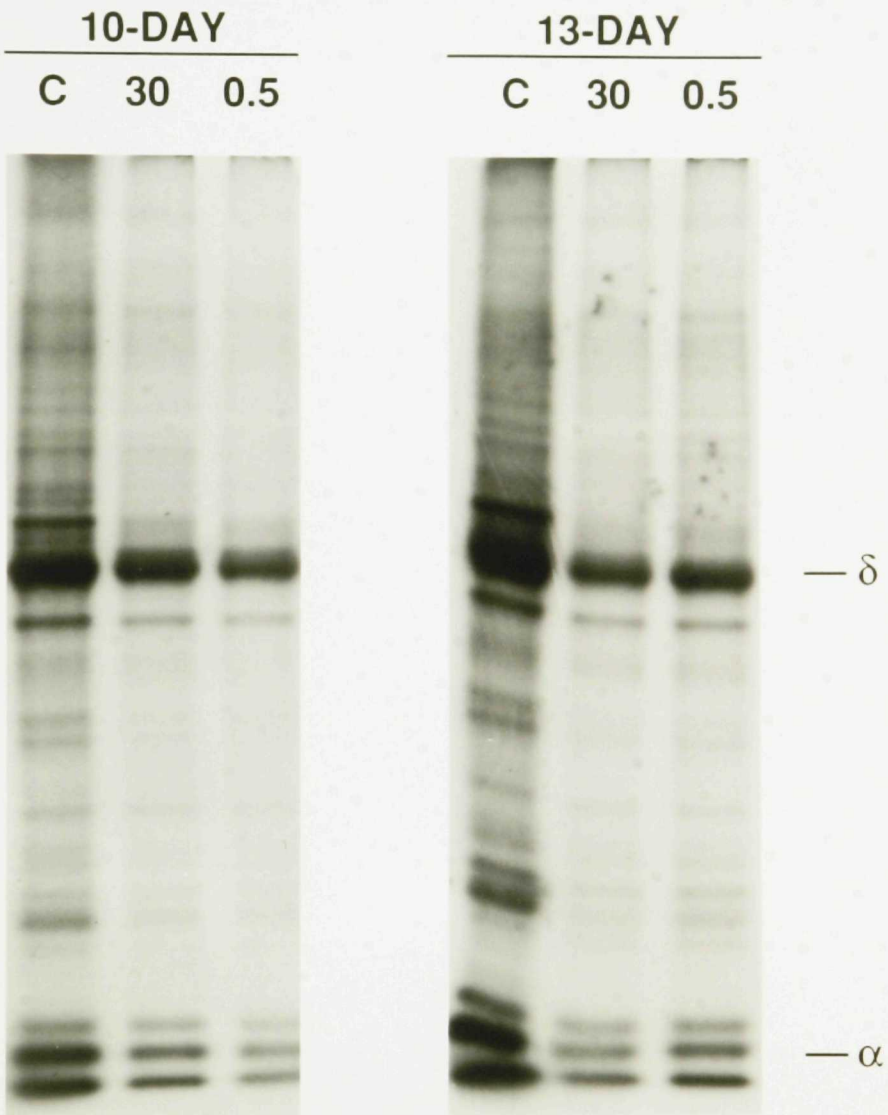
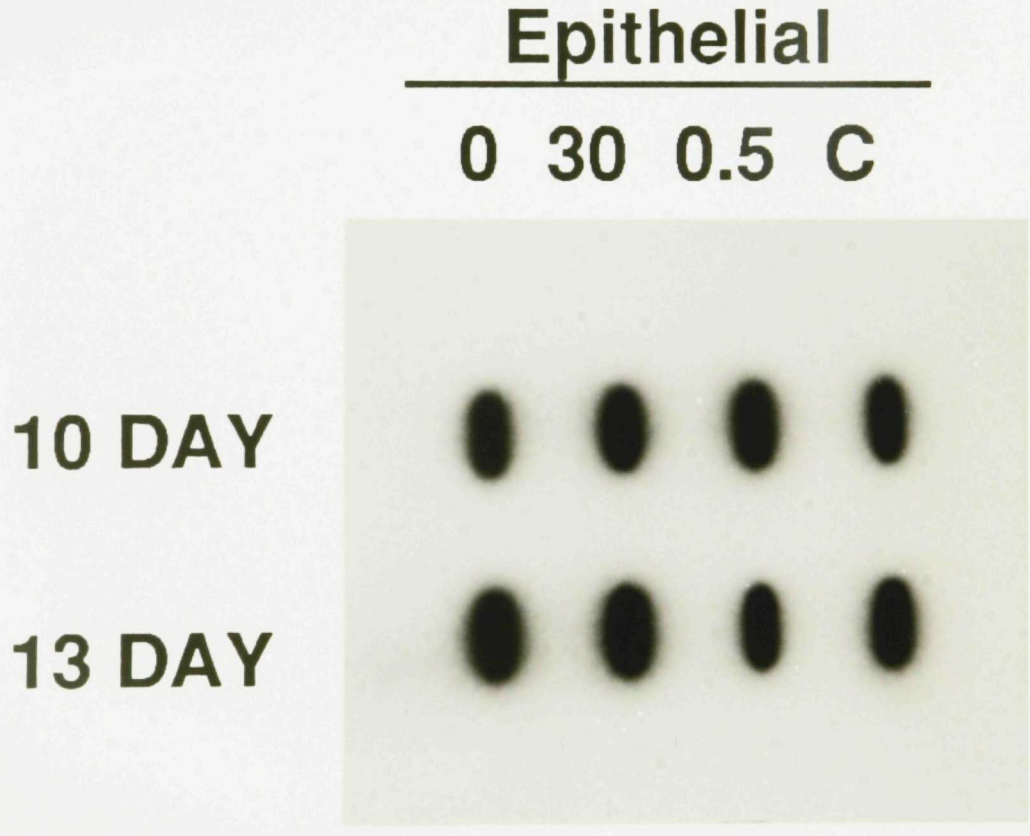

Pulse Labelling
10-DAY
C
30
0.5
13-DAY
C
30
0.5
— δ
— α

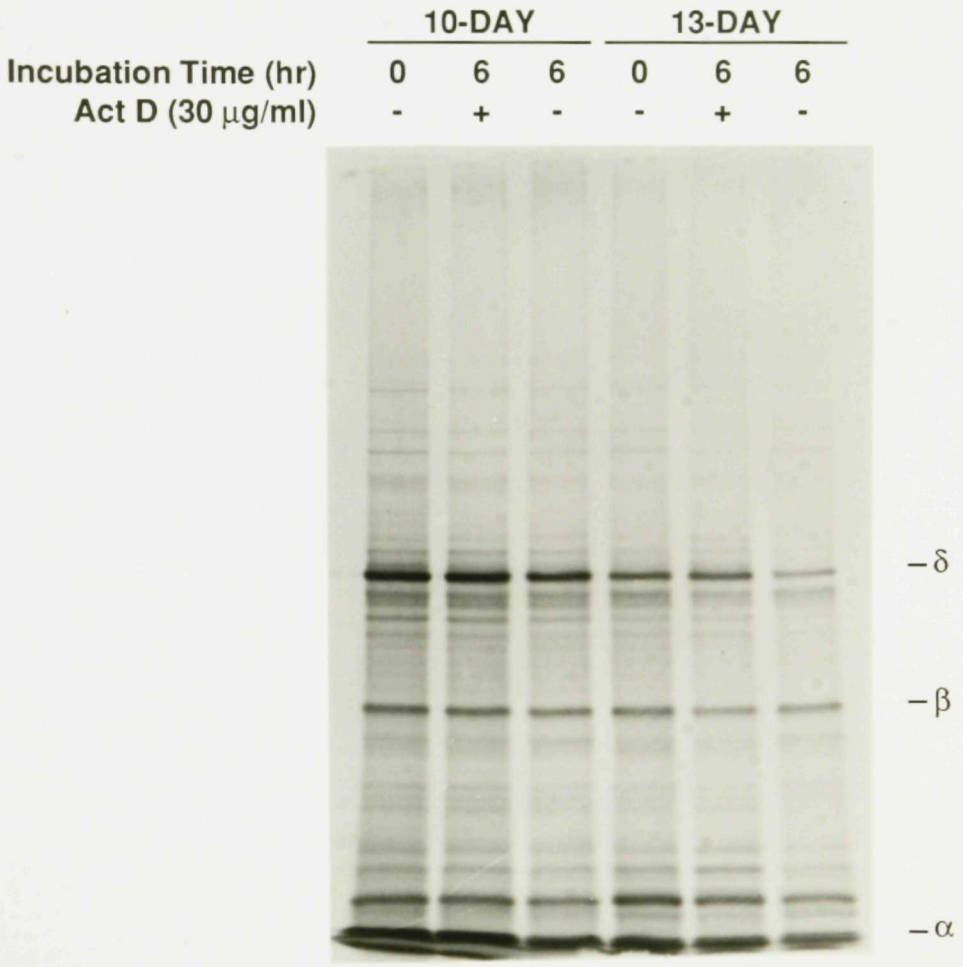
Fig. 12. **Hybridization of a labeled δ-crystallin cDNA probe to mRNA extracted from 10- and 13-day old embryonic lenses.** Total RNA was extracted from lens epithelia and fibers immediately after removal of the lens from the embryo (0) or after culture of the lens in Ham's F-10 medium for 6 hr (C) or after culture for 6 hr in the same medium with the addition of 30 µg/ml (30) or 0.5 µg/ml (0.5) actinomycin D. The autoradiogram was exposed for 20 hr.


Epithelial
0 30 0.5 C
10 DAY
13 DAY

crystallin mRNA sequences from lens epithelial cells. However, it is possible that these mRNAs were partially degraded during the incubation period. In this case they would have been detected by hybridization, but would not have been active as templates for protein synthesis. To resolve this issue, mRNAs were extracted from 10- and 13-day-old lens epithelia that were freshly dissected or had been cultured for 6 hr with or without actinomycin D. Total RNA was used as a template in a cell-free translation system and the products of translation were analyzed by polyacrylamide gel electrophoresis. The results are shown in Fig. 13. There was no obvious difference in the kind or relative amount of polypeptides synthesized from RNA extracted from lens epithelia treated with actinomycin D and controls. This is particularly evident when one compares the first and second and fourth and fifth lanes of the gel. These pairs of lanes have nearly identical band patterns, although they represent mRNA extracted from freshly-dissected lenses and lenses cultured in actinomycin D for 6 hr.

When the autoradiograms were quantitated by densitometry, δ-crystallin accounted for between 3.4 and 5.4% of total protein synthesis. No consistent effect of actinomycin on the percentage of δ-crystallin was detected. Thus, differential mRNA degradation in drug treated cells was not evident. In addition, the amount of δ-crystallin made from RNA extracted from drug-treated cells exceeded the amount produced fromcontrols in all cases. This is in general agreement with

Fig. 13. **Autoradiogram of proteins labeled with [^{35}S]-methionine in a reticulocyte lysate primed with total RNA from lens epithelial cells of 10- or 13-day-old chicken embryos.** RNA was extracted from groups of 10 freshly-isolated lens epithelia or from the same number of epithelia dissected from lenses cultured for 6 hr in the absence or presence of actinomycin D (30 μg/ml). Autoradiograms were exposed for 7 days. The percentage of total label incorporated into the δ-crystallin band varied by less than 2% whether or not cells had been treated with actinomycin D.


10-DAY
13-DAY
Incubation Time (hr)
0 6 6 0 6 6
Act D (30 μg/ml)
- + - - + -
δ
β
α

the data obtained by hybridization and suggested that, if anything, δ-crystallin mRNA may have been stabilized in actinomycin-treated cells.

Discussion

Most experiments aimed at studying mRNA stability in the lens have used actinomycin D to block transcription. At successive times after administering the drug, protein synthesis was measured. Decreases in protein synthesis were assumed to be due to the decay of mRNA. Results of these experiments suggested that mRNA turnover was lower in lens fibers than in lens epithelial cells (Scott and Bell, 1965; Reeder and Bell, 1965; Stewart and Papaconstantinou, 1967) and that mRNA stabilization occurred in chicken embryo lens epithelial cells between 11 and 13 days of development (Yoshida and Katoh, 1971, 1972). However, while experiments with actinomycin D can give information about mRNA turnover, this is an indirect approach. Interpretation of these experiments can be complicated by unforseen effects of the drug on other cellular processes and by compensatory changes in cellular metabolism in response to inhibition of RNA synthesis (Gross and Cousineau, 1964; Singer and Penman, 1973; Steinberg, Levinson and Tomkins, 1975; Gong and Brandhorst, 1988).

The studies of Yoshida and Katoh are among the most provocative in this series. They suggested that the majority of lens epithelial mRNAs are unstable during the early

development of the chicken embryo. During the 24 hr period between 11½ and 12½ days, these mRNAs appeared to become much more stable. Because of my interest in the control of lens mRNA stability, I began my studies by repeating these experiments. My results were quite different from those reported earlier.

