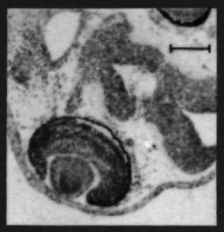
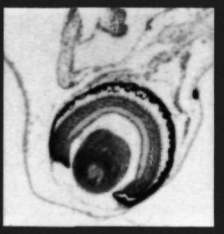
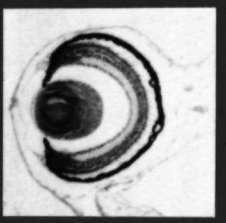
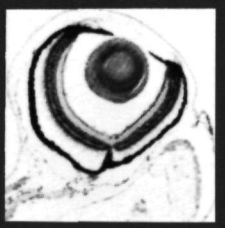
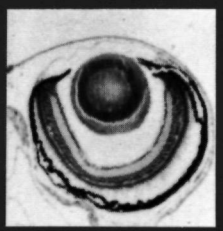






STRUCTURE OF LENS PROTEINS:
A MOLECULAR GENETIC APPROACH





Ruud H. Brakenhoff

STRUCTURE OF LENS PROTEINS:

A MOLECULAR GENETIC APPROACH

Een wetenschappelijke proeve op het gebied van de
natuurwetenschappen,
in het bijzonder de biologie

PROEFSCHRIFT
ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
vrijdag, 10 januari, 1992
des namiddags te 1.30 uur precies

door

RUDOLF HENRIKUS BRAKENHOFF
geboren op 24 januari 1959
te Arnhem

Promotor: Prof. Dr. J. G. G. Schoenmakers

Co-promotor: Dr. N. H. Lubsen

Ter nagedachtenis aan mijn vader,

Theodorus Paulus Brakenhoff, 1927-1976.

DANKWOORD

Mijn dank gaat uit naar iedereen, zeker niet allen hier genoemd, die heeft bijgedragen aan de totstandkoming van dit proefschrift. Ik wil zowel Eldine Jacobs als Robin Ruuls dank zeggen voor de vele genoeglijke uren rondom een emmer *Xenopus*. Een hele prettige tijd heb ik ervaren met mijn collegae promovendi Henk Aarts, Harry Kraft en Ron Peek. I will certainly remember Graham Elliott, who told me everything about the english 'pub' language which is not listed in the Oxford Dictionary.

De laatste groep die ik met name wil noemen zijn de hoofdvak studenten Frans Reek, Hans Henskens en Jack Vossen, en daarnaast de HLO stageaires Caroline van Alebeek, Maarten van Rossum en Robin Ruuls, die allen een daadwerkelijke ondersteuning van het hier beschreven onderzoek hebben geleverd. Ik denk met de beste herinneringen terug aan de vakgroep Moleculaire Biologie.

Behalve de gehele vakgroep Moleculaire Biologie ben ik dank verschuldigd aan Ron Engels en Tony Coenen van de vakgroep Experimentele Dierfysiologie, en tevens Ir. F. Schut, HTS stageaire Ron Smeets en Ruud Gelsing van het Technisch Centrum Nijmegen.

Mijn vrouw Monique ben ik in ieder geval dank verschuldigd voor haar geduld inzake experimenten na zevenen zonder bellen, experimenten in het weekend, voortdurend geratel van toetsenborden en wat dies meer zij.

CONTENTS

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Brakenhoff, R.H.

Structure of lens proteins : a molecular genetic approach
/ R.H. Brakenhoff. - [S.l. : s.n.]. - Ill.
Thesis Nijmegen. - With ref. - With summary in Dutch.
ISBN 90-9004532-5
Subject heading: crystallins.

CHAPTER 1

INTRODUCTION

THE LENS

As a rule, living matter is opaque. The eye lens is an obvious exception, the mature lens fibre cells are transparent. The transmission of light through the lens is only possible because elaborate measures have been taken. First of all, all large cell organelles, such as nuclei and mitochondria, are removed during maturation of the lens cell (Piatigorsky, 1981). This assures that the macromolecules and cell organelles which remain in the lens fibre cell have a diameter that is smaller than the wavelength of the passing light. Otherwise the light will be scattered instead of transmitted (Bettelheim, 1985). Secondly, the lens is packed with proteins, up to 20-60 % of the wet weight of the lens (for review, see Wistow & Piatigorsky, 1988). These proteins are laid down in a concentric concentration gradient, with the highest protein concentration at the centre and the lowest at the periphery of the lens (Sun *et al.*, 1984; Carper *et al.*, 1985; Hejtmancik *et al.*, 1985; Murer-Orlando *et al.*, 1987; Van Leen *et al.*, 1987; Aarts *et al.*, 1989b). As a result optical aberrations are corrected (Bettelheim, 1985; Delaye & Tardieu, 1983). Finally, the protein concentration also determines the elastic properties of the lens. A low protein/high water content yields a soft lens, which can accommodate, while a high protein/low water content is found in hard lenses which cannot change shape (De Jong, 1981; Lindley *et al.*, 1985). The optical demands thus constrain the morphological and biochemical properties of the eye lens.

THE DEVELOPMENT OF THE LENS

The lens develops from the ectoderm. In *Xenopus'* development part of the head ectoderm is induced to form the lens placode by the presumptive anterior neural plate (Henry & Grainger, 1990). A similar inductive process occurs in other vertebrates. The lens placode invaginates and forms a lens vesicle. The posterior cells of the lens vesicle differentiate to the primary fibre cells and form the lens nucleus. A layer of epithelial cells remains on the anterior side of the lens. These cells can differentiate to (secondary) fibre cells at the equatorial region (Piatigorsky, 1981; Maisel *et al.*, 1981). The lens thus grows by the apposition of fibre cells in the equatorial region. The stimulus for epithelial cell differentiation is provided by factors secreted by the retina (McAvoy, 1981). The most important lens morphogen in the rat is fibroblast growth factor (McAvoy & Chamberlain, 1989).

During the course of differentiation, the fibre cells elongate, express a specific set of genes, and finally lose their cell organelles (Piatigorsky, 1981). Due to the removal of the transcription and translation apparatus, accumulation of the lens protein

constituents gradually ceases and a renewal of the cellular components is not further possible. The proteins, accumulated in the fibre cells, are present for life, which means that they should be thermodynamically stable.

THE CRYSTALLINS

As mentioned above, the lens has a very high protein content. The bulk of the lens proteins consists of crystallins, defined as abundant water-soluble lens proteins. The crystallins are divided in two groups: ubiquitous crystallins and taxon-specific crystallins (Wistow & Piatigorsky, 1988). The ubiquitous crystallins are classified in three subdivisions, the α-, β- and γ-crystallins (Piatigorsky & Wistow, 1989). The α-crystallins are large aggregates composed of two different polypeptides: αA and αB chains (Quax-Jeuken *et al.*, 1985a; Van den Heuvel *et al.*, 1985). The β-crystallins form dimer and higher order aggregates, composed of βA(cidic) and/or βB(asic) chains (Berbers *et al.*, 1982; Ramaekers *et al.*, 1982). The β-crystallin division consists of four acidic and three basic polypeptides in all investigated species (Hejtmancik & Piatigorsky, 1983; Inana *et al.*, 1982; Ramaekers *et al.*, 1982; Berbers *et al.*, 1984). The γ-crystallins are monomeric (Wistow & Piatigorsky, 1988). In rat six γ-crystallins (γA-γF) were detected, whereas in man only two polypeptides, and traces of a third, could be found (Siezen *et al.*, 1987; Siezen *et al.*, 1988). The γ-crystallin gene family contains an additional ubiquitous member, γs. This crystallin was long thought to be the only monomeric member of the β-crystallin division (denoted βs), but based upon its protein and gene structure it appears to be more related to the γ-crystallin division and was renamed (Van Rens *et al.*, 1989). For brevity's sake, in this thesis γ-crystallin (family) is used to denote the closely related γA-γF proteins. The γs-crystallin is indicated specifically as γs.

The ubiquitous crystallins are present in the lenses of all vertebrate species examined. In some species, the crystallin make-up is completed by an exceptionally high concentration of an enzyme or a protein still closely related to an enzyme (Table 1). Their role in the eye lens is thought to be of structural, and not enzymatic, importance (Piatigorsky & Wistow, 1989). As these "structural" proteins are present only in specific taxa or species, they are called the taxon-specific crystallins (Piatigorsky & Wistow, 1989). Apparently, throughout evolution the lens has recruited housekeeping proteins to serve as crystallins (Stapel *et al.*, 1985; Carper *et al.*, 1987; Huang *et al.*, 1987; Wistow *et al.*, 1987; Hendriks *et al.*, 1988; Watanabe *et al.*, 1988).

Table 1. Recruitment of proteins as eye lens crystallins.

CR	related protein	amount	occurrence
α	small heat shock protein *Schistosoma* egg antigen *Mycobacterium* antigen	up to 50%	all vertebrates
β	*Myxococcus xanthus* protein S *Physarum polycephalum* 3a	up to 70%	all vertebrates
γ	*Myxococcus xanthus* protein S *Physarum polycephalum* 3a	up to 40%	all vertebrates
δ	δ2=argininosuccinate lyase	up to 70%	birds, reptiles
ε	=lactate dehydrogenase B	up to 23%	many birds, crocodiles
η	aldehyde dehydrogenase	24%	elephant shrew
ζ	alcohol dehydrogenase	10%	guinea pig
λ	hydroxyacyl-CoA-dehydrogenase	8%	rabbits, hare
μ	?	up to 10%	wallaby, kangaroo
ρ	aldose/aldehyde reductase prostaglandin F synthetase	12%	frogs (genus *Rana*)
τ	=α-enolase	up to 10%	lamprey, some fishes, birds, reptiles
SIII	glutathione S-transferase		squid

In the first column, the eye lens crystallin (CR) is denoted, in the second column the protein identical with (indicated by =) or related to that particular crystallin. In the third column the amount of protein found in the lens relative to the total amount of protein, and in the fourth column the occurence of the crystallin in the animal kingdom are listed. References are given in Wistow (1990); Wistow & Piatigorsky (1987); De Jong *et al.* (1989); Wistow *et al.* (1990).