The effect of actinomycin on protein synthesis in lens epithelia

In my studies, protein synthesis in 11-day-old embryonic chicken lens epithelial cells was inhibited 30-40% after treatment of the lenses for five hr with actinomycin D. In contrast, Yoshida and Katoh (1971) found nearly complete inhibition of protein synthesis under similar conditions. When I preceded the period of protein labeling with a 5 hr treatment with the drug, labeling was inhibited to a greater extent. However, even under these conditions inhibition of protein synthesis was similar in 10- and 13-day-old lens epithelia. Thus, my studies did not agree with the previous observation that protein synthesis became resistant to the effects of actinomycin during the twelfth day of development.

My results were more similar to those obtained previously by Craig and Piatigorsky (1973) and Laurent, et al. (1987). Craig and Piatigorsky found that treatment of 6-day-old lens epithelia for a short period, two hours, with concentrations of actinomycin D that inhibited nearly all RNA synthesis had little effect on protein synthesis. They

concluded from these studies that embryonic chicken lens epithelial cells had relatively stable mRNAs at this early stage of embryonic development. Laurent and co-workers found that actinomycin D inhibited lens epithelial protein synthesis in 11- and 18-day-old chicken embryo lenses by only 49% and 36%, respectively. In their studies lenses were labeled for two hours following a two hour preincubation in actinomycin. The extent of inhibition of protein synthesis in their work was very similar to that obtained in the present study and much less than was seen by Yoshida and Katoh (1971, 1972).

The effect of actinomycin D on δ-crystallin mRNA levels

Hybridization of lens epithelial RNA with a labeled δ-crystallin cDNA probe revealed no major difference in the quantity of δ-crystallin mRNA between control and actinomycin-treated lens epithelia or fibers from either 10- or 13-day-old embryos. These data suggested that δ-crystallin mRNA was stable at both ages. Alternatively, mRNA turnover may have been inhibited by the block of transcription, as has been described in other studies (Gross and Cousineau, 1964; Steinberg, Levinson and Tomkins, 1975). In either case, our studies suggested that a decrease in the level of δ-crystallin sequences did not contribute to the decline in protein synthesis seen in actinomycin-treated cells.

This conclusion was tested further by translating the mRNAs from untreated and actinomycin-treated lens epithelia in an *in vitro* protein synthesizing system. It was possible

that, in actinomycin-treated cells, mRNAs were partially degraded, but still detectable by nucleic acid hybridization. However, our studies demonstrated that the mRNAs from control or actinomycin-treated, 10- or 13-day-old embryonic lens epithelia were translated with comparable efficiency. This was true for crystallins and non-crystallin proteins.

Upon further examination of their data, one aspect of Yoshida and Katoh's experiments was puzzling. In their studies, actinomycin D nearly abolished protein labeling in 11⅓-day-old lens epithelia during 5 hr of culture. If actinomycin D reduced protein synthesis solely by preventing new RNA synthesis, the half-life of the all of the mRNAs in these cells would have had to have been exceedingly short. If not, a substantial amount protein synthesis would have occurred during the early portions of the labeling period, while the majority of the mRNAs were still intact. Because protein labeling was nearly undetectable in these experiments, it is possible that the preparation of actinomycin D that Yoshida and Katoh used for their studies had non-specific toxic effects on lens epithelial protein synthesis. If this were true, lens epithelia from embryos that were 12⅔ days old were much more resistant to this effect.

In my study, actinomycin D appeared to inhibit protein labeling primarily by reducing translation, rather than by its effects on transcription. Although the drug effectively blocked lens epithelial RNA synthesis, we detected no change in the relative amounts of proteins synthesized when mRNAs

from untreated or actinomycin-treated lenses were used to prime an *in vitro* translation system. We also showed that δ-crystallin sequences persisted in a translatable form in actinomycin-treated lens epithelial cells. These observations make it likely that the decrease in protein labeling in cultured lens epithelia caused by treatment with actinomycin D was not due to the decay of other, non-δ-crystallin, messages. If this had occurred, the translation of δ-crystallin mRNA would have accounted for the synthesis of a larger percentage of the proteins produced *in vitro*. Because this was not observed, it is most likely that all of the major mRNAs had a similar half-lives in the actinomycin D-treated cells. The decline in protein labeling that resulted from treatment with actinomycin D was probably due to either a direct effect of the drug on translation (Goldstein and Penman, 1973; Singer and Penman, 1973), or to the rapid decay of a critical component of the translation machinery that depended on new RNA synthesis.

CHAPTER IV

Testing New Approaches to Study mRNA stability

Introduction

Regulation of RNA metabolism can include the alteration of synthesis, modification, processing, function and degradation. The mechanisms for regulation of RNA metabolism are understood only to a limited extent. A principal reason for this is the lack of good experimental techniques (Atwater, Wisdom and Verma, 1990).

Inhibitors of RNA synthesis and modification are often used to study the mechanisms controlling mRNA levels. However, these drugs disrupt the metabolism of all mRNAs in a cell and, therefore, may have deleterious effects on cell function. These drugs also alter the rate of translation or degradation of mRNA in some cases (Goldstein and Penman,1973; Steinberg, Levinson and Tomkins, 1975; Mullner and Kuhn, 1988). My studies also showed that actinomycin D has other inhibitory effects, in addition to blocking transcription (Chapter III, Li and Beebe, 1991). The precise effects of these drugs should be established in each system in which they are used. However, this is often not possible or practical.

The optimal method for the study of mRNA metabolism would be the pulse-chase technique. This method has been used

successfully to study the turnover of some mRNAs, notably that for vitellogenin (Brock and Shapiro, 1983), but is difficult to apply widely (Atwater, Wisdom and Verma, 1990). This is because it is difficult to rapidly equilibrate intracellular UTP pools and because the low sensitivity of this method makes it difficult to apply to mRNAs of low abundance. In spite of these potential difficulties, I did try to use this method for my project (see below).

Another method is to selectively label newly synthesized mRNA with analogs, like 4-thiouridine and 6-thioguanosine. After labeling, labeled mRNA can be separated from the pre-existing mRNA by affinity chromatography on mercurated agarose. This method had also been tested for my project (see below).

An efficient and general method for measuring mRNA metabolism would be of great use to molecular biologists. I have combined one of the oldest techniques in molecular biology, density labeling, with one of the newest, the polymerase chain reaction (PCR) to provide a new way of measuring mRNA metabolism. My approach is to density-label newly-synthesized RNA using nucleoside precursors containing ^{13}C and ^{15}N following methods described by Bowman and Emerson (1980). Density-labeled RNA can then be separated from the pre-existing RNA by equilibrium density gradient centrifugation (Winkles, and Grainger, 1985). After separation, the newly-synthesized, density-labeled RNA is reverse-transcribed and quantitated using PCR (Saiki, et al,. 1988). This method

permits one to measure the rate of synthesis, processing, and degradation of any mRNA for which a sequence is available.

Use of An RNase Protection Assay to Measure δ-crystallin mRNA Metabolism

Introduction

After reexamination of the actinomycin D experiment, I found that RNA synthesis inhibitor, actinomycin D, was not an ideal drug for investigating RNA stability. Its many deficiencies and side effects were described above.

Based on the preliminary studies, I first planned to quantitate the accumulation of δ-crystallin mRNA in chicken lens fibers and epithelia at different stages of development and during differentiation in vitro using an RNase protection assay. Second, I planned to estimate the stability of the δ-crystallin mRNA in lens fiber cells and epithelial cells using [^{3}H]uridine pulse labeling technique. Once these data were available, δ-crystallin mRNA accumulation, turnover and synthesis could be calculated.

Results

A 492 bp fragment of the full-length δ-crystallin cDNA (pδCR17, a gift from Dr. Piatigorsky) was subcloned into the pGEM blue vector, which contains both SP6 and T7 promoters and which can be used for in vitro transcription. The full length cDNA was cut from the plasmid, pδCR17, with the restriction enzyme *Hpa* II (see Fig. 6B). Then the 492 bp cDNA fragment

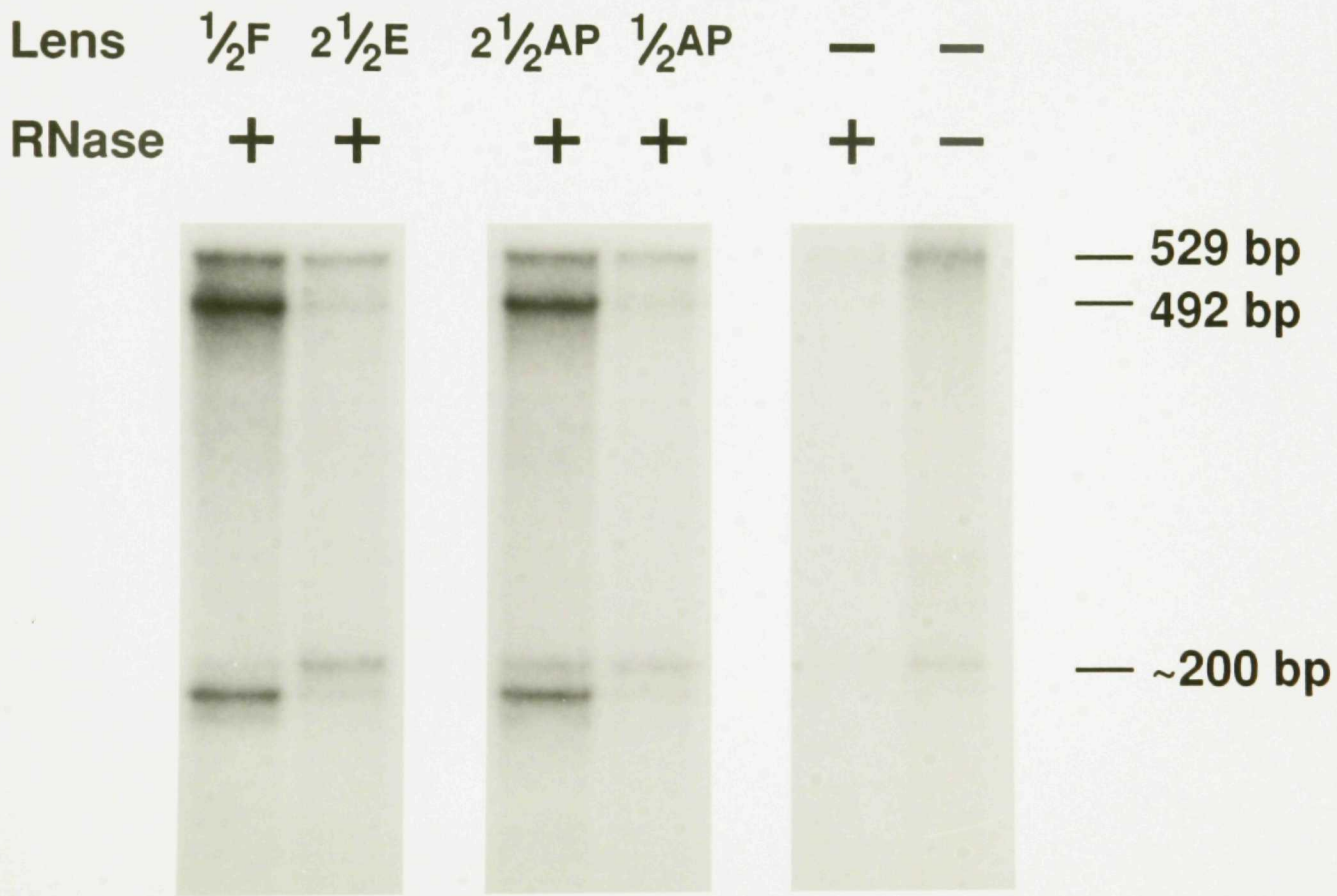
(572 bp to 1063 bp, see Fig. 6A) was cut with *Pst* I and subcloned into the *Pst* I cloning site of pGEM blue vector (see Fig. 6C).

This vector, containing 492 bp of δ-crystallin cDNA, was linearized with *Sma*I or *Hind* III (for Sp6 or T7 polymerase respectively), and transcribed with SP6 and T7 polymerase to produce [^{32}P]rUTP labeled RNA probes of opposite polarity. Sense or antisense RNA was determined by hybridization with RNA extracted from the chicken lens. The sense RNA probe did not hybridize to the lens RNA (the vector was linearized with *Hind* III and transcribed with T7 polymerase), but antisense RNA did hybridize (when the vector was linearized with *Sma*I and transcribed with SP6 polymerase, data not shown).

After making an RNA probe, the RNase protection assay was performed. The total RNA in different regions of the lens (epithelium, fiber and annular pad) was extracted from 6-day-old chicken embryos and hybridized to the [^{32}P] labeled antisense RNA probe in solution. The results were not fully satisfactory (see Fig. 14). The probe made from <u>in vitro</u> transcription had two bands. One band, 529 bp, is the full length probe, of which 492 bp was the δ-crystallin cDNA fragment (see method of riboprobe construction) and 37 bp was Sp6 promoter. Another band, ~200 bp, was also formed. This band was seen in every experiment that I performed. It was difficult to explain.