The latest recruits are often encoded by the same gene as the housekeeping protein and retain enzymatic activity (Wistow *et al.*, 1987; Piatigorsky *et al.*, 1988; Wistow *et al.*, 1988). The ubiquitous crystallins also still show remnants of structural similarity with other polypeptides from various sources of the animal kingdom and probably

represent early recruits (Ingolia & Craig, 1982; Wistow *et al.*, 1985; Nene *et al.*, 1986). These proteins are now encoded by lens-specific duplicates of the ancestral housekeeping gene (note that the αB gene is widely expressed outside the lens as well: Bhat & Nagineni, 1989; Dubin *et al.*, 1989; Dubin *et al.*, 1991). The ubiquitous crystallins in different species are very similar and their three-dimensional structure, to the extent known, is well conserved (Blundell *et al.*, 1981; Wistow *et al.*, 1983; Summers *et al.*, 1986; Lubsen *et al.*, 1988). The evolutionary conservation of the ubiquitous crystallins suggests that little variation can be accommodated by the lens (De Jong & Hendriks, 1986; Den Dunnen *et al.*, 1986a; Aarts *et al.*, 1988; Aarts *et al.*, 1989a). Yet, in apparent contradiction, the lens is capable of accepting the accumulation of different taxon-specific crystallins without adverse affects on transparency. Clearly we do not yet know what structural properties make a protein acceptable as a crystallin, nor do we know what roles the various ubiquitous crystallins play in establishing a supramolecular order on the lens proteins.

THE β/γ-CRYSTALLIN SUPERFAMILY

The three-dimensional structure of the γ- as well as the β-crystallins has been solved by X-ray analysis (Blundell *et al.*, 1981; Wistow *et al.*, 1983; Bax *et al.*, 1990). Both proteins contain four topologically equivalent "Greek Key" motifs, with pairs of motifs organized in two domains (Summers *et al.*, 1986,; White *et al.*, 1989; Fig. 1). The homology in structure indicates that the β- and γ-crystallins belong to a β/γ superfamily (Wistow *et al.*, 1981; Inana *et al.*, 1983; Lubsen *et al.*, 1988). The β-crystallins, however, have additional N- and C-terminal extensions, which are believed to be involved in the higher order (tetramer) interactions of these proteins (Bax *et al.*, 1990; Fig. 1). The close resemblance in motif structure is reflected by a close resemblance in amino acid sequence. The β/γ-crystallin motif is between 39 and 50 amino acids long. Each motif contains conserved amino acids at the positions 6 (Tyr, Phe or rarely Trp), 7 (Glu or Asp) and 13 (Gly), while a Ser is found 6 or 7 residues before the end of the motif (Summers *et al.*, 1986; Lubsen *et al.*, 1988; White *et al.*, 1989). These residues are implicated in the folding and stabilization of the hairpin within the "Greek key" motif (Summers *et al.*, 1986). Substitutions of those amino acids are predicted to have adverse effects on protein folding, protein structure and protein function. However, a structure-function relationship has not yet been proven experimentally.

The γ-crystallins are thought to be critical determinants of the water content of the lens fibre cell. The concentration of these proteins is inversely correlated with the water

content of the fibre cell (De Jong, 1981; Lindley *et al.*, 1985). For example, γ-crystallins are mainly found in the low water region of the lens, the lens nucleus (McAvoy, 1978; Carper *et al.*, 1985; Murer-Orlando *et al.*, 1987; Van Leen *et al.*, 1987; Siezen *et al.*, 1988; Voorter *et al.*, 1990). Furthermore, the concentration of the γ-crystallins differs widely between species. Hard lenses, e.g. rodent lenses, contain a high concentration of γ-crystallins (41 % in the central part of the lens: Siezen *et al.*, 1988), whereas soft human lenses have a low concentration (11 %: Thomson & Augusteijn, 1985; Siezen *et al.*, 1987).

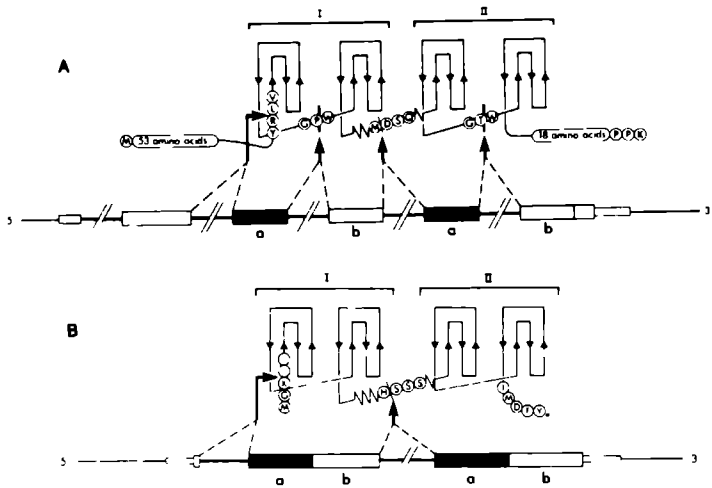


Figure 1. Schematic representation of the correlation between protein and gene structure of the rat βB1- (A) and γE-crystallin (B). For the gene, coding exonic sequences are indicated by broad bars, non-coding regions by narrow bars. The internal sequence similarity in the subregions is shown by the shading of the coding regions. Intronic sequences are drawn with thick lines; gene flanking sequences by thin lines. For the protein, the two domain (I, II)/ four motif (a, a', b, b') structure is indicated. At the amino- and carboxyl-terminal ends of the protein as well as around the splice sites individual amino acids are shown. The connecting peptide is indicated by a zig-zag line. The structure of the Greek Key motif in the protein is shown schematically (Taken from Den Dunnen *et al.*, 1986b).

The β-crystallin gene family is remarkable for its diversity in sequence and in expression pattern (Lubsen *et al.*, 1988; Aarts *et al.*, 1989a,b). Some members of the β-crystallin gene family are only expressed during early lens development, while the expression of other β-crystallin genes is limited to the postnatal lens. This expression pattern is conserved: the βB2-crystallin is late in rat or man as well as *Xenopus laevis* (Aarts *et al.*, 1989b; Jiang *et al.*, 1989; this thesis). The functional significance of the

wide range in developmental expression patterns of the different β-crystallins may perhaps be found in the fact that different β-crystallins combine to differently sized aggregates (Siezen *et al.*, 1986). The size of the β-crystallin aggregates in a fibre cell is thus controlled by the exact complement of β-crystallins in that fibre cell. The size of the β-crystallin aggregates could be of importance in packing of the lens protein. The dense packing in the nucleus may require a different size distribution of protein aggregates than the looser packing in the lens cortex.

The hypotheses about the possible function of the β- and γ-crystallins shortly outlined above need to be experimentally tested. The role of the crystallins in the packing interactions is probably critically dependent on the natural environment in a living lens and the presence of the natural complement of crystallins, parameters which cannot be mimicked *in vitro*. Hence, the phenotypic effect of changes in the expression or structure of the genes encoding these proteins may well be seen only *in vivo*. At present, there are basically two approaches to the study of gene function *in vivo*: "classical genetics" and genetic disease, and "reverse genetics".

CLASSICAL GENETICS

The genetic approach to the study of gene function relies on the analysis of the effect of mutations in that gene on the phenotype of an organism. Classically, one needs to screen randomly generated mutants, either spontaneous or induced, to find a mutation in the desired gene.

The first step in such a screen is the selection of phenotypes predicted to possibly result from a mutation in the gene under study. The most straight forward method to further analyze the involvement of the gene dysfunction in the phenotype is the investigation of the presence, absence or change of the gene product in the affected tissue. Many α- and β-globin gene mutations have been traced by the protein analysis of blood of anaemia patients (Collins & Weismann, 1984). Similarly, the abnormal behaviour of βB2-crystallin in the lens of the Philly mouse indicated the involvement of the βB2-crystallin gene in the cataract of this mouse strain, although the formal genetic proof, i.e. linkage of the cataract locus with the βB2-crystallin gene, is not established as yet (Russell & Chambers, 1990).

When the affected tissue cannot be examined directly, as often the case in human disease, then a molecular genetic screen must be used. A cloned copy of the gene, suspected but not proven to be involved in the particular phenotype, is used as a chromosomal marker to investigate the linkage with that phenotype. The first step here is the detection of a Restriction Fragment Length Polymorphism (RFLP; nucleotide

sequence variations visualized by restriction enzyme mapping and Southern hybridization, reviewed by Gusella, 1986; Botstein *et al.*, 1980) with a probe of the gene of interest, followed by the analysis of the segregation of the allelic RFLPs with the phenotypic disease. The assignment of genes and aberrant phenotypes to chromosomal loci facilitates this approach enormously (Gusella, 1986). Cloning of the damaged gene should then unravel the mechanisms which affected the gene, and the effects of the damage on the gene function. Using this approach, the linkage of a hereditary "Coppock-like" cataract with the human γ-crystallin gene family was discovered (Lubsen *et al.*, 1987). A mutant arrangement of Taq I restriction fragments was shown to be indicative of the cataract phenotype, and suggested that a mutation in one of the γ-crystallin genes caused the hereditary lens opacity.

An analogous approach can be used to identify the genetic basis for a phenotypic change, for which an obvious biochemical basis is lacking. The linkage of an aberrant phenotype with known chromosomal markers as blood groups and isozyme patterns (Renwick & Lawler, 1963), or at present RFLPs mark the chromosomal region in which a putative mutation, responsible for the aberrant phenotype, is located. After the cloning of that region, genes can be located by hybridization of Northern blots and Zoo Southern blots or by exon trapping (Koenig *et al.*, 1987; Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). The isolated gene sequences can then be used as probe for changes in the affected cells or tissues.

REVERSE GENETICS

The research on gene function using classical genetics is clearly limited in that the genome is not manipulated at will. Therefore, in the last ten years of recombinant DNA research an alternative methodology has been developed, known as reverse genetics (Landel *et al.*, 1990). Essentially, in reverse genetics experiments foreign, manipulated DNA is introduced in an organism. If the exogenous DNA stably integrates in the germline chromatin, so-called transgenic animals are obtained (Palmiter & Brinster, 1986). Using transgenesis in animal model systems, it is possible to express genes in an alternative tissue, at an alternative time, and, moreover, to inhibit the endogenous gene expression by exogenous antisense constructs (reviewed by Stout & Caskey, 1987). This latter procedure mimics the situation in which a gene is deleted or turned off by a mutation. In the usual transgenic experiments, the endogenous genes remain intact and the mutant constructs introduced must thus function in a wild-type background. Recently, an promising addition to reverse genetics techniques has been developed using homologous recombination in embryonic stem cells. Mouse

embryonic stem cells are cultured *in vitro*, one or more gene(s) are modified by homologous recombination with transfected mutant copies, and the cells injected into developing blastocysts. If these cells invade the gonads in later developmental stages, transgenic offspring with the desired mutations can be obtained (Capecchi, 1989). With this laborious (and expensive) technique the equivalents of "classical" mutations, i.e. mutations in the endogenous genes, can be produced. These will provide an important tool for the study of gene function, and the relationship with genetic disease.

Several animal models for *in vivo* studies are available: *Xenopus laevis* (Gurdon & Melton, 1981), mice (Palmiter & Brinster, 1986; Landel *et al.*, 1990), rats (Hammer *et al.*, 1990), rabbits (Hammer *et al.*, 1985), sheep (Hammer *et al.*, 1985) and pigs (Hammer *et al.*, 1985), and have been used with varying amounts of success. Research on genes involved in (the regulation of) early development is most easily performed in *Xenopus*, as *Xenopus* develops just in tap water and embryonic development can be followed continuously (McMahon & Moon, 1989). A limitation of transgenesis in *Xenopus* is caused by the very low frequency of germline integration and therefore, only the effect of transient expression of the introduced DNA can be studied (Gurdon & Melton, 1981). Mice are suitable for most experiments, and have a high frequency of germline integration. Stable transgenic lines are thus easily obtained (Palmiter & Brinster, 1986). However, mice do not always provide the correct model system for man. Bechterew disease (degeneration of the intervertebral discs) could not be mimicked in transgenic mice, whereas transgenesis in rats succeeded (Hammer *et al.*, 1990).

HUMAN CRYSTALLIN GENES AND CATARACT

A lens opacity, i.e. a region scattering light, is called a cataract. Cataract is due to cytoplasmic changes (protein aggregation, syneresis, phase separation), membrane degradation and/or disorientation of cytoskeletal elements in the lens fibre cells (reviewed by Bettelheim, 1985). The incidence of cataract increases with age. As the crystallins or other lens proteins cannot be replaced, the lens ages: the proteins are damaged and start to aggregate, resulting in senile cataract (Hoenders & Bloemendal, 1983). Cataract is also often associated with metabolic disease, such as diabetes (Spector, 1985). Finally, cataract is sometimes found as (part of) a hereditary syndrome (Clayton, 1985). Mutations in the crystallin genes would be expected to cause hereditary cataract. However, at present, there are only two cases described where cataract is the result of a mutation in a crystallin gene. In the Philly mouse the βB2-crystallin gene is partially deleted, and in a guinea pig mutant part of the ζ-

crystallin gene is lacking (Russell & Chambers, 1990; Borras *et al.*, 1990). In man, as yet only two loci of hereditable forms of cataract have been assigned to chromosomes, the "Coppock-like" cataract locus to the γ-crystallin gene family on chromosome 2 (Lubsen *et al.*, 1987) and a second cataract locus to chromosome 1 (Renwick & Lawler, 1963).

The human eye lens contains the regular set of ubiquitous crystallins, α, β, and γ. No taxon-specific crystallin has been identified in man. Most of the human genes encoding the α-, β- and γ-crystallins have been cloned and mapped on the human genome (Table 2).

Table 2. The chromosomal localization of the human crystallin genes

crystallin gene	chromosomal localization	references
αA	21q22.3	Quax-Jeuken *et al.*, 1985b. Hawkins *et al.*, 1987.
αB	11q22.3-23.1	this thesis. Ngo *et al.*, 1989.
βA1/A3	17q11.2-q12	Law *et al.*, 1986. Sparkes *et al.*, 1986.
βA2	unknown	not yet cloned.
βA4	22	Van Rens *et al.*, in prep.
βB1	22(?)	this thesis.
βB2-1 βB2-2 βB3	22q11.2-q12	Hogg *et al.*, 1987. Hulsebos *et al.*, 1991.
γA-γG	2q33-q36	Den Dunnen *et al.*, 1985a. Shiloh *et al.*, 1986.
γS	3	Van Rens *et al.*, 1989.

In the first column the human crystallin gene is denoted, in the second column the chromosomal locus, and in the third column the reference to the assignment is given.

The human αA- and αB-crystallin genes are single copy genes, as they are in all species examined thus far. Within the β-crystallin gene family, most genes are single copy genes, except for the βB2 gene which is duplicated in the human genome (Aarts

et al., 1987). Man is the only species known in which this gene is duplicated. It must be noted that the human β-crystallin proteins are poorly characterized and that in most cases the exact protein products of the genes listed in Table 2 have not been identified.

When the studies described in this thesis were initiated, six members of the human γ-crystallin gene family had been cloned, the γA through γF genes (Den Dunnen *et al.*, 1985b; Meakin *et al.*, 1985). Two of these genes, the ψγE and ψγF genes, contain in-frame stopcodons and are pseudogenes. The presence of additional γ-crystallin genes in the human genome was suggested by Southern hybridization experiments (Den Dunnen *et al.*, 1985b; Meakin *et al.*, 1985).

Only two members of the human γ-crystallin gene family encode abundant protein, the γC and γD genes (Siezen *et al.*, 1987). Thus of the potential seven or eight genes only two are used. In contrast, the rat orthologs of the γA through γF genes are all active (Van Leen *et al.*, 1987; Siezen *et al.*, 1988). How this differential pattern of γ-crystallin gene expression between rat and man is correlated with lens function is still unclear.

Although a few structural details still have to be elucidated, our rapidly accumulating knowledge of the ubiquitous crystallin genes in man during the last years has provided the required framework for studies on the function of the crystallins, in particular their role in assuring the transparency of the ocular lens and the causal involvement of the crystallins in cataract, the major cause of blindness in man.

OUTLINE OF THIS THESIS

Natural variation between species or between individuals within a species is a source of crystallin mutations. Analysis of the effects of these mutations on eye lens function provides some insight into the correlation between structure and function.

Inter-species variation is found in the copy number of the β- as well as the γ-crystallin genes. The observation of a duplication of the βB2 gene in man, thus increasing the gene dosage twofold, raised the question whether the expression level of βB2 was increased twofold as well. However, as described in Chapter 3, the βB2-2 gene is a pseudogene.

The inter-species variation in the γ-crystallin genes is examined in Chapter 4. The remaining human gene(s) were cloned, and the expression pattern of the complete family was established. Using model systems, the cause of the difference in expression of orthologous genes in rat and man was traced.

Intra-species variation, i.e. crystallin mutations, could in some cases be responsible for hereditary eye aberrations. An informed guess as to whether a particular crystallin gene is involved in a particular hereditary syndrome is easily made if both loci are mapped. Most crystallin genes have been assigned to a chromosomal region. The mapping of one of the remaining unknowns, the αB-crystallin gene, is reported in Chapter 2. In the case of the hereditary "Coppock-like" cataract, the cataract locus has been shown to be closely linked to the γ-crystallin gene cluster. The cloning of γ-crystallin genes from the mutant chromosome and the detailed molecular analysis of these genes is described in Chapter 5.

Classical genetics, the study of natural variants, does not allow one to "design" mutations. Therefore the research was extended to "reverse genetics". In Chapter 6 the suitability of transgenesis in *Xenopus* for lens research was investigated. In order to analyze the effect of manipulations on *Xenopus* lenses, an apparatus to measure lens opacities in very small lenses was developed and tested. The results are described in Chapter 7.

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CHAPTER 2

HUMAN αB-CRYSTALLIN (CRYA2) GENE MAPPED TO CHROMOSOME 11q12-q23

The results described in this Chapter were published in Hum. Genet. **85**, 237-240 (1990).