In addition, labeling with [^{3}H]uridine was unsatisfactory (data not shown). Selected lenses from 6-day-

Fig. 14. **Distribution of δ_1-crystallin mRNA in different regions of the lens.** A solution hybridization/RNase protection assay was performed using a δ_1-crystallin antisense RNA as described in Materials and Methods. Total RNA extracted from the central epithelial (E), annular pad (equatorial epithelial, AP), and whole fiber masses (F) of 6-day-old embryonic chicken lenses. The autoradiogram shown in this figure represents 7 days of exposure.


Lens
½F
2½E
2½AP
½AP
—
—
RNase
+
+
+
+
+
—
529 bp
492 bp
~200 bp

old chicken embryos were incubated in 2 ml of basal incubation medium (Ham's F-10) containing 100 μCi [^{3}H]uridine for 4 to 6 hr (the lens became cloudy if the incubation time was longer than 6 hr). After labeling, the lenses were washed three times with fresh ice-cold medium and dissected. Total RNA was extracted and counted in the scintillation counter. It was found that incorporation of [^{3}H]uridine was low. Preliminary calculations suggested that the δ-crystallin mRNA would have no more than 200 total cpm, a number that was too small for statistical significance.

I decided to abandon this method. This decision was based on uncertainly about the probe and the low [^{3}H]uridine counts, as well as concern about the time needed to saturate the uridine pool.

Separation of Newly-Synthesized, Thiol-substituted RNA by Phenylmercury Affinity Chromatography for the Study of δ-Crystallin mRNA Metabolism

Introduction

I next decided to separate newly-synthesized mRNA from pre-existing mRNA by labeling RNA with thiol-containing precursors. Total RNA was labeled with 4-thiouridine or 6-thioguanosine, followed by isolation of the thiol-substituted, newly-synthesized RNA by phenylmercury affinity chromatography (Woodford, *et al*. 1988; Melvin and Keir, 1977). Logically, under in *vitro* conditions, once the pool of the UTP or GTP in the cells was saturated by 4-thiouridine or 6-thioguanosine,

the thiol-containing RNA precursors should be incorporated into newly-synthesized RNA. The rate of appearance of thiol-substituted RNA should represent the rate of RNA synthesis. The rate of the synthesis and degradation can be calculated by measuring the amount of newly-synthesized RNA and "old RNA" after labeling for increasing periods of time. Newly synthesized, thiol-substituted RNA can be recovered by Affi-Gel 501 phenylmercury affinity chromatography (Woodford, et al., 1988).

Results

Lens epithelia were removed from 10- or 13-day-old chicken embryos and explanted and the lens epithelia incubated in the basal (Ham's F-10) medium with 20 μCi of [^{3}H]uridine and 75 μM 4-thiouridine of 3 μM 6-thioguanosine for 4, 6, or 24 hr at 37°C. At the end of the labeling period, the explanted lens epithelia were washed and RNA was extracted. The thiol-substituted RNA (4-thiouridine or 6-thioguanosine labeled) was isolated by Affi-gel 501 phenylmercury agarose chromatography. The fractions were collected and the radioactivity present in samples were determined by liquid scintillation counting. RNA was recovered by precipitation for RNA blot or Northern blot.

The results of preliminary studies were again not satisfactory. First, the incorporation of [^{3}H]uridine was very poor. Four, six, or even twenty four hours labeling was not enough to get substantial counts into newly synthesized

RNA. Second, thiol-substituted RNA was very poorly bound to the affinity column. The data showed that only the background counts appeared in the elution solution, which should have contained thiol-substituted RNA. Instead, most counts appeared in the wash solution. These experiments were repeated with many modifications, but the results were always technically inadequate.

In order to attempt to increase the amount of uridine incorporation, 20 mM glucosamine was added at beginning of the incubation period (Levis and Penman, 1977). Glucosamine depletes the endogenous, intracellular UTP pool by stimulating the production of UDP-glucosamine. This increases the ability of added uridine to saturate the UTP pool and be incorporated into RNA. After a 30 min pretreatment with glucosamine, 75 μM 4-thiouridine and [^{3}H]uridine were added. Unfortunately, the incorporation of [^{3}H]uridine in the sample with glucosamine was much lower than in the control sample without glucosamine (data not shown). I then tested different time of glucosamine pretreatment, including 0 min, 30 min, and 60 min. Labeling time was always 4 hr and [^{3}H]uridine tracer was always added at the beginning of the experiments. The results showed that incorporation of the [^{3}H]uridine in all the samples with different times of glucosamine pretreatment were lower than the control without glucosamine.

All the data described above suggested that thio-uridine inhibited RNA synthesis in lens epithelial cells. For this reason and because of the poor binding of the thiol-

substituted RNAs to the affinity column, this method, too, had to be abandoned.

Use of Density Labeling and PCR to Study δ-crystallin mRNA Metabolism

Introduction

As I mentioned before, in most cells, the details of mRNA regulation are understood only to a limited extent, due to the lack of adequate techniques with which to isolate species arising from individual steps in RNA metabolism from those that precede or follow it (Atwater, Wisdom and Verma, 1990). These limitations are most difficult to manage when limited numbers of cells are available.

The level of a specific mRNA can often be accurately determined by one of several methods. These include northern blotting, RNase or S_1 protection assays and quantitative PCR. However, if the amount of this RNA changes, it is often difficult to determine whether the change was due to alteration of its rate of synthesis, processing, degradation, or a combination of these steps. Methods to measure the synthesis or degradation of specific mRNAs are available. However, these usually require homogeneous preparations of large numbers of cells. For instance, analysis of mRNA metabolism by pulse-chase or equilibrium labeling methods requires that detectable levels of radioactivity be incorporated into a single kind of mRNA. If this message represents a relatively small proportion of the total mRNA pool, high levels of

radioactivity and large numbers of cells may be required to reliably detect alterations in synthesis or degradation. Similarly, run-on transcription assays, which permit one to estimate the rate of RNA synthesis, depend on the isolation of large numbers of nuclei.

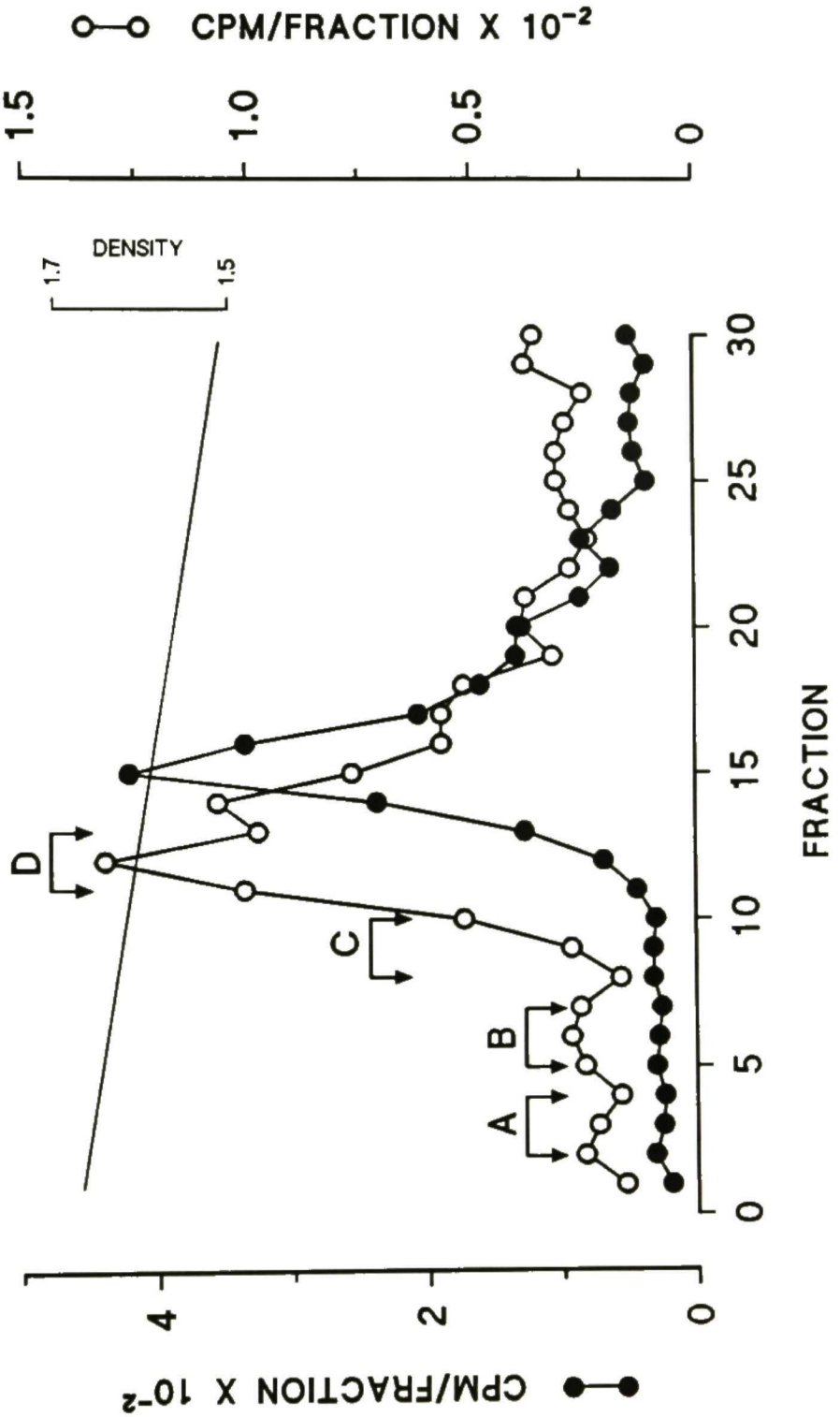
A method was needed which would permit investigators to follow the metabolism of a specific mRNA in a relatively small number of cells. I have used density labeling, followed by equilibrium density gradient centrifugation, to separate newly-synthesized RNA from pre-existing RNA. Messenger RNA levels were then assayed using the exceptional sensitivity of the polymerase chain reaction.

Results

Separation of density-labeled and light RNA from lens fiber cells on a NaI/KI equilibrium density gradient.

Lenses from 14-day-old chicken embryos were labeled for four hours with ^{3}H-uridine in the presence or absence of 10^{-4}M $^{13}C^{15}N$-labeled ribonucleosides. Figure 15 shows the pattern of ^{3}H-uridine incorporation into density-labeled and control lens fiber RNA separated on a NaI/KI equilibrium density gradient. The peak of the density-labeled RNA was shifted toward the bottom of the gradient. For light RNA, only background levels of radioactivity were present in fraction 11 or below, while nearly half of the counts in density-labeled RNA were present in fraction 12 or below. I also analyzed RNA distribution in the fractions of NaI/KI

Fig. 15. **Separation of density-labelled and light RNA from 14-day-old embryonic lens fiber cells on a NaI/KI equilibrium density gradient.** O–O density-labelled RNA; ●–● control (light) RNA. RNA was extracted from 2 lens fiber masses cultured for 4 hr in medium containing 100 µCi [^{3}H]uridine in the absence or presence 10^{-4}M $^{13}C^{15}N$-labelled ribonucleosides. After the RNA was centrifuged to equilibrium in NaI/KI gradients, the fractions were collected as A (fraction 2-4), B (fraction 5-7), C (8-10), and D (11-13), of which 15 µl of each fraction was taken for ethanol precipitation.


CPM/FRACTION X 10⁻²
1.5
1.0
0.5
0
DENSITY
1.7
1.5
A
B
C
D
0
5
10
15
20
25
30
FRACTION
CPM/FRACTION X 10⁻²
0
2
4

equilibrium density gradient by agarose electrophoresis (Fig. 16). Most of the RNA was located between fraction 13 and 16. The different scales used in Fig. 15 reflect the dilution of the specific activity of the ^{3}H-uridine by density-labeled uridine. The position of light RNA in the gradient in different experiments was extremely reproducible.

Amplification of density-labeled mRNA purified by equilibrium density gradient centrifugation

After separation, I recovered RNA from groups of three fractions in the dense region of the gradient (A - D in Fig. 15). RNA from these fractions was reverse transcribed using primer #139 (see Fig. 17), and amplified by PCR using the 5′ and 3′ exon primers, #137 and #139. These oligonucleotides bind to identical sequences in δ_1- and δ_2-crystallin mRNA. Fig. 18 shows an electrophoretic analysis of the PCR products from fractions A-D of Fig. 15. In the density-labeled samples a 229 bp band was detected in samples A - D. In the control samples the 229 bp band was present only in fraction D. This was consistent with the distribution of light RNA in Fig. 15. PCR reactions that did not contain template never contained amplified DNA. Several repetitions of this experiment gave similar results.

Fig. 19 shows electrophoretic analysis of PCR products from the same fractions shown in Fig. 18. In this case, the 5′ intron-specific primer, #999, and the 3′ primer, #139 were used to reamplify an aliquot of the first PCR reaction. Both

Fig. 16. **Electrophoretic analysis of RNA distribution in the fractions of the NaI/KI equilibrium density gradient.** Total RNA was extracted from two fiber masses of 14-day-old chicken embryonic lenses labelled with 10^{-4}M $^{13}C^{15}N$-labelled ribonucleosides. After the RNA was centrifuged to equilibrium in NaI/KI gradients, the fractions were collected and every two fractions were combined and precipitated in ethanol, then analyzed on the denaturing formaldehyde agarose gel and photographed.