ABSTRACT

The αB-crystallin gene (CRYA2) encodes the abundant lens protein αB-crystallin. A panel of human/rodent hybrid cell lines, derived from five different parental combinations, was characterized with respect to human chromosomal content and the presence of well established human chromosome-specific markers. This panel was screened for the presence of CRYA2, using the third exon of the hamster αB-crystallin gene as a probe. The patterns of segregation of CRYA2 with individual human chromosomes show the highest degree of concordance between CRYA2 and chromosome 11. Using cell hybrids containing translocated and/or partially deleted human chromosomes, the CRYA2 gene was localized to 11q12-11q23.

INTRODUCTION

The abundant water-soluble structural proteins found in the human lens, the α-, β- and γ-crystallins, are presumably involved in the establishment and maintenance of the transparency as well as the refractive index gradient of the lens (Bloemendal, 1982; Lindley *et al.*, 1985). Mutations in the genes encoding these proteins might thus be a cause of hereditary cataract. Indeed, close linkage between the γ-crystallin genes and a locus for a hereditary "Coppock-like" cataract was found in one family (Lubsen *et al.*, 1987). Similar involvement of other crystallins in other forms of cataract can be investigated if the formal genetics of the relevant genes is understood.

The α-crystallins are encoded by two closely related genes, the αA- and αB-crystallin genes. These strongly conserved genes are found not only in man, but in all vertebrates examined. It is generally accepted that these two genes have evolved from a common ancestral gene, early in the evolution of vertebrates (Piatigorsky, 1984). The elucidation of the chromosomal localization of these genes in man, as well as in other species, will help in tracing the evolutionary history of this family and in the establishment of syntenic groups. The αA gene has been mapped to human chromosome 21 (Quax-Jeuken *et al.*, 1985). We report here that the αB gene is located on the long arm of chromosome 11.

MATERIALS AND METHODS

Materials

Restriction enzymes, *E. coli* DNA polymerase I (Kornberg) and *E. coli* DNA polymerase I large fragment (Klenow), were from Boehringer Mannheim. Agarose was from Sigma, NA agarose from Pharmacia, formamide from Fluka, nitrocellulose from Schleicher and Schüll and [α-^{32}P]dATP (3000 Ci/mmol) and [α-^{32}P]dCTP (3000

Ci/mmol) from Amersham. The multiprime kit for labelling DNA fragments was from Amersham.

Methods

Cell lines and growth conditions

Hybrid cell lines were isolated after fusion of leukocytes from various donors with hypoxanthine phosphorybosyl transferase (HPRT$^-$) WEHI-TG mouse cells or thymidine kinase deficient (Tk$^-$) Chinese hamster a3 cells, as reported previously (Geurts van Kessel *et al.*, 1983). For the regional localization studies described in this paper we used hybrid cell lines obtained after fusion of leukocytes from a patient with chronic myeloid leukemia carrying a complex Philadelphia translocation (9;11;22)(q34;q12;q11) (Geurts van Kessel *et al.*, 1984), leukocytes from an aniridia-Wilms' tumour patient, carrying a deletion 11p12-p15.1 (Mannens *et al.*, 1987), and an acute leukemia cell line carrying a specific translocation (4;11)(q21;q23) (Sacchi *et al.*, 1987). All cell lines were grown in F10 or RPMI medium supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and fetal calf serum (10%).

Human chromosome content of hybrid cells

Air-dried chromosome spreads were R-banded with acridine orange after heat denaturation. At least 16 metaphases were analyzed per hybrid cell line. Various chromosome-specific iso-enzyme markers were analyzed by cellulose acetate (Cellogel) electrophoresis (Meera Khan, 1971). The cells used for chromosome analysis and DNA extraction were always derived from the same culture batch.

DNA isolation

Cultured cells were suspended in 3 ml 0.9 % NaCl. TNE buffer (10 mM TRIS HCl pH 7.5, 150 mM NaCl, 25 mM EDTA) was added, followed by SDS to 1% and proteinase K to 0.2 mg/ml in a final volume of 20 ml. After 30 minutes incubation at 37 °C, the mixture was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1). After centrifugation the aqueous phase was removed and the DNA precipitated with 0.4 volumes 5 M ammonium acetate and two volumes isopropanol. The DNA was spooled out with a bent glass rod, and washed with 70% ethanol. The DNA was redissolved in 10 mM TRIS HCl, 1 mM EDTA (pH 7.8) at 4 °C overnight. The solution was treated with RNAse (20 μg/ml) for one hour followed by proteinase K incubation, phenol/chloroform/isoamyl alcohol extraction and isopropanol precipitation as described above. After washing with 70% ethanol, the DNA was dissolved in 10 mM TRIS HCl, 1 mM EDTA (pH 7.8) at approximately 0.5 mg/ml.

DNA probes

The DNA probe used to detect the human αB-crystallin sequences was the 220 bp

BamH I/Hind III fragment of the third exon of the hamster αB-crystallin gene (Quax-Jeuken *et al.*, 1985). The fragment was excised from the vector by restriction enzyme digestion and separated by electrophoresis on NA agarose. The DNA fragment was cut out and isolated by the "freeze-squeeze" method. The fragment was labelled with [α-^{32}P]dCTP to a specific activity of about 10^9 dpm/μg using the multiprime labelling method.

Southern blotting and hybridization

15 μg of DNA was digested to completion with the indicated restriction endonucleases under the conditions recommended by the supplier. DNA fragments were separated by electrophoresis on 0.7% agarose gels in E buffer (40 mM TRIS, 20 mM sodiumacetate, 2 mM EDTA, pH 8.0), and transferred to nitrocellulose as described by Maniatis *et al.* (1982). The filters were hybridized for 16 hours at 42 °C in 6xSSC (1xSSC = 150 mM NaCl/15 mM sodiumcitrate, pH 7.0), 50% formamide, 5x Denhardt's solution (0.4% bovine serum albumin, 0.4% polyvinylpyrolidone, 0.4% Ficoll), 0.1 mg/ml salmon sperm DNA, 0.1% SDS. After hybridization the filters were washed twice with 2xSSC, 0.1% SDS and twice with 0.2xSSC, 0.1% SDS at 42 °C. Autoradiography was performed for 60 hours using Kodak X-AR 5 film and intensifying screens.

RESULTS AND DISCUSSION

When Bgl II or EcoR I restriction enzyme digests of human genomic DNA were hybridized with a third exon fragment of the human αB-crystallin gene, only a single band was detected and the hybridizing human fragment could easily be separated from the hybridizing mouse of hamster fragment in human/rodent cell hybrids. For example, the αB probe hybridized with a 5.0 kb Bgl II human genomic DNA fragment (see Fig. 1, lane hu) while mouse DNA yielded a 1.5 kb fragment and hamster DNA a 6.7 kb fragment (see Fig. 1). Therefore Bgl II or EcoR I digests of cell hybrid DNA were used to screen a cell panel of 18 human/mouse or human/hamster hybrids for the presence of human αB-crystallin sequences. The results obtained are summarized in Table I. These suggested that the αB-crystallin gene was located on chromosome 11 with a discordance of 6%.

To map the αB-crystallin locus more regionally, a further panel of seven cell lines containing partial deletions of chromosome 11 was screened. As those cell lines that lack the 11q12-11q23 region failed to hybridize with the αB-crystallin probe (see schematic drawing in Fig. 2), we conclude that the αB-crystallin gene is located within this region. These results thus confirm the observation made by Quax-Jeuken *et al.*

(1985), viz. that the αB-crystallin gene is not linked to its close relative, the αA-crystallin gene. The latter gene was shown by the same authors to be located on chromosome 21 (Quax-Jeuken *et al.*, 1985). Based on sequence homology, the genes appear to have arisen at least 500 million years ago by gene duplication (Piatigorsky, 1984). Later in evolution the duplicates were separated, possibly by extensive chromosomal rearrangements.

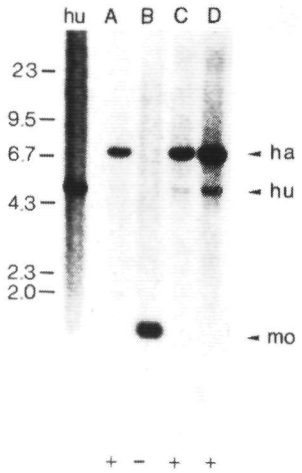

Figure 1. Hybridization of the αB-crystallin probe to a somatic cell hybrid panel. DNA was isolated, digested and hybridized as described in materials and methods. The origin of the hybridizing fragments is indicated as mo = mouse, hu = human and ha = hamster. The cell lines shown are: lane A = A3RS-27B, lane B = Wegli 9, lane C = A3BI-4C, lane D = A3BI-1C. Lane hu = human genomic DNA. Molecular weight is indicated in kilo basepairs. The presence of the human αB-crystallin fragment is designated as + (present) or - (absent).

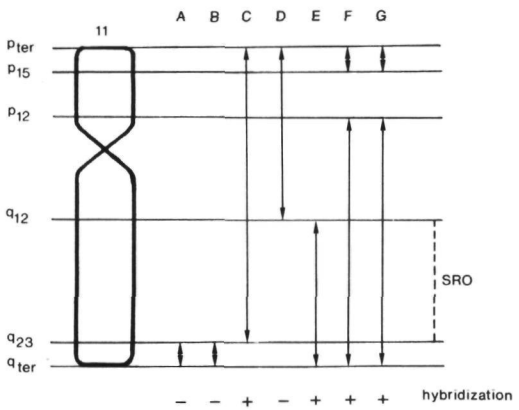

Figure 2. Schematic drawing of chromosome 11. The arrows show the region present in the cell lines used. The shortest region of overlap is denoted by SRO.