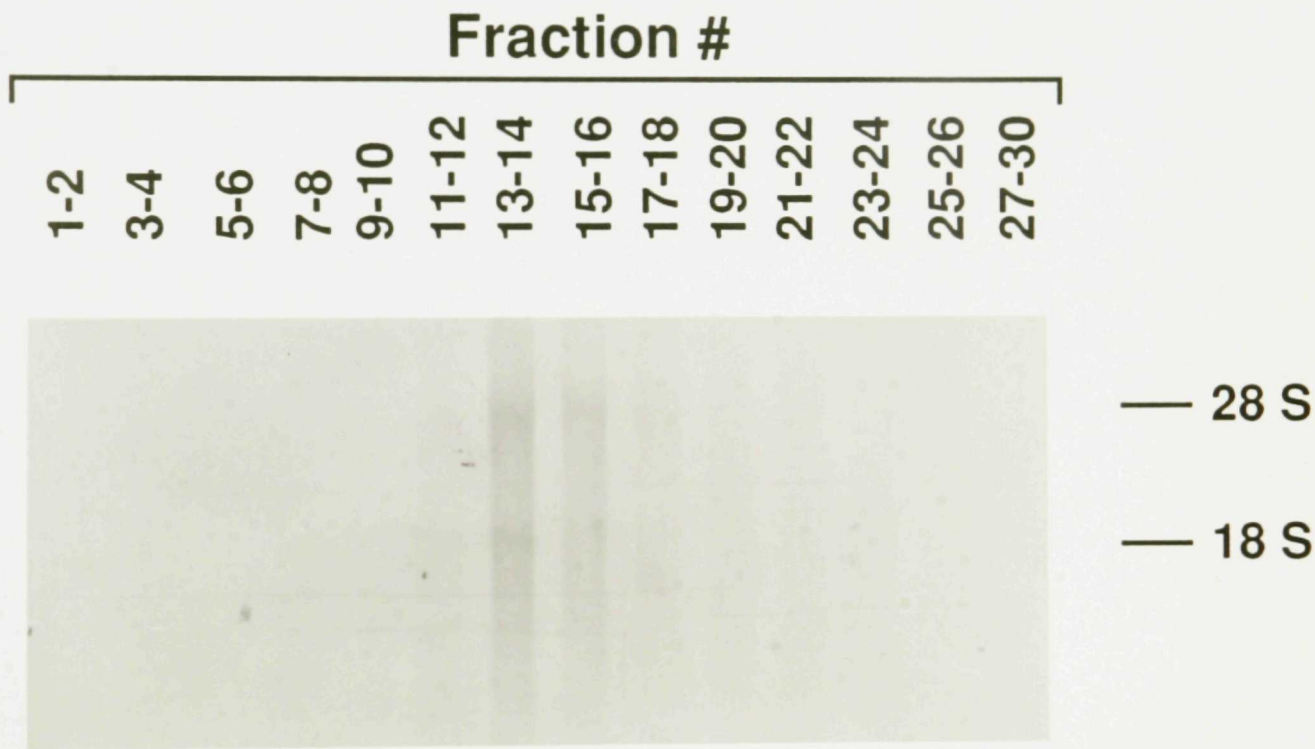

Fraction #
1-2
3-4
5-6
7-8
9-10
11-12
13-14
15-16
17-18
19-20
21-22
23-24
25-26
27-30
28 S
18 S

Fig. 17. **Schematic diagram of the region of The δ_1- & δ_2-crystallin genes encoding exons 12-14.** The oligonucleotide primers used to amplify mature and unprocessed mRNA are shown. Primers: #999, an intron-specific, sense-strand primer identical to sequences in intron L of the δ_1- and δ_2-crystallin genes; #137, a sense-strand primer identical to sequences in exon 12 of both δ-crystallin genes; #139, an antisense primer complementary to identical sequences in exon 14 of both δ-crystallin genes; #513, a sense-strand 35-mer specific for a sequence in exon 13 of the δ_1-crystallin gene; #492, a 20-mer specific for exon 13 of the δ_2-crystallin gene.

PCR PRIMERS AND PRODUCTS

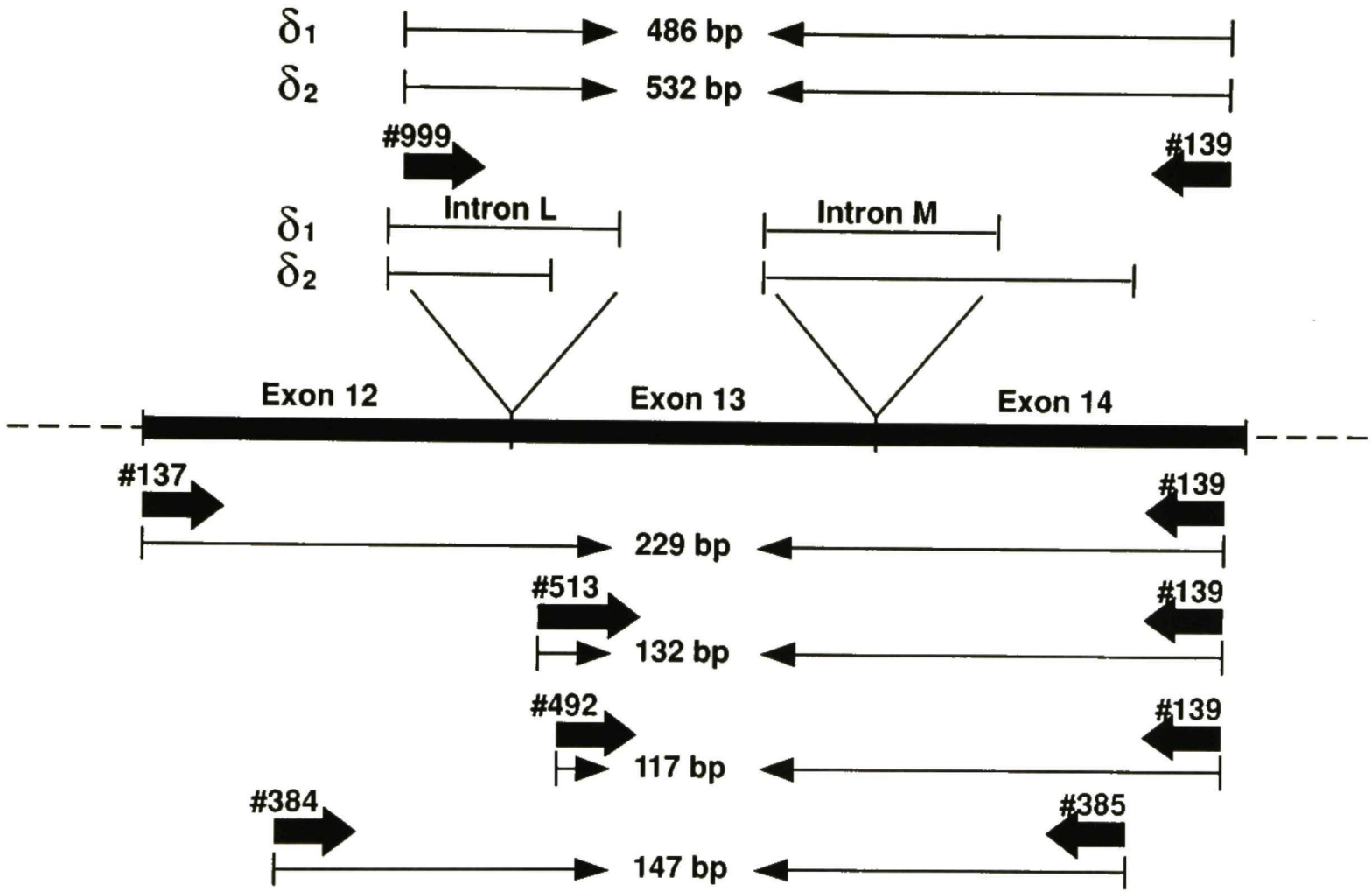


Fig. 18. **Electrophoretic analysis of PCR products from fractions A-D of Fig. 15.** Each lane represents the final reaction product derived from total RNA from fractions A, or B, or C, or D. Mature δ-crystallin mRNA sequences were detected in the dense region of the gradient only when the RNA was density-labelled. Primers were #137 and #139, hybridize to exons yielding a PCR product of 229 bp, which is identical in both the δ_1- and δ_2-crystallin matured mRNA.

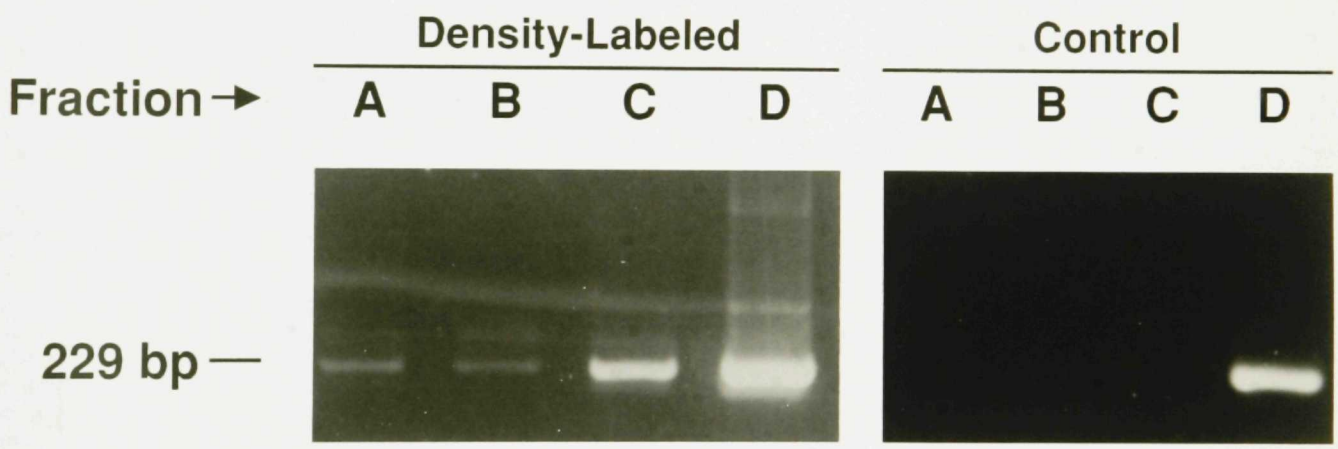

Density-Labeled
Control
Fraction →
A
B
C
D
A
B
C
D
229 bp —

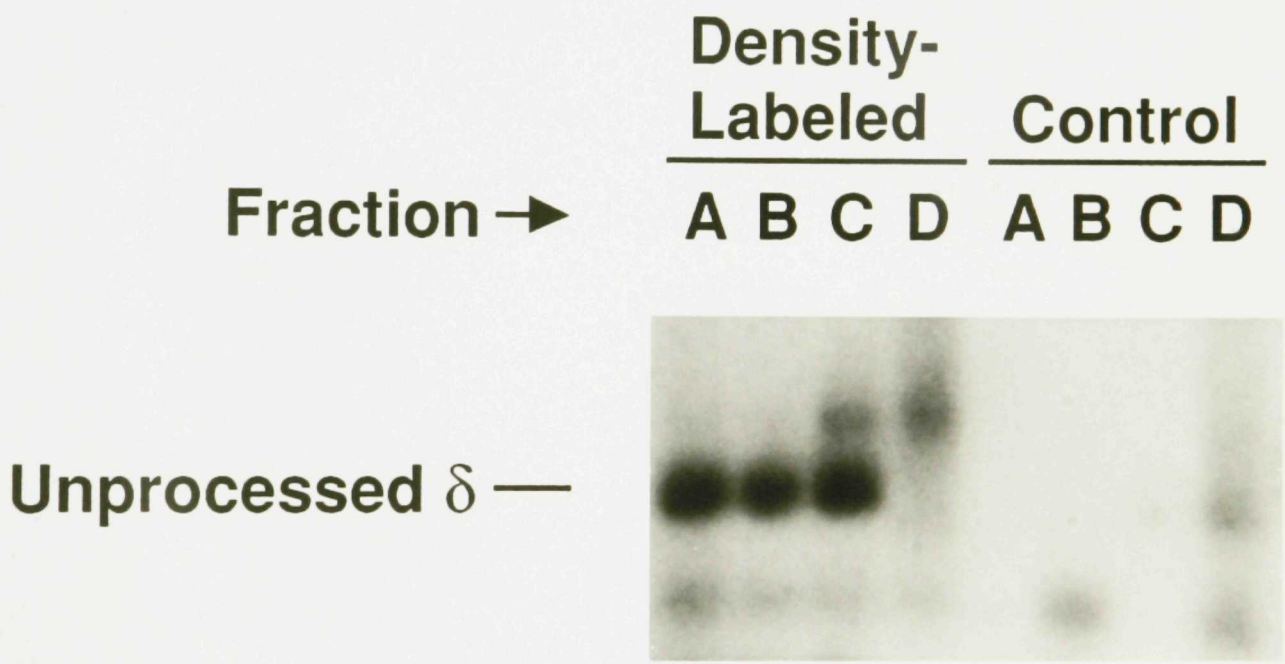
Fig. 19. **Electrophoretic analysis of PCR products from fractions A-D using an intron-specific primer.** Lane G represents the PCR products when genomic DNA was used as template. The lane marked STD contains a 1 kb standard DNA markers (BRL). Unprocessed δ-crystallin mRNA was detected in fractions A-C only in samples containing density-labelled RNA. Only δ_1 sequences were detected in these studies, suggesting that transcription of the δ_1 gene predominated over δ_2. The 5′ intron specific primer #999 and the 3′ exon primer #139 were employed. Unprocessed δ_1-crystallin mRNA is predicted to yield 486 bp, while unprocessed δ_2-crystallin mRNA would yield a 532 bp fragment.


STD
Density-Labeled
Control
Fraction →
A
B
C
D
A
B
C
D
G
517
506
— δ2 532 bp
— δ1 486 bp

primers bind to sequences that are identical in unprocessed δ_1- and δ_2-crystallin mRNA. However, because introns L and M are of different length in the two genes, these primers yield PCR products of different sizes for δ_1 and δ_2 (Nickerson, et al., 1985, 1986). Unprocessed δ-crystallin mRNA was readily detected in fractions A-C only in samples containing density-labeled RNA. Very small amounts of the 486 bp δ_1-crystallin product were detected in Fraction D in both density-labeled and "light" RNA. The origin of the lightly-stained band migrating just ahead of the 486 bp δ_1-crystallin product is not known. It did not appear to hybridize to an oligonucleotide containing a sequence from exon 14, (see Fig. 20).

It was interesting that more unprocessed δ-crystallin mRNA was amplified from fractions A and B than from C or D. This was the opposite of what was seen when mature mRNA was amplified (Fig. 18). These results may be caused by several factors. Unprocessed mRNA is expected to have a relatively short half-life. Therefore, the unprocessed mRNA detected after 4 hr of labeling was probably synthesized during the latter part of the labeling period. Since it also takes time for the nucleoside triphosphate pools to become saturated with density-labeled precursors, RNA synthesized at the end of the labeling period should contain the largest percentage of density label. This predicts that RNA that is rapidly turned over would distribute to the densest regions of the gradient. Thus, mature mRNA would first accumulate in fractions of intermediate density, while unprocessed mRNA would be most

Fig. 20. **Autoradiogram of a Southern blot hybridization of PCR products from Fig. 19.** The ^{32}P end-labelled oligodeoxynucleotide probe was primer #385 (see Fig. 17). It binds to a sequence present in exon 14 of the δ_1- and δ_2-crystallin genes. The primer was ^{32}P end-labeled using T4 polynucleotide kinase.


Density-
Labeled
Control
Fraction →
A B C D A B C D
Unprocessed δ —

abundant in the densest fractions.

If this interpretation is correct, it predicts that over labeling periods longer than 4 hr, a larger amount of mature mRNA should accumulate near the bottom of the gradient and the separation between light and density-labeled RNA should increase. This is consistent with the results of Bowman and Emerson (1980) and Winkles and Grainger (1985), who achieved greater resolution between light and density-labeled RNA in cells labeled for longer periods. Because, over short labeling periods, stable and rapidly-degraded mRNA are expected to distribute differently in the gradient, the methods used in my studies might be useful for revealing precursor-product relationships in RNAs.

Fig. 20 shows an autoradiogram of a Southern blot hybridization of the PCR products from Fig. 19. The oligodeoxynucleotide probe was a ^{32}P end-labeled primer which binds to identical sequences in δ_1- & δ_2-crystallin mRNA. The autoradiogram indicates that the abundant PCR product of 486 bp from δ_1-crystallin hybridized to the probe, as anticipated. My data showed that DNA did not contaminate the RNA samples that were amplified in these studies. DNA should not be found in the density-labeled RNA samples at the bottom of the gradient, because DNA is of lower density than RNA. However, because the gradients were fractionated by passing a micropipette through the gradient from the top, I felt it was important to rule out contamination by DNA. There is one copy of each of the δ-crystallin genes per chromosomal haplotype

(Hawkins, et al., 1984). If DNA had been present in my samples, both δ-crystallin sequences would have been detected. This is illustrated by the δ-crystallin markers seen in Fig. 19, lane G, which were prepared by PCR amplification of chicken genomic DNA using this same primer set used for amplifying unprocessed δ-crystallin mRNA (#999 and #139). However, only sequences comigrating with the δ_1-crystallin band were detected in fractions A and B in these studies. In further tests, RNA samples pretreated with RNase-free DNase showed similar results to those presented in Figs. 19 and 20. Therefore, the methods outlined in this study permit the analysis of unprocessed mRNA or mRNA from intronless genes, without the need for pretreatment with DNase.

The absence of detectable, unprocessed δ_2-crystallin transcripts in fractions A and B suggests that either the δ_1-crystallin gene is transcribed much more efficiently than the δ_2-crystallin gene, that δ_2-crystallin transcripts are quickly degraded after transcription, or that δ_2-crystallin mRNA is processed to mature mRNA much more rapidly than δ_1-crystallin mRNA. I have compared the levels of mature, newly-synthesized δ_1- and δ_2-crystallin transcripts in fractions A-D by PCR amplification with gene-specific primers. δ_1-Crystallin sequences were much more abundant than δ_2-crystallin sequences, suggesting that rapid processing was not sufficient to account for the low levels of δ_2-crystallin sequences in unspliced mRNA (see below). Regulation of the synthesis of the two δ-crystallin genes will be addressed in greater detail

in chapter VI.

CHAPTER V

Validation of Specificity and Quantitative Accuracy of PCR

Introduction

PCR has proved useful in amplifying specific mRNAs, especially those in low abundance (Frohman et al. 1988). Recently, quantitation of mRNA by PCR has been used widely, but the accuracy of the quantitation and specificity of PCR have been questioned by many investigators. The techniques I used for quantitating the ratio of δ_1- to δ_2-crystallin mRNA faced the same problems.

In order to assure the specificity of δ_1- and δ_2-crystallin gene specific primers, #513, and #492 (Fig. 17), serially-diluted, cloned δ_1-crystallin cDNA was amplified with both primers (Zintz and Beebe, unpublished). The results of this test are described below.

Many factors can influence the quantitative accuracy of PCR, such as different efficiency of the specific primers, too low or widely different amounts of starting mRNA, different optimal Mg^{+2} concentrations for different primers etc. To determine the amplification efficiency of the δ_1 and δ_2 gene specific primers, genomic DNA was amplified with each primer. Because there is one copy of each δ-crystallin gene, equal amounts of the two PCR products should result. The optimal Mg^{+2} concentration for each primer was also titrated.

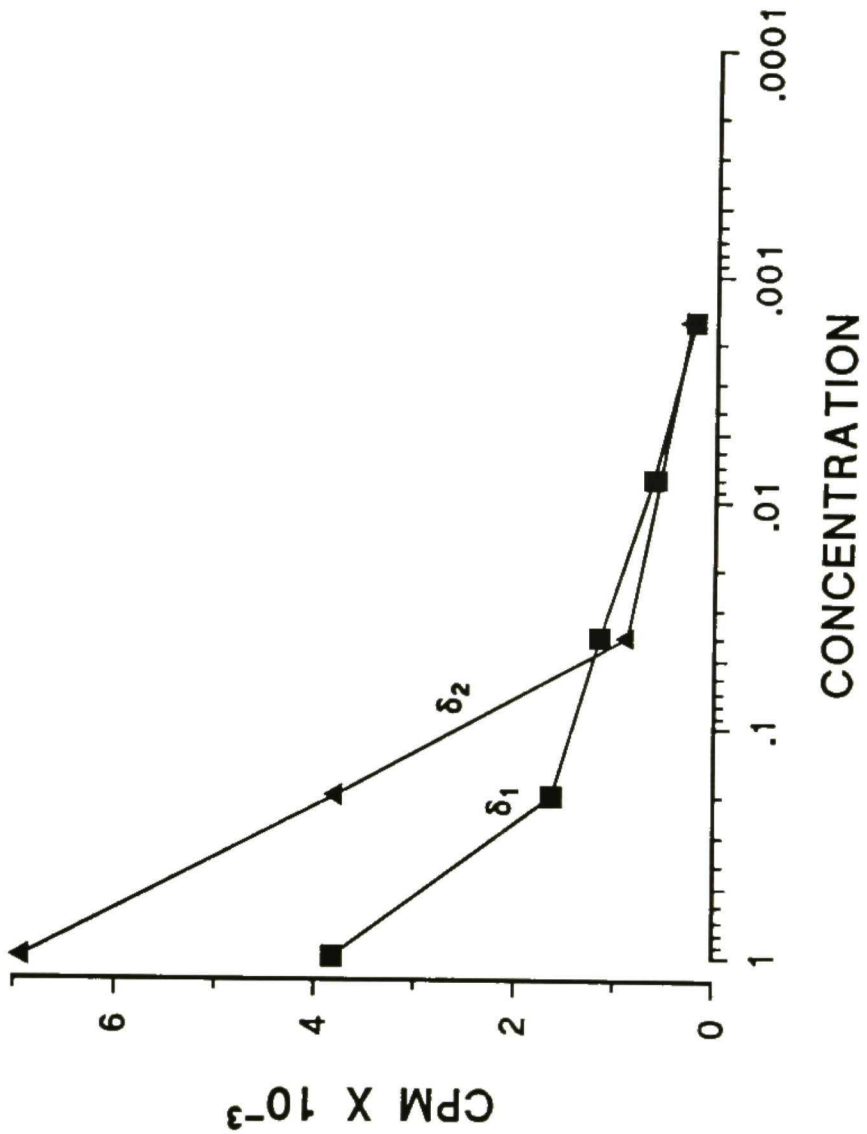
Results

Primer #513 is a δ_1-specific, 3-prime 35-mer, identical to sequences in exon 13 of δ_1 mRNA and primer #492 is a δ_2-specific 3-prime 20-mer, identical to sequences in exon 13 of the δ_2 gene. The length of two PCR products produced with these primers using mRNA or cDNA as substrate is different (see Fig. 17) and these products can be separated by gel electrophoresis. The δ_2-specific primer differed from the δ_1-specific primer at only three positions. However, the amplification of cloned δ_1 cDNA showed that the δ_1 primer was more than 1,000X as effective as the δ_2 primer (Zintz and Beebe, unpublished). In addition, each primer amplified only its cognate gene from chromosomal DNA (data not shown). This could be determined because the region between the two primers is of different length in the two genes in the chromosome, due to differences in the size of their introns (Li, Zintz and Beebe, in preparation; see Fig. 17). These results show that the specificity of these two δ-crystallin primers is reliable.

To measure the relative efficiency of PCR using the δ_1 and δ_2 specific primers, genomic DNA was amplified with both primers sets. Figure 25 shows that under the same conditions, amplification of genomic DNA with the δ_2-specific primer, #492, was about 5 times more efficient than with the δ_1-specific primer, #513. This suggested that primer #492 is more efficient in the PCR reaction than the δ_1-specific primer, #513.

The other primers used in my studies were tested in a

Fig. 25. **Amplification efficiency of δ_1- and δ_2-crystallin gene- specific primers.** PCR was performed for 28 cycles. The template was genomic DNA from 15-day-old chicken embryo blood. The original concentration of the genomic DNA was 0.3 µg before serial dilution. The δ_2 primers were about 5 times more effective than the δ_1 primers.


CPM X 10⁻³
0
2
4
6
1
.1
.01
.001
.0001
CONCENTRATION
δ2
δ1

similar manner. These were primers #137 and #139, which bind to sequences that are identical in both processed and unprocessed δ-crystallin mRNA and primers #999 and #139, which bind to sequences that are identical in unprocessed δ_1- and δ_2-crystallin mRNA. The results showed that all the primers which bind to sequences that are identical in both δ-crystallin mRNAs had similar amplification efficiency when genomic DNA was used as a template (the ratio of δ_1 to δ_2 was about 1, data not shown). Again, the δ_1- and δ_2-specific products could be separated because the intron sequences lying between the primer-binding regions were of different length in the two genes.