Table 1. Segregation of human chromosomes and/or chromosome-specific DNA markers and CRYA2 in rodent-human somatic cell hybrids[a]

Hybrids	Chromosomes[b]																						CRYA2
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
MaY1	+		+	+	+			+	+			+		+	+	-				+	+			
MaY2	+									+	+	+	+		+	+				+	+	+		
MaY3												+	+											
MaY4	+				+	NT		+		+		+	+	+	+		+	+	NT		+	+	+	
MaY5	+				+	+			+				+	+	-		+	+	+		+	+		
MaY6	+		+								+	+			+				+		+		+	+
MaY7	+		+					+			+	+			+				+		+		+	+
MeA1							+		+	+	+			+		+	+		+	+	+	+	+	+
MeB1		+	+	+			+	+			+	+	+	+		+	+			+	+	+	+	+
MpM1			+	+		+		+				+		+		+	+				-		+	
MpM2			+	+	+	+	+	+	+	+	+	+	+	+	-		+			+			+	+
MpM3						+											+				+			
MpM4		+	+		+	+		+		+	+	+	+	+	+	+	+	+		+	+		+	+
MpM5			+	+		+		+		+		+		+				+				+	+	
MpM6			+	+	+	+	+		+	+	+			-	+		+	+				+	+	+
WEBE-3	+	+		+	+	+	+	+			+	+	+	+	+			+		+	+	+	+	+
WEGROTH-D2																						+		
X/3-F2																	+		+	+	+			
Chromosome/CRYA2[c]																								
+/−	4	0	3	3	4	4	0	4	2	3	1	6	4	5	4	3	5	3	3	3	7	5	3	
−/+	5	5	2	4	4	4	3	3	5	4	0	2	4	2	2	5	3	5	5	3	2	4	0	
Discordance (%)	50	28	28	39	44	44	17	39	39	39	6	44	44	39	33	44	44	44	44	33	50	50	17	

a) CRYA2 is the locus symbol proposed for the human gene coding for αB-crystallin. The hybrids WEBE-3, WEGROTH-D2 and X/3-F2 have been described by Geurts van Kessel *et al.* (1983). Details of the other hybrids included in the present panel, and the set of DNA probes used to screen them for chromosome-specific markers, will be reported separately (M. Oldenburg, J.T. Wijnen, P. Meera Khan, in preparation)

b) Chromosome-specific markers, G-banding, Giemsa-11 and/or Q-banding techniques were used to screen the hybrids for the presence (symbol +) of individual chromosomes. NT, not tested

c) The categories of discordants (chromosome present/CRYA2 absent and vice versa) were counted separately

With the elucidation of the locus for the αB-crystallin gene, all but one of the known human crystallin genes have now been mapped (De Jong & Hendriks, 1986; Van Rens *et al.*, 1989). Information is still lacking about the chromosomal localization of the βB1-crystallin gene, but preliminary data suggest that this gene is located on chromosome 22 (unpubl.). Unfortunately, mapping data on human cataract are still scarce: only two human hereditary cataract loci have been assigned to a chromosome, one to chromosome 1 and one to chromosome 2 (Renwick & Lawler, 1963; Lubsen *et al.*, 1987). Further mapping studies of human hereditary cataracts are required to show whether a mutation in the αB-crystallin gene is a possible cause of this disease. The only eye aberration known to be linked to chromosome 11 at present is the aniridia often seen as a part of the Wilms' tumour syndrome. However, the gene(s) responsible for this syndrome is (are) linked to 11p (Kazagian & Junien, 1987) and our results thus exclude the possible involvement of the αB-crystallin gene in the eye aberrations of this syndrome. Recently it has been shown that the αB-crystallin gene is expressed not only in the eye lens but also in heart muscle, and to a lesser extent also in skeletal muscle and brain (Bhat *et al.*, 1988; Dubin *et al.*, 1989). The functional relevance of the expression of this gene in these tissues, if any, is unknown. However, these observations suggest that mutations in the αB-crystallin gene could have phenotypic effects in tissues other than the eye lens.

ACKNOWLEDGMENTS

The authors would like to thank E. van Drunen for technical assistance. This work was supported in part by the Netherlands Cancer Society (KWF), and has been carried out in part under the auspices of the Netherlands Foundation for Chemical Research (SON), with financial aid of the Netherlands Organization for the Advancement of Pure Research (NWO).

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CHAPTER 3

THE SECOND HUMAN βB2-CRYSTALLIN GENE IS A PSEUDOGENE

The results described in this Chapter will be published in
Exp. Eye Res. (in press)

ABSTRACT

Comparison of the partial sequences of the human βB2-1- and βB2-2-crystallin genes with orthologous rat or calf sequences shows that the fourth exon sequence of the human βB2-2 gene contains an one triplet deletion and a mutated splice acceptor site. No transcripts from the βB2-2-crystallin gene could be detected in the human lens. These data suggest that the human βB2-2-crystallin gene is a pseudogene.

INTRODUCTION

One of the three gene families which encode the abundant mammalian lens-specific proteins is the β-crystallin gene family. Characterization of human and rat genomic clones of one of the members of the β-crystallin gene family, the βB2-crystallin gene, revealed that the human genome contained two copies of the βB2-crystallin gene, while only a single copy is present in the rat genome (Aarts *et al.*, 1987). As a doubling of the gene dosage could upset the delicate balance between protein composition and concentration thought to be responsible for the optical properties of the lens, we have investigated whether both human genes could yield a functional βB2 protein product. The peculiar sequence of the human βB2-2 gene as well as the lack of detectable transcripts suggests that this gene is inactive.

MATERIALS AND METHODS

Isolation and sequencing of human βB2-crystallin coding sequences

Appropriate restriction fragments of the human genomic clones λHeB2-3 and λHeB2-4 (Aarts *et al.*, 1987) were subcloned in M13mp vectors. M13 recombinants containing βB2-crystallin coding sequences were selected by hybridization with a calf (Quax-Jeuken *et al.*, 1984) or rat (Aarts *et al.*, 1989) βB2-crystallin cDNA probe as previously described (Aarts *et al.*, 1987). The M13mp clones were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977).

Sequence comparisons

Sequences were aligned using the programs of Staden (1982). The extent of substitution was calculated according to Miyata & Yasunaga (1980) and corrected for back mutations by the method of Jukes & Cantor (1969). In these calculations a TCC triplet was introduced in the exon 4 (motif II) sequence of the βB2-2-crystallin gene to obtain an exact alignment with the other human as well as the calf and rat sequences.

RNA analysis

Total lens RNA was isolated by using the hot-phenol method as described by Maniatis *et al.* (1982). For Northern blot analysis lens RNA was separated on a 1%

agarose-formaldehyde gel. After electrophoresis the gel was soaked in 20x SSC for 5 min and the RNA was blotted to nitrocellulose (Schleicher & Schüll). The βB2- and βB3-crystallin probes were obtained by radiolabeling the inserts of the corresponding cDNA clones (Quax-Jeuken *et al.*, 1984; Den Dunnen *et al.*, 1985) with [α-^{32}P]dATP through nick-translation. Hybridization and washing conditions were as described (Aarts *et al.*, 1989).

In the primer extension experiments the following two (end-labelled) oligonucleotides were used: prβ2-1: 5'-CTTCGGCTGCTGGTCCATG-3'; and prβ2-2: 5'-CTCTGGCTGCTGGTCCACC-3'. Both oligonucleotides are complementary to a region (see also Fig. 1) of the putative 4th exon of the human βB2-crystallin genes. Annealing of the oligonucleotide to the RNA and the primer extension reaction were performed according to Geliebter *et al.* (1986). The primer extension products were analyzed on a 6% polyacrylamide sequencing gel.

Differential hybridization of oligonucleotides

Dot blots of 2 μg of 22 months postnatal human total lens RNA or 1 and 0.1 μg of denatured plasmid DNA with inserts containing the 4th exon of either the βB2-1 or the βB2-2 gene, were prepared according to Maniatis *et al.* (1982). The dot blots were hybridized with the end-labelled oligonucleotides prβ2-1 and prβ2-2 in 6x SSC, 2.5x Denhardt's solution, 0.1% SDS, 10 μg/ml denatured salmon sperm DNA and 10 μg/ml yeast rRNA, at 42 °C for 16 hours. The blots were washed three times with 6x SSC at room temperature, followed by a final wash with prewarmed 6x SSC at 61.0 °C for 15 minutes. The conditions for this differential hybridization were determined empirically on dot blots of βB2-1 and βB2-2 clones.

RESULTS AND DISCUSSION

Comparison of the human βB2-1- and βB2-2-crystallin sequences

The partial sequence of the human βB2-1- and βB2-2-crystallin genes is given in Fig. 1. A high overall sequence correspondence is noticed when both sequences are compared with orthologous rat or calf sequences. However, striking differences are found in the sequences of the putative 4th exons. Compared to the βB2-1- (and the rat or calf βB2-; Hogg *et al.*, 1987; Aarts *et al.*, 1989) crystallin sequence, the βB2-2-crystallin gene lacks the triplet encoding the serine residue involved in maintaining the characteristic "Greek key" protein fold. In addition, this exon of the βB2-2-crystallin gene has a mutated splice acceptor site. The effect of the one amino acid deletion on the protein structure is not clear. However, the mutation at the splice acceptor site is predicted to shift the splice site two nucleotides upstream, which would

result in a shift in the reading frame and premature termination at the third triplet (TGA) after the splice site.