CHAPTER VI

Regulation of the Differential Expression of the Two δ-crystallin Genes During Lens Development

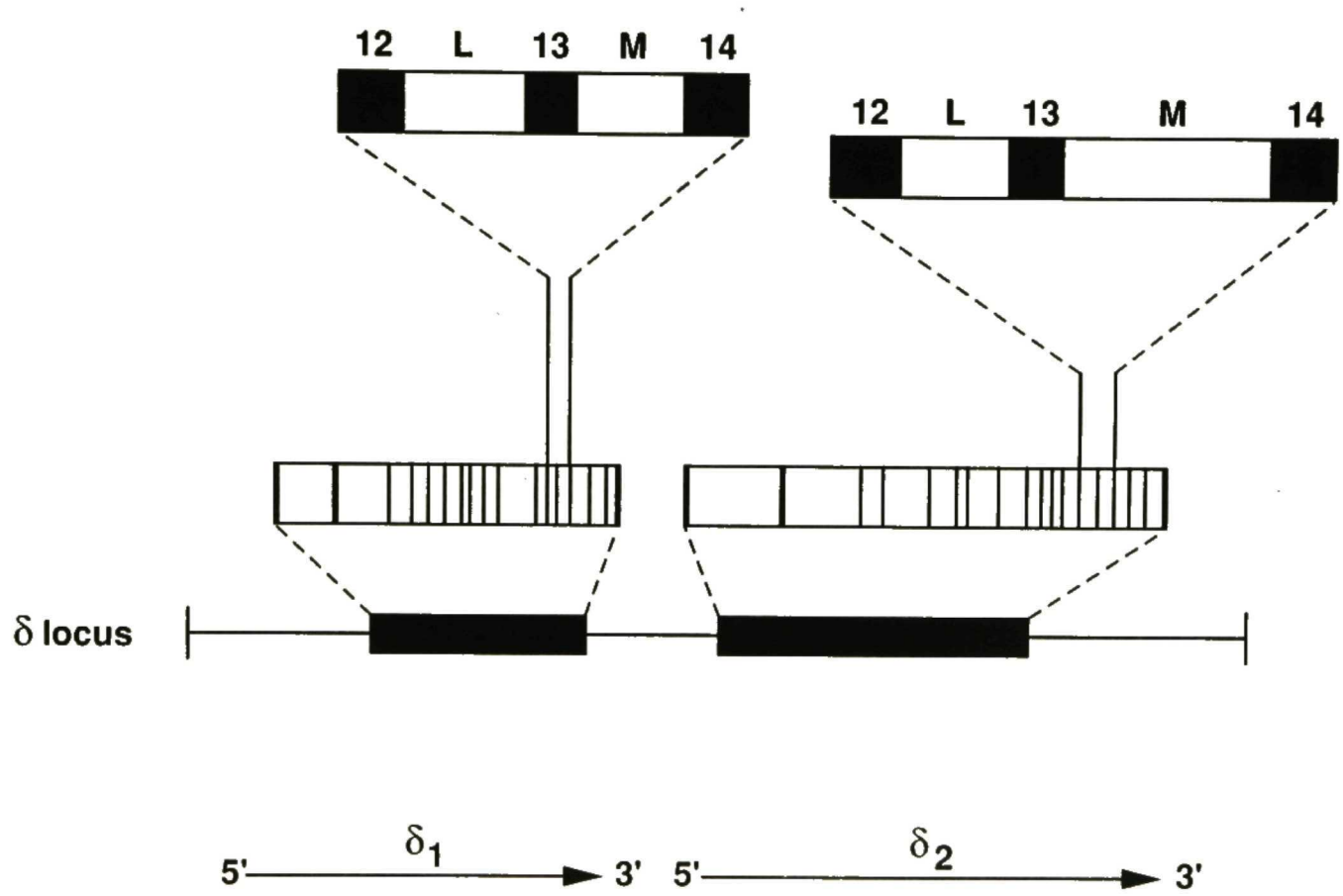
Introduction

Recent studies have shown that, in the later stages of embryonic life in chickens, the amount of the δ_1 and δ_2 mRNAs are very different in lens epithelial and fiber cells (Thomas, et al. 1990). In the epithelium at some stages the two mRNAs are present in nearly equal amounts. In the fibers there may be 100 times more δ_1 mRNA than δ_2 mRNA (Thomas, et al. 1990). There was little evidence to indicate whether the differential expression of the δ-crystallin genes was regulated by differential transcription, processing, or mRNA stability. I was interested in determining the mechanisms that regulated the differential expression of the two δ-crystallin genes during lens development.

The region of the δ-crystallin genes that I chose for this study includes exons 12, 13 and 14, including introns L and M, in both δ-crystallin genes (see Fig. 21). The sequences of exons 12 and 14 are nearly identical in both genes. The major difference between the two genes is in exon 13. Primers specific for a region in exon 13 of each gene were selected and shown to be gene specific for PCR amplification (Chapter V). Because the length of intron L and M in two δ-crystallin

Fig. 21. **The organization of the chicken δ-crystallin locus.** The δ_1 and δ_2 genes are represented by the two rectangles. The dark areas represent exons, while the light areas are introns.

Structure of the Two Delta-Crystallin Genes



genes is different, PCR products of unprocessed mRNAs of both genes can be distinguished by electrophoresis.

For these studies I first used the method I developed, in which density labeling and equilibrium density gradient centrifugation are used to separate newly-synthesized transcripts from pre-existing RNA. After separation, the polymerase chain reaction (PCR) was used to quantitate the levels of δ_1- and δ_2-crystallin mRNAs in the newly-synthesized and "old" mRNA.

For subsequent studies, exon- and intron-specific primers for δ_1- & δ_2- were used to determine the δ_1/δ_2 ratio in processed and unprocessed RNA from lens epithelial, annular pad and fiber cells of 14-day-old embryos. The δ_1/δ_2 ratio in mature (processed) mRNA from lens central epithelial, and fiber cells of 6-, 10-, and 14-day-old embryos also was tested by the same methods.

Results

Differential δ_1- and δ_2-crystallin gene mRNA levels during lens development

The ratio of δ_1- and δ_2-crystallin mRNAs was determined by PCR, as described above, using δ_1- or δ_2-crystallin gene-specific primers. Total RNA was isolated from different lens regions (epithelium, annular pad and fiber) of 6-, 10-, and 14-day-old embryonic chicken lenses. The 5′ primers, #513, CTCAT GGTTC TCAAA GGAAT TCCAA GCACC TTCAG, a sense-strand 35-mer specific for a sequence in exon 13 of the δ_1-crystallin

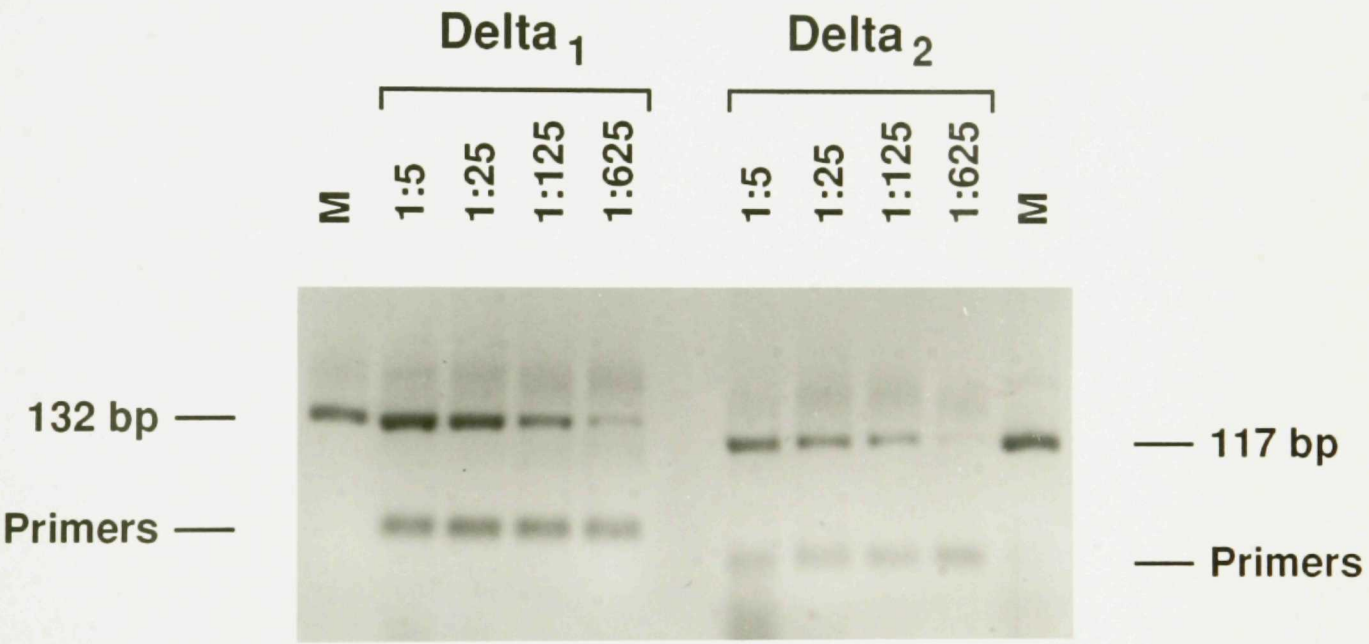
gene, and #492, GGACT TCCAA GCACC TACAA, a 20-mer specific for exon 13 of the δ_2-crystallin gene, were coupled with the 3′ primer, #139, CTGGA GGGTA GAAAT CACTC, a common antisense 20 mer oligodeoxynucleotide complementary to identical sequences in exon 14 of both δ-crystallin genes (Fig. 17). Serially diluted cDNAs, which were reverse transcribed from the total RNA of different lens regions at different embryonic ages, were used to represent the relative concentration of the total RNA in the PCR reaction. PCR products were analyzed by agarose electrophoresis, stained with ethidium bromide and photographed (see Fig. 22A). The bands containing amplified DNA were cut out and counted in a scintillation counter. The results were ploted on the semi-logarithmic plot shown in Fig. 22B. For PCR quantitation, comparisons were made only between samples within the same experiment.

The results are shown as Table 1. The δ_1/δ_2 mRNA ratios were similar in the central epithelial cells at all ages and increased from 19 to 100 in the whole fiber cells between 6 and 14 days of development. These results were generally consistent with the reported results of Thomas et al. (1990) and my Northern blot results (Fig. 23).

Differential accumulation of unprocessed and processed δ_1- and δ_2-crystallin mRNAs during lens differentiation

Total RNA was isolated from central epithelial cells, annular pad cells, cortical fiber cells and central fiber cells of 14-day-old embryonic chicken lenses. These different

Fig. 22. **Quantitation of PCR products by serial dilution.** (A) Agarose gel electrophoresis of the PCR products. PCR was performed as described as Fig 18. The template for PCR was derived from serially-diluted cDNA derived from total RNA of the cells of the annular pad of 14-day-old chicken embryonic lenses. The δ_1- or δ_2-specific 5′ oligodeoxynucleotide primers, #513 or #492, and a common 3′ primer, #139, were used for these studies (see Fig. 17). (B) Semi-logarithmic plot of the counts of the DNA bands of the PCR that labeled with [^{32}P]dCTP shown in B. The horizontal line and vertical arrows illustrate the method that was used for quantitation, i. e., the difference in the amount of diluted cDNA required to generate the same number of counts of PCR product was taken as the difference in the relative concentration of δ_1 or δ_2 cDNA present in the initial cDNA sample.


Delta 1
Delta 2
M
1:5
1:25
1:125
1:625
1:5
1:25
1:125
1:625
M
132 bp
Primers
117 bp
Primers