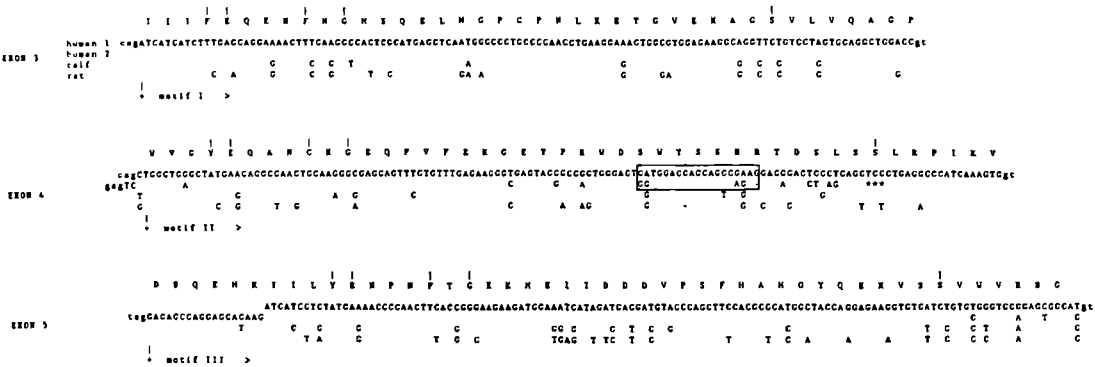

Figure 1. Comparison of human βB2-crystallin sequences with orthologous sequences from rat and calf. The sequence of exons 3, 4, and 5 of the human βB2-crystallin gene is given completely, for the other sequences only the nucleotides that differ are shown. Identical nucleotides are indicated by a dash The deletion of the triplet TCC in the βB2-2-crystallin gene is indicated by three asterisks. The residues involved in the characteristic "Greek key" folding of the protein are indicated with an (|). The numbering of the exons is by analogy with that of the rat βB1-crystallin gene (Den Dunnen *et al.*, 1986). The sequences of the putative fifth exon of the human genes were taken from Aarts *et al.* (1987), the calf sequence is from Hogg *et al.* (1987). The regions complementary to the primers used in the primer extension experiments are boxed.

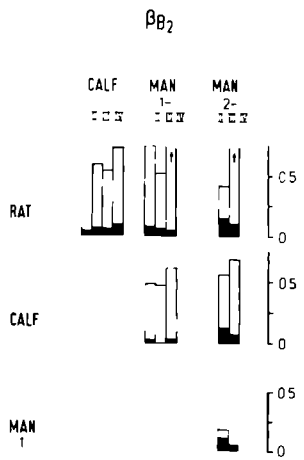

Figure 2. Extent of synonymous and non-synonymous substitutions in paralogous human [βB2-1 (-1-), βB2-2 (-2-)] and orthologous human, calf and rat βB2-crystallin genes. Open bars indicate the extent of synonymous substitution, filled bars the extent of nonsynonymous substitutions. Arrows show where the fraction of nucleotides synonymously substituted is greater than one. Roman numerals indicate motif regions I to IV and correspond to the four motif coding regions. The first two nucleotides from the fourth exon of the human βB2-2-crystallin gene were excluded from the comparisons.

Hence from this region of the coding sequences we would predict that only the βB2-1-crystallin gene can yield a functional protein product. The suggestion that the

βB2-2-crystallin gene is a pseudogene is supported by the apparent relaxation of the selective pressure on this sequence: the extent of change of the coding sequence of the βB2-2-crystallin gene relative to the calf or rat sequence is almost twice as high as that of the βB2-1-crystallin gene (Fig. 2).

Transcription of the *β*B2-1- and *β*B2-2-crystallin genes

In the rat the βB2-crystallin gene is a "late" crystallin gene, i.e. its transcripts accumulate only during postnatal development (Aarts *et al.*, 1989). The same situation pertains in man. The βB2-crystallin transcripts are found mainly in the postnatal human lens (Fig. 3), while transcripts of the closely linked βB3-crystallin genes as well as the transcripts of most other crystallin genes are abundant in the embryonic human lens (Fig. 3; Brakenhoff *et al.*, 1990).

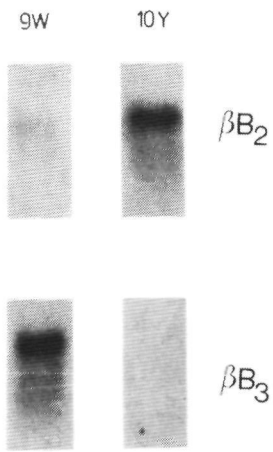

Figure 3. Expression of the *β*B2- and the *β*B3-crystallin genes in the human lens. Lens RNA (2 μg), isolated either from 9 weeks old embryonic or 10 yr old postnatal lenses, was electrophoresed, Northern-blotted and hybridized with the nick-translated inserts of the calf *β*B2- or the rat *β*B3-crystallin cDNA clone as described in Materials and Methods. Note that the *β*B2-crystallin probe should hybridize equally well to the transcripts of either human *β*B2-crystallin gene.

We therefore used RNA isolated from postnatal lenses in experiments designed to detect a transcript from the βB2-2-crystallin gene. To discriminate between the two human βB2-crystallin transcripts oligonucleotides specific either for the βB2-1- or for the βB2-2-crystallin transcripts were used (see Fig. 1 and 4). In initial dot blot experiments a hybridization signal was found only with the βB2-1-crystallin specific oligonucleotide. To confirm this result a primer extension experiment was performed. With the βB2-1-crystallin oligonucleotide an extension product about 280 nt long was found (Fig. 4). This size is in agreement with the length predicted from the Northern blot experiments. No extension product was seen using the βB2-2-crystallin oligonucleotide (Fig. 4). Hence, if the βB2-2-crystallin gene is transcribed, the

concentration of its transcript must be much lower than that of the βB2-1-crystallin gene.

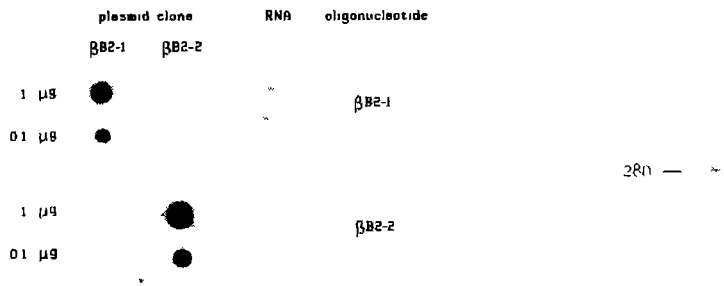

Figure 4. Detection of the transcripts from the βB2-crystallin genes.
(left) Hybridization with gene-specific oligonucleotides. DNA (left) or RNA (right) dot blots (see also Materials and Methods) were hybridized with either the βB2-1 (top) or the βB2-2 (bottom) specific oligonucleotide.
(right) Primer extension analysis of βB2-crystallin transcripts. Either the βB2-1 (a) or the βB2-2 (b) specific oligonucleotide was extended on RNA isolated from 22 months old human lenses as described in Materials and Methods. The length in nucleotides of the primer extension product is indicated.

From the results presented above we conclude that the human βB2-2-crystallin gene is inactive by two criteria: no transcript is detected and it contains a mutated splice site. In addition, its rate of change is much higher than that of the βB2-1-crystallin gene. It is not known whether the βB2-2-crystallin gene is a complete gene, as we have as yet not succeeded in cloning the first exonic regions of either one of the two human βB2-crystallin genes.

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CHAPTER 4

part A

THE HUMAN γ-CRYSTALLIN GENES: A GENE FAMILY ON ITS WAY TO EXTINCTION

The results described in Chapter 4, part A, were published in
J. Mol. Biol. **216**, 519-532 (1990).

ABSTRACT

During hominoid evolution the γ-crystallins of the lens have decreased in quantity as well as complexity, a change correlated with an increased water content of the lens. To trace the molecular basis for the decrease in γ-crystallin gene expression, we have characterized the structure and expression of the human γ-crystallin gene family. We show that the human γ-crystallin gene family consists of six complete genes (γA, γB, γC, γD, ψγE and ψγF) and one second exon fragment, the γG gene. Model experiments showed that, although the γG sequence is bordered by consensus splice sites, it is most likely transcriptionally inactive in the lens.

In the human embryonic lens the γC and γD genes accounted for 81% of the γ-crystallin transcripts, the γA gene contributed 14% and the γB gene only 5%. The composition of the γ-crystallin mRNA pool changed only after birth, with the γD transcript as the only detectable transcript at 10 years of age.

The relative activities of the γA, γC and γD promoters in a transient expression system were in agreement with the ratio of their *in vivo* RNA levels, suggesting that the difference in accumulation of these transcripts is due to differences in the rate of transcription. The γB promoter was much more active than expected and had lost its tissue-specificity. Model experiments showed that the low yield of the γB transcript is due to post-transcriptional processes, most likely RNA instability mediated by third exon sequences.

Together with previous data, our results show that the decrease in expression of the γ-crystallin genes in the human lens is the consequence of gene loss (γG), inactivation of coding sequences (ψγE and ψγF), decrease in rate of transcription (γA), increase in rate of RNA turn-over (γB) and a delay in the onset of transcription during development.

INTRODUCTION

The γ-crystallins are a closely related family of abundant lens-specific proteins. They are monomeric proteins, highly symmetrical in shape, which is thought to allow the close packing of protein molecules required for lens transparency and establishment of the proper refractive index gradient (Delaye & Tardieu, 1983). The distribution of the γ-crystallins is inversely correlated with the water content of the lens: they are found primarily in the dehydrated lens nucleus and are much less abundant in the cortex. Similarly, the γ-crystallins are particularly prominent in species with (hard) lenses having a low water content, such as fish and rodents (De Jong, 1981; Maisel, 1985), but are less well represented in species with (soft) lenses having a high water

content. The hard, spherical rat lens, for example, contains six abundant γ-crystallins, which constitute between 34% (lens cortex) to 41% (lens nucleus) of the total lens protein (Siezen *et al.*, 1988). In contrast, the soft, ovoid human lens has only two abundant (and some unidentified minor) γ-crystallin species (Siezen *et al.*, 1987). Furthermore, the γ-crystallin fraction of the human lens comprises but 11% of total protein (Thomson & Augusteyn, 1985).

At present then, there is a distinct difference between the rat and human γ-crystallin population. Evolutionary evidence indicates that the common ancestor of man and rat contained six active γ-crystallin genes (Lubsen *et al.*, 1988). In the rodent lineage the functional activity of these six genes has been maintained (Moormann *et al.*, 1985; Murer-Orlando *et al.*, 1987; Van Leen *et al.*, 1987b), while partial or complete loss of function of four out of the six parental γ-crystallin genes must have occurred in the hominoid lineage. The human γ-crystallin gene family thus provides an unique opportunity to study the mechanisms by which genes are functionally inactivated during evolution.

Previous studies have shown that at least six γ-crystallin genes (the γA through γF genes) are present in man with the possibility of additional genes suggested by the presence of as yet unassigned γ-crystallin sequences in the human genome (Den Dunnen *et al.*, 1985; Meakin *et al.*, 1985). We show here that the human gene complement consists of six and one quarter genes. Of the six genes, two, the γE and γF genes, are known to be inactive by virtue of an in frame stopcodon (Meakin *et al.*, 1985). We have detected transcripts of the other four genes in the human lens, but the transcripts of the γA and γB genes are present at relatively low levels. Model experiments indicate that the low activity of the γA gene is the result of a poor rate of transcription, while the lack of accumulation of the γB transcript is due to its instability.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from Boehringer Mannheim, Amersham or Pharmacia. DNA polymerase (Klenow fragment), T4 polynucleotide kinase, S1 nuclease and T4 DNA ligase were from Boehringer Mannheim, AMV reverse transcriptase was from Stratagene and mung bean nuclease from Promega or Pharmacia. The reaction conditions were chosen as recommended by the supplier. [^{32}P]-labeled nucleoside triphosphates (3000 Ci/mmol) and [^{14}C]chloramphenicol (50 mCi/mmol) were purchased from Amersham.

Methods

Isolation of genomic clones

A human gene library (obtained from Dr. P. Leder), made by partial digestion of human DNA with Mbo I and cloning in the BamH I sites of λCh28, stock 2270, was screened with a mixture of [^{32}P]dATP labeled rat γ-crystallin cDNA fragments and the [^{32}P]dATP labeled 3.5 kb Hind III fragment containing the human γF gene. Clones were plaque purified and identified by their restriction and hybridization pattern. Clone λhuγG, which contains a hybridizing 3.5 kb EcoR I fragment, was studied in detail. A 0.7 kb EcoR I/Xba I fragment of λhuγG, which comprised all sequences hybridizing with γ-crystallin probes, was subcloned in M13mp10 and -mp11 for sequence analysis. All sequences given in this report were determined by the dideoxy chain termination method on both strands. The sequence has been submitted to the EMBL Data Library (accession number: X52628).

RNA isolation, Northern blotting

The lens RNA used in primer extension assays and Northern blotting was isolated as follows: 3-5 lenses were homogenized by vortexing in 0.5-1 ml RNA lysis buffer (100 mM NaCl, 50 mM Tris.HCl, 50 mM EDTA, 0.2% SDS and 2% Triton-X100, pH 7.5). An equal volume of phenol, saturated with TE (10 mM Tris.HCl, 1 mM EDTA, pH 8.0), was added and the mixture was vortexed intensively. An equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added, the mixture was vortexed and the phases were separated by centrifugation. The aqueous phase was reextracted with phenol and chloroform/isoamyl alcohol as described, and then extracted once with chloroform/isoamyl alcohol. The RNA was recovered by ethanol precipitation.

Lens RNA used in the S1 nuclease protection assays, and RNA from tissue culture cells, was isolated by the guanidinium chloride method according to Cheley & Anderson (1984), followed by resolubilization in NTES (100 mM NaCl, 10 mM Tris.HCl, 1 mM EDTA, 0.1% SDS, pH 7.5), phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and ethanol precipitation, or according to Gough (1989).

For Northern blot analysis, lens RNA was separated on an 1% agarose-formaldehyde gel in 25 mM phosphate buffer, pH 7.0. After electrophoresis, the gel was soaked in 20x SSC for 5 min. and the RNA was blotted to nitrocellulose (Schleicher & Schüll).

The blots were hybridized in 50% formamide, 6x SSC, 2.5x Denhardt's solution, 0.1% SDS, 10 μg/ml salmon sperm DNA and 10 μg/ml yeast rRNA at 45 °C for 16 hrs. Filters were washed twice with 2x SSC/0.1% SDS and twice with 0.2x SSC/0.1% SDS at 45 °C.

DNA isolation, Southern blotting and hybridization

DNA was isolated from human blood essentially as described by Bell *et al.* (1981). The DNA was purified further by centrifugation through an 1 M NaCl cushion at 60,000g for 16 hrs at 15 °C according to Sambrook *et al.* (1989). Prior to loading on the NaCl cushion, sarcosyl was added to 1%, and the solution was incubated at room temperature for 10 min.

15 μg of genomic DNA was digested to completion with restriction enzymes, loaded on a 0.7% agarose gel and electrophoresed in TAE buffer according to Sambrook *et al.* (1989). The DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 45 min., and the gel was neutralized by soaking twice in 1M NH_4Ac, 0.02 M NaOH for 20 min. The DNA was blotted to nitrocellulose in neutralization buffer for 16 hrs.

The blots were hybridized in 50% formamide, 6x SSC, 2.5x Denhardt's solution, 0.1% SDS and 10 μg denatured salmon sperm DNA, at 42 °C for 16 hrs. Filters were washed twice with 2x SSC, 0.2% SDS and twice with 0.2x SSC, 0.2% SDS at 42 °C.

Probes

Construction and synthesis of the αA- and γ-crystallin SP6 probes have been described by Van Leen *et al.* (1987a). All other probes were made by labeling the isolated inserts of cDNA clones or genomic clones with [α-^{32}P]dATP through nick translation (Sambrook *et al.*, 1989), or multiprimed elongation (Feinberg & Vogelstein, 1983).

Oligonucleotide and primer extension

The following 18 mer oligonucleotide was used:

prγex12 : 5' GAAGGTGATCTT(C/T)CCCAT 3'

This oligonucleotide, which has been described by Van Leen *et al.* (1986), is complementary to the last 9 nucleotides of the first exon and the first 9 nucleotides of the second exon of the human γA-, γB-, γC-, and γF-crystallin gene, and with one mismatch (at nucleotide 7 of the second exon) complementary to the same region of the human γD- and γE-crystallin genes.

Annealing of the oligonucleotide to the RNA and the primer extension reaction were performed according to Geliebter *et al.* (1986).

S1 nuclease protection assay

Appropriate fragments of the γA-, γB-, γC- and γD-crystallin genes were subcloned in M13mp vectors. Continuously labeled extension products from these clones, prepared by elongating the universal M13 primer in the presence of [α-^{32}P]dATP, were hybridized with 0.5 μg of human total lens RNA, and treated with S1 nuclease as described previously (Moormann *et al.*, 1985). Usually, 10 ng of probe (10^6 cpm) were

used in the hybridization. After gel electrophoresis and autoradiography, the amount of protected fragment was determined by densitometric scanning of the autoradiograms according to Van Leen *et al.* (1987b). The values were corrected for the percentage of dAMP in the protected fragments. All relative values were determined within one single experiment. All experiments were performed at least in duplo.

CAT constructs

Restriction fragments containing the γ-crystallin promoter sequences, were blunt-ended by mung bean nuclease treatment and inserted (in either orientation) in the Sma I site of the supercat vector. In all cases but one, the Nco I site surrounding the ATG start codon was used for cloning, whereby the ATG was removed by the mung bean nuclease treatment. The following constructs were generated: the 1.9 kb EcoR I/Nco I fragment of the γA gene, the 0.5 kb Xba I/Nco I fragment of the γC gene and the 0.6 kb Taq I/Nco I fragment of the γD gene. The latter clone was constructed by ligating the Taq I/blunt-ended Nco I fragment into the Acc I/blunt-ended Hind III supercat vector. The γB promoter was cloned as a blunt-ended 0.44 kb Hind III/Pst I fragment in supercat. The Pst I site is located at position -6 according to Den Dunnen *et al.* (1985b).

Cell culture, transfection and CAT assay

Transdifferentiating chicken neural retina cells were cultured essentially as described by Okada *et al.* (1975), except that M199 medium, supplemented with 10% fetal calf serum, was used. After 8 days of culture the cells were transfected by calcium phosphate co-precipitation followed by a 25% DMSO shock (Graham & Van der Eb, 1973). Cells were harvested 48 hrs later and assayed for CAT activity as described by Gorman *et al.* (1982). Radioactive decay of the acetylated [^{14}C]chloramphenicol was measured by densitometric scanning of the autoradiograms, or by liquid scintillation counting. The values were corrected for cell density by measurement of the protein concentration in the cell lysate (BioRad protein assay). Transfection efficiency was monitored by measuring the activity of pSV2CAT transfected in parallel experiments.