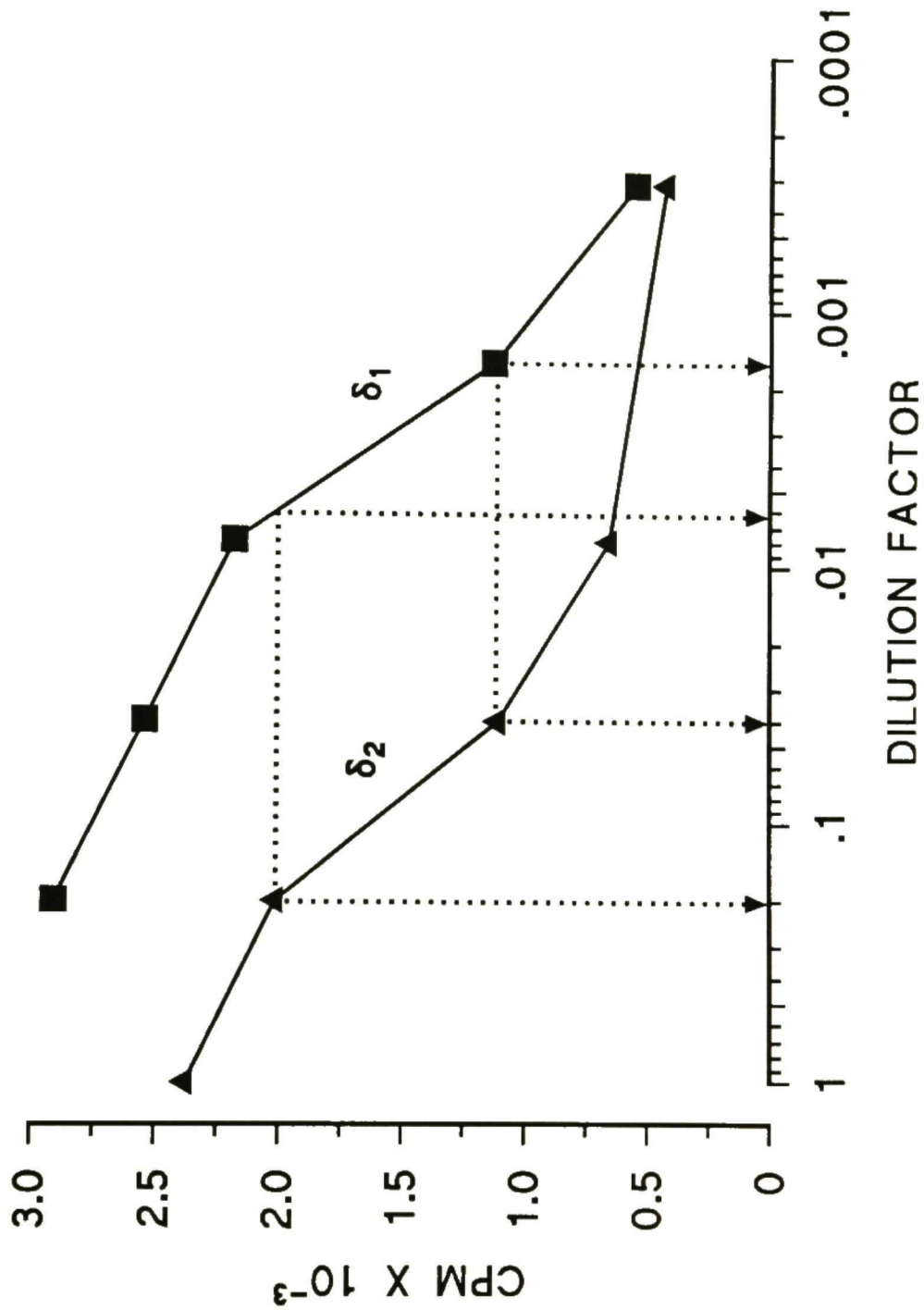

CPM X 10⁻³
3.0
2.5
2.0
1.5
1.0
0.5
0
1
.1
.01
.001
.0001
DILUTION FACTOR
δ1
δ2

Fig. 23. **Autoradiogram of a Northern blot hybridization of δ_1- & δ_2-crystallin mRNAs during lens development.** Total RNA was isolated from the central epithelia (EP), equatorial epithelia (annular pad, AP), whole fiber masses (WF), central fiber masses (CF), and peripheral fiber masses (PF) of 6-day-old (250), and 14-day-old (150) embryonic lenses. Each RNA sample was subjected to electrophoresis on a formaldehyde gel, transferred onto a Nytran filter, and hybridized with [^{32}P]ATP-end-labelled oligonucleotide probes [A 20 mer from exon 2 in the δ_1 & δ_2-crystallin genes (Thomas, et al. 1991)]. Filters were exposed for 3 days.

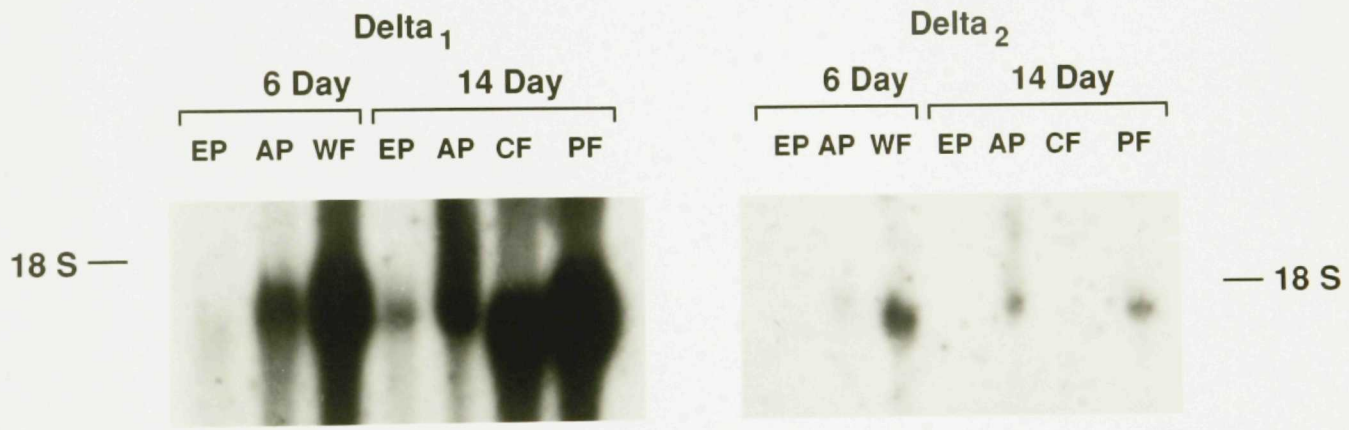

Delta 1
6 Day
14 Day
EP AP WF EP AP CF PF
18 S —
Delta 2
6 Day
14 Day
EP AP WF EP AP CF PF
— 18 S

Table 1. **Ratio of processed δ_1- and δ_2-crystallin mRNA in the lens.** Total RNA was extracted from five freshly-dissected lens central epithelial cells or whole lens fiber masses of 6-, 10-, and 14-day-old chicken embryos. The δ_1/δ_2 ratio was estimated by quantitation of the PCR products as described in Fig. 22.

RATIO OF PROCESSED δ_1 AND δ_2–CRYSTALLIN mRNA IN THE LENS

	RATIO δ_1/δ_2	
AGE (DAY)	Central Epithelium	Whole Fibers
6	5.7	18.9
10	4.1	18.2
14	4.6	99.5

regions of the lens represented different stages of lens cell differentiation. The ratio of processed δ_1/δ_2 mRNA was obtained by the method as described above. To determine the relative amount of the unprocessed mRNA, I used the 5′ primer, #999, TAAAG AAAGA AGAGG GGCAG G, an intron-specific 21 mer, sense-strand primer identical to sequences in intron L of the δ_1- and δ_2-crystallin genes, coupled with the 3′ primer, #139 (see Fig. 17), which is a common primer for all the reverse transcription and PCR reactions. Because the length of intron L is different in the two genes, the bands containing amplified DNA could be identified by agarose electrophoresis (see Fig. 22A). The δ_1/δ_2 ratios in mRNA from these regions are shown in Table 2.

The δ_1/δ_2 ratio in unprocessed messages was similar to the ratio in mature mRNA in the different lens regions. The ratio of the two crystallin mRNAs increased from the central epithelium toward the cortical fibers in both mature mRNA and unprocessed mRNA, from about 10 to about 300. The ratio of these two crystallin mRNA in the central fibers was about 100 in the mature mRNA, but unprocessed message could not detected in this region. These data suggest that no mRNA is synthesized in the central region of the 14-day-old chicken embryo lens. In addition, the ratio of the two processed δ-crystallin mRNAs in central part of the lens fibers was lower than in the cortex (about 100 in the central fibers, but about 300 in the cortical fibers). It is known that the central lens fibers are made at an early stage of development. The

Table 2. **Ratios of δ_1- and δ_2-crystallin mRNA sequences measured in unprocessed and mature mRNA.** Unprocessed mRNA was amplified by primers #999 and #139 and the amount of δ_1- or δ_2-specific products was determined after separating the 532 bp δ_2 band from the 486 bp δ_1 band by agarose gel electrophoresis. Mature mRNA was quantitated by separately amplifying the δ-crystallin mRNA sequences using the 5′ primers, #513 or #492, for δ_1 and δ_2, respectively. Estimation of the δ_1/δ_2 ratio was performed after diluting the cDNA as described in Fig. 22.

RATIO OF PROCESSED AND UNPROCESSED δ_1– AND δ_2–CRYSTALLIN mRNA IN THE LENS

	RATIO δ_1/δ_2	
LENS REGION	MATURE mRNA	UNPROCESSED
Central Epithelium	9	8
Annular Pad	28	29
Cortical Fibers	296	333
Central Fibers	94	ND

ratio of δ_1/δ_2 was lower in the fibers of younger embryos (Thomas et al., 1990 and Table 2). Thus, the δ_1/δ_2 ratio in the lens center reflects the ratio of these messages at the time when these fiber cells were produced.

The data in Table 2 provided evidence that the differential expression of the two δ-crystallin genes is regulated at the level of transcription. In each lens region, the δ_1/δ_2 ratio was similar in unprocessed, presumably newly-synthesized, mRNA as in the processed mRNA that had accumulated in the cells. Differential mRNA degradation or differential processing would be unlikely to account for these results.

Differential accumulation of newly synthesized δ_1- and δ_2-crystallin mRNA in lens fiber cells

The accumulation of the newly synthesized δ_1- and δ_2-crystallin mRNAs was determined from the density labeling experiments using gene-specific PCR. This method was described in Chapter V. Total RNA was isolated from 14-day-old embryonic chicken lens fiber cells which had been labeled for 4 hr with dense (^{13}C and ^{15}N) ribonucleosides. After equilibrium density centrifugation, the density-labeled, newly synthesized RNA was separated from the light, pre-existing, "old" RNA (Fig. 15). After recovery of the density-labeled RNA, δ-crystallin mRNA was reverse transcribed, serially diluted and quantitated by gene-specific PCR. The results are shown in Table 3. The ratios of mature, newly-synthesized δ_1-

Table 3. **The δ_1/δ_2 ratio in newly-synthesized lens fiber RNA.** Density-labelled RNA from fractions A-C of Fig. 3 was reverse transcribed, the cDNA was serially diluted and δ_1- and δ_2-specific sequences were amplified using the 5′ primers, #513 or #492 (see Fig. 17).

RATIO OF δ_1- AND δ_2-CRYSTALLIN SEQUENCES IN DENSITY-LABELED LENS RNA

GRADIENT FRACTION	δ_1/δ_2 RATIO
A	13
B	38
C	59
D	40

and δ_2-crystallin mRNA was not significantly different in the dense portions of the gradient (fractions B and C) from the ratio in the lighter portion (fraction D). These data demonstrated that newly-synthesized δ-crystallin mRNA had a similar ratio to "old" mRNA. This further suggested that the differential expression of two δ-crystallin genes is controlled at the level of transcription.

Discussion

Previous studies have demonstrated that transcriptional regulation has a major role in the control of crystallin gene expression (Piatigorsky, 1989), but there is little evidence for transcriptional regulation of the δ_1- and δ_2-crystallin genes (Thomas, et al., 1990). My studies strongly suggested that the differential expression of two δ-crystallin genes was regulated at the level of transcription. This conclusion was made from following evidence:

1. The density labeling experiments showed that newly-synthesized, unprocessed δ_2-crystallin mRNA was in very low abundance in the most dense region of the density gradient, while unprocessed δ_1-crystallin mRNA was readily detectable. Southern blot analysis confirmed these observations. Thus, either δ_2-crystallin mRNA is processed much more rapidly than δ_1, δ_2 mRNA is selectively degraded during synthesis, or the δ_1 gene is transcribed more frequently than the δ_2 gene. This finding was consistent with the results from previous studies (Thomas, et al., 1990), which found that about 100 times more

δ_1 than δ_2 mRNA accumulated in whole fiber masses of 14-day-old chicken embryos.

2. In the density labeling experiment, more processed δ_1 than δ_2 mRNA was detected in the dense region of the gradient. This again suggests that the difference in the accumulation of these mRNAs is not due to differences in their stability. However, the δ_1/δ_2 ratio was almost ten times less than was found in total mRNA (12 - 50, rather than 100 - 300). These results suggest that either some of the excess δ_1 mRNA that accumulates in lens fiber cells is due to differential mRNA processing, or that my estimates of the δ_1/δ_2 ratio were not accurate. There is some evidence for the latter interpretation.

When the amplification efficiency of the two gene-specific PCR primers was compared, the δ_2 primer was about five times more effective than the δ_1 primer. Only small amounts of mRNA are available for amplification in the dense region of the gradient, because the mature mRNA in this region must have been synthesized and processed during the labeling period. Given that the RNA precursor pool must also have accumulated sufficient density-labeled nucleoside to shift the newly-synthesized mRNA to a denser region of the gradient, the RNA in this region may represent only a small fraction of the total RNA synthesized during the labeling period. To detect this small amount of δ-crystallin mRNA, more extensive amplification is required. Because the primers are not equally efficient, the more amplification needed, the more the

δ_2 sequences will be overrepresented in the product. This may partly account for the discrepancy between the δ-crystallin mRNA ratios in newly-synthesized, density-labeled mRNA and total δ-crystallin mRNA accumulated in fiber cells.

3. Additional evidence for transcriptional control of the δ-crystallin genes came from comparison of the δ_1/δ_2 ratio in unprocessed and processed mRNA from different regions of the lens. In all regions in which unprocessed δ-crystallin mRNA could be measured, the ratios were similar. While it may be possible that these data could be explained by immediate degradation of a portion of the δ_2-crystallin mRNA immediately after its synthesis, the simplest explanation for these data is that the differential expression of two δ-crystallin genes was due to different rates of transcription.

4. Northern blot analysis of δ_1- and δ_2-crystallin mRNA distribution in the different regions of the 6- and 14-day-old embryonic lens was consistent with the results that I obtained using PCR. This observation, along with the tests of PCR specificity and quantitative accuracy performed in my studies, suggest that the methods used to compare the abundance of the δ-crystallin transcripts gave reasonable estimates of δ-crystallin gene expression.

CHAPTER VII

Expression of the Two Delta-Crystallin Genes in Cultured Lens Cells

Introduction

Lens epithelial explants or primary cultures of lens cells have been used to study important aspects of lens function and development. In many cases, these cultured cells closely mimic normal lens cell function and differentiation (Piatigorsky, 1981). Aspects of lens cell behavior that have been studied using lens cells in vitro include the influence of the ocular environment on lens cell differentiation (Philpott and Coulombre, 1968), the identification of mitogens for lens epithelial cells (Reddan, and Dziedzic, 1982; Reddan, Dziedzic and McGee, 1982; Reddan and Wilson-Dziedzic, 1983; McAvoy and Chamberlain, 1989), the discovery of factors that stimulate lens fiber cell differentiation (Philpott and Coulombre, 1965; Piatigorsky, 1973; Beebe, Jebens and Feagans, 1980; McAvoy, 1980; McAvoy, Campbell and Walton, 1985), the role of the cytoskeleton in lens fiber cell elongation (Piatigorsky, 1975; Beebe, et al., 1979) and the activation of lens fiber-specific gene expression (McAvoy, 1980; Lok, et al., 1985).

Recently, epithelial explants and primary cell cultures have been used to study the factors that control the

expression of the crystallin genes. These studies are most often performed by transfecting the cultured lens cells with plasmids containing crystallin gene regulatory sequences coupled to reporter genes, like chloramphenicol acetyl transferase (CAT) or β-galactosidase (Borras et al., 1985; Chepelinsky et al., 1985; Lok et al., 1985; Borras et al., 1988; Peek, et al., 1990; for review see Piatigorsky and Zelenka, 1991). In short-term cultures, the lens cells used in these studies elongate considerably, continue to express lens crystallins and crystallin mRNAs at high levels and may produce lentoid bodies, which are small aggregates of fiber-like cells (Piatigorsky, 1981; Borras et al., 1988; Piatigorsky and Zelenka, 1991).

One recent use of cultured lens cells has been to study the differential expression of the δ_1-and δ_2-crystallin genes. Transfection of δ_1 and δ_2 promoter/CAT constructs into cultured cells revealed little specificity for expression in the lens (Borras, et al., 1985). In addition, the two promoters appeared to have similar activity in lens cells, suggesting that promoter strength was not responsible for the differential expression of these genes (Borras, et al., 1985; Hayashi, et al., 1987). A strong, lens-specific enhancer was later found in the third intron of the δ_1 (Hayashi, et al., 1987) and δ_2 genes (Thomas, et al., 1990). Transfection studies in which the promoter and enhancer sequences of the two δ-crystallin genes were interchanged, showed that these regulatory sequences were functionally equivalent (Thomas et

al., 1990). From these data, it was proposed that the differential expression of two chicken δ-crystallin genes may be due to post-transcriptional mechanisms, rather than different rates of transcription (Thomas, et al., 1990; Piatigorsky and Zelenka, 1991).

As I reported above, my data suggested that differential transcription may be important in regulating the relative expression of the δ-crystallin genes in chicken embryo lenses. To investigate the differences between the conclusions derived from my studies and those performed in other laboratories, I examined δ-crystallin mRNA levels in lens cells prepared and cultured as described previously for in vitro transfection studies (Borras, et al., 1985; Chepelinsky, et al., 1985; Thomas, et al., 1990). Surprisingly, I found that, unlike lens fiber cells, these cultured lens cells accumulated similar amounts of δ_1- and δ_2-crystallin mRNA. This was true whether cultured lens epithelial explants or dissociated lens cells were assayed.

Results

I used gene-specific primers and the polymerase chain reaction to determine the ratio of δ_1- and δ_2-crystallin mRNA in the explants and cell cultures. The relative amount of each δ-crystallin mRNA accumulated in the lens cells cultured under four different in vitro conditions were compared with freshly-dissected lens fiber and epithelial cells. The δ_1/δ_2 ratio in cultured lens epithelial explants or primary cultures

of dissociated lens cells (PLE) was decreased from that found in freshly-dissected lens epithelia (Fig. 24 and Table 4). The ratio of δ_1 to δ_2 mRNA in explants cultured in Coon's modified medium and PLE cells even appeared to inverted, compared to freshly-dissected central epithelial cells (Fig. 24E and F; Table 4).

These results show that lens cells cultured under the conditions used for previous _in vitro_ transfection studies did not behave like lens fiber cells with respect to the relative accumulation of δ-crystallin transcripts. Even stimulation of lens fiber-like differentiation with vitreous humor, the stimulus most like that seen by lens cells _in vivo_, did not increase the δ_1/δ_2 ratio. This was in spite of the fact that vitreous humor is known to increase δ-crystallin synthesis and δ-crystallin mRNA accumulation in 6-day-old lens explants under the similar culture conditions (Beebe, _et al._, 1980; Hejtmancik, _et al._, 1985). Similarly, vitreous humor and Coon's medium caused explanted lens central epithelial cells to elongate. Dissociated, cultured, lens cells (PLE) also formed lentoid bodies (data not shown). Both behaviors have previously been taken as evidence for authentic lens fiber cell differentiation. Thus, although all or many of the cells in these cultures resembled lens fiber cells, as a whole, the cultured cells did not accumulate δ-crystallin mRNA in a manner that resembled lens fiber cells that had differentiated _in vivo_.

Discussion

Fig. 24. **Relative content of δ_1- and δ_2-crystallin mRNA in cultured lens central epithelia and dissociated lens cells.** Gene-specific PCR primers were used to amplify the cDNA for each of the two δ-crystallin mRNAs (see Fig. 18). For the experiments shown RNA was extracted from: (A) Four freshly-dissected central lens epithelia from 14-day-old embryos, (B) Two freshly-dissected peripheral lens fibers from 14-day-old chicken embryos, (C) Four lens central epithelial explants from 14-day-old embryos cultured for three days in Ham's F-10 medium, (D) Four lens central epithelial explants from 14-day-old embryos cultured for three days in Ham's F-10 medium supplemented with 50% vitreous humor, (E) Four lens central epithelial explants from 14-day-old embryos cultured for three days in Coon's Modified F-12 medium and (F) Four partially dissociated lenses (PLE; primary patch lens cells) from 14-day-old embryos cultured for three days in D-MEM medium supplemented 10% FCS.

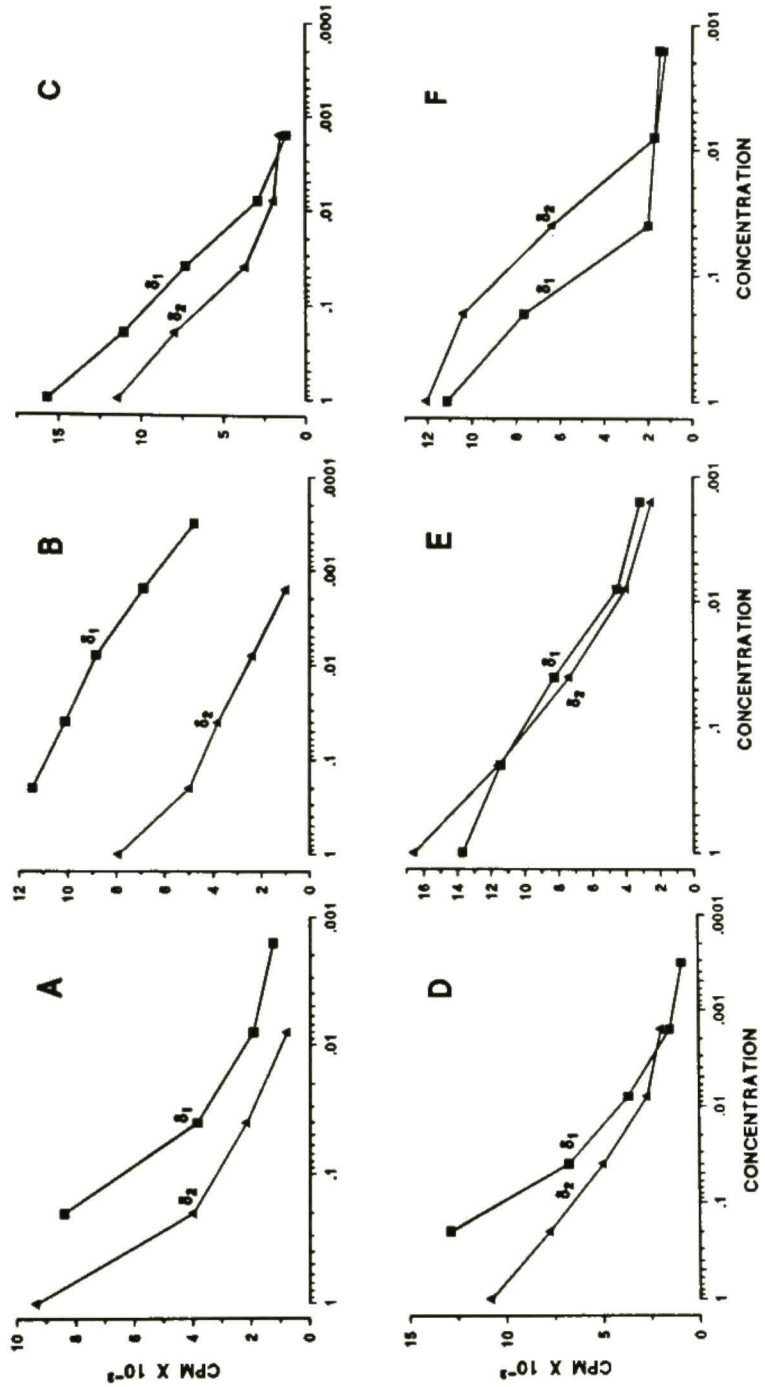

A
B
C
D
E
F
CPM X 10⁻³
CONCENTRATION
CONCENTRATION
δ1
δ2

Table 4. **Ratio of δ_1- and δ_2-crystallin mRNA in cultured lens cells.** The δ_1/δ_2 ratio was estimated by quantitation of the PCR products as described in Fig. 6. δ_1- and δ_2-specific sequences were amplified using the 5′ primers, #513 or #492 (see Fig. 17).

RATIO OF δ_1- AND δ_2-CRYSTALLIN mRNA IN CULTURED LENS CELLS[1]

Age (Day)	Culture Style	Culture Medium	Ratio δ_1/δ_2
11	[2]PLE	D-MEM (10% FCS)	0.4
14	PLE		0.3
14	Explants	Ham's F-10	4
14	Explants	50% Vitreous	3
14	Explants	20% FCS	6
14	Explants	Coon's modified F-12	1

(1) The δ_1/δ_2 ratio in freshly-dissected 14-day central lens epithelial cells and peripheral fiber cells was approximately 5 and 300, respectively.

(2) Primary Patched Lens Cells.

Lens epithelial cells have been used by many investigators to study gene regulation during lens fiber cell differentiation. In most cases this approach has been useful for demonstrating lens-specific regulatory elements and for identifying the specific sequences required for the efficient expression of the crystallin genes.

In the case of the δ-crystallin genes, this approach has provided evidence for positive and negative regulatory elements in the δ_1-crystallin promoter (Borras, Peterson and Piatigorsky, 1988) and has demonstrated the presence of a tissue-specific enhancer elements in the third intron of both δ-crystallin genes (Hayashi et al., 1987, Thomas, et al., 1990).

This approach has also been used to study the differential expression of the two δ-crystallin genes. Borras et al. (1985) found little difference in the two promoter regions when chimeric reporter genes were used as a test system. Similarly, Thomas et al. (1990) showed that any combination of δ-crystallin promoter and enhancer sequences was expressed approximately equally after transfection. From these studies it was concluded that either the sequences that had been tested were not the ones responsible for differential expression of these genes or that their expression was regulated after transcription by differential processing or mRNA stability (Thomas, et al., 1990; Piatigorsky and Zelenka, 1991).

My studies provide a different view of δ-crystallin

gene regulation. First, there is now good evidence for transcriptional control of these genes *in vivo*. Second, the cultured cells used for transfection studies do not express the δ-crystallin genes in a manner similar to that seen *in vivo*. Therefore, data from these transfection experiments would not be relevant to understanding δ-crystallin expression in lens fiber cells.

My data show that δ-crystallin expression in lens epithelial cells does not resemble the regulation of these genes in lens fibers, even when the cells are stimulated to become like fiber cells by several other criteria. This suggests that the program of δ-crystallin gene expression seen in epithelial cells may be quite stable. At this time it is not clear whether this is because the epithelial cells have lost the ability to form "authentic" fiber cells during development or whether the *in vitro* culture conditions used in my studies did not permit the cells to fully express this phenotype.

Examination of the pattern of DNA methylation within and around the δ-crystallin genes or the use of DNA protection assays might reveal the nature of control transcriptional mechanisms which regulate the tissue-specific expression of the δ-crystallin genes. These are important areas for future study.

It is also my hope that the methods developed in my studies, especially the use of density labeled nucleosides and the quantitation of unprocessed mRNA using intron-specific PCR

primers, will be valuable to future studies of lens gene expression and to molecular biologists interested in the mechanisms that regulate other genes.

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