Rat lens epithelial cells were isolated, cultured and transfected as described by Peek *et al.* (1990) for mouse lens epithelial cells. COS and Ltk$^-$ cells were cultured in DMEM enriched with 10% newborn calf serum, and transfected as described for chicken neural retina cells.

Construction and analysis of the pSVγD, pSVγD/γG(+) and pSVγD/γG(-) clones

A 3.2 kb Nco I (filled in with Klenow polymerase to preserve the ATG initiation codon)/EcoR I fragment containing the γD gene, was inserted in the Hind III (blunt-ended)/EcoR I vector fragment of pCH110 (Pharmacia; Fig. 1). The resulting clone, pSVγD, contains the SV40 origin of replication, the SV40 enhancer sequence, and the SV40 early promoter region in front of the γD gene. Subsequently, a 514 bp Alu I fragment containing the γG gene fragment (see also Fig. 3) was inserted in both orientations in the unique EcoR V restriction site, located in the 2.0 kb long second intron (0.4 kb upstream of the 3' splice site) of the γD gene, to yield the clones pSVγD/γG(+) and pSVγD/γG(-) (Fig. 1). The orientation of the γG fragment was determined by restriction enzyme digestion, using an unique Ava I site in the 514 bp Alu I fragment (see also Fig. 3). The pSVγD, the pSVγD/γG+ (sense) and the pSVγD/γG- (anti-sense) clones were transfected into COS cells as described above. The transcripts were characterized by Northern blotting and hybridization with a second exon probe, and further by S1 nuclease protection assays, using a continuously labeled second exon fragment of the γD gene as probe.

Construction and analysis of the pSVγB and pSVγD/γB clones

The pSVγB clone was assembled as follows: a pSVmp vector was constructed by inserting a mp10 multiple cloning site between the Hind III and EcoR I sites of pCH110. A 4.2 kb Pst I/Hpa I fragment containing the γB-crystallin gene from position -6 up to 0.5 kb downstream of the poly-A addition site (Den Dunnen *et al.*, 1985b), was cloned in the Pst I and Sma I restriction sites of the pSVmp vector.

Clone pSVγD/γB was constructed by swapping the 1.2 kb EcoR V/EcoR I fragment containing the third exon of the γD gene with the 1.5 kb Sma I/EcoR I fragment containing the third exon of the γB gene (Den Dunnen *et al.*, 1985b; see Fig. 1). The original pSVγD clone, the pSVγB clone and the pSVγD/γB construct were transfected into COS cells and the transcripts were analyzed by Northern blotting and hybridization. The amount of γD versus γD/B RNA was determined by densitometric scanning of the autoradiogram of the Northern blot hybridized with a second exon probe, and the amount of γB versus γD/B RNA by similar measurement of the Northern blot hybridized with a third exon probe. Variation in hybridization signal due to different sequence similarity with the probes could thus be avoided. The hybridization signal obtained with a ribosomal probe was used as an internal standard for the amount of RNA on the blot. The transfection efficiency was monitored by co-transfection with pSV2CAT plasmid in a ratio of 1 to 10, and determination of the CAT activity/μg protein as described above.

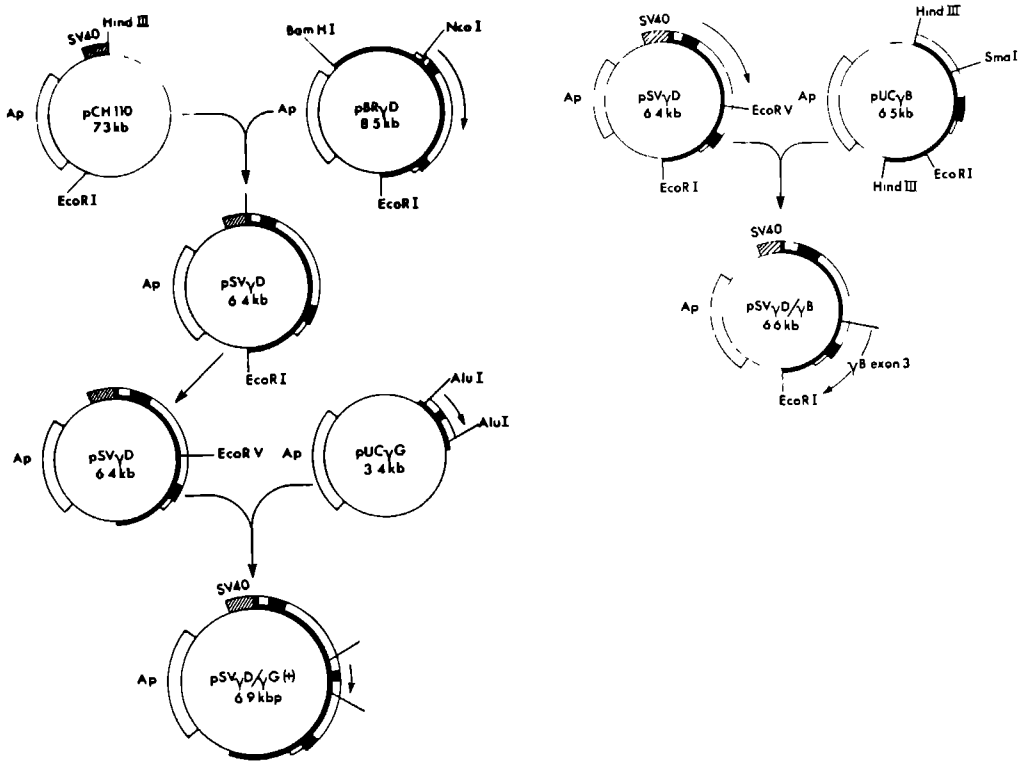


Figure 1. (left) Schematic representation of the construction of the pSVγD/γG clones. Plasmid sequences are drawn with a thin line. The open box denoted Ap, represents the ampicillin resistance gene. The inserts of the plasmids are drawn with a thick line. The dashed box represents the SV40 promoter/enhancer/ORI sequences. The black boxes represent the coding regions, the open boxes the introns, and the small open boxes the 5' and 3' non-coding regions of the γ-crystallin genes. The direction of transcription as compared with the γ-crystallin genes is indicated by an arrow.
(right) Schematic representation of the construction of the pSVγD/γB clone. Functional elements are indicated as in the legend to Fig. 1, left. The restriction map of the γB gene is given in Den Dunnen *et al.* (1985).

The structure of the human γ-crystallin gene family

The genomic organization of the six previously characterized human γ-crystallin genes is outlined in Fig. 2 (Den Dunnen *et al.*, 1985b; Meakin *et al.*, 1985).

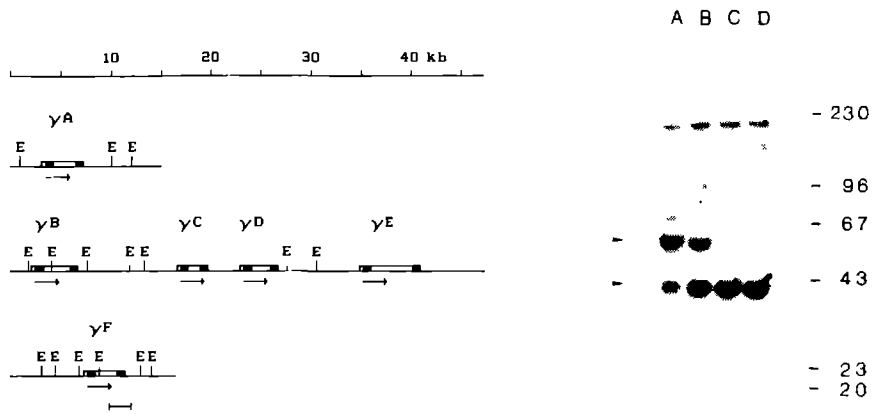


Figure 2. (left) Physical map of the human γ-crystallin gene cluster. Only the EcoR I restriction sites (E) are shown. The nomenclature of the genes is as proposed by Aarts *et al.* (1988). Note that the order of the γB, γC, γD and γE genes is the same in rat and man. The γA gene has been placed upstream from the γB gene by analogy with the gene order in the rat (Aarts *et al.* 1988). The exact location of the human γA and γF genes with respect to the four gene cluster is unknown. The heavy bars indicate exons or putative exons; open bars denote introns. The direction of transcription is shown by arrows. The 1.3 kb Pvu II fragment of the ψγF-crystallin gene, which was used as probe in the hybridization experiment shown in Fig. 2, right, is indicated by a bar.
(right) Changes in the γ-crystallin hybridization pattern of human genomic DNA with increasing EcoR I digestion. 15 μg of human DNA was digested with 18 (lane A), 36 (lane B), 54 (lane C) or 72 (lane D) units of EcoR I restriction enzyme for one hour. After electrophoresis, the DNA was blotted and hybridized with the nick-translated Pvu II fragment of the ψγF gene as described in Materials and Methods. The mobilities of phage λ-DNA Hind III fragments used as molecular weight marker are indicated. The 4.0 kb and the 5.6 kb hybridizing fragments are indicated by arrows. Note that only the 4.0 kb digestion product of the 5.6 kb fragment hybridizes with the probe. The other bands visible after complete digestion (lane D) are due to cross-hybridization of the probe with the γA through γE genes.

This map accounts for all hybridizing EcoR I fragments in the human genome except for one of 3.5 kb and one of 5.6 kb (Meakin *et al.*, 1985). Hence, an additional γ-crystallin gene could be present within the human genome. As we wished to examine the expression of the entire human γ-crystallin gene family, we set out to characterize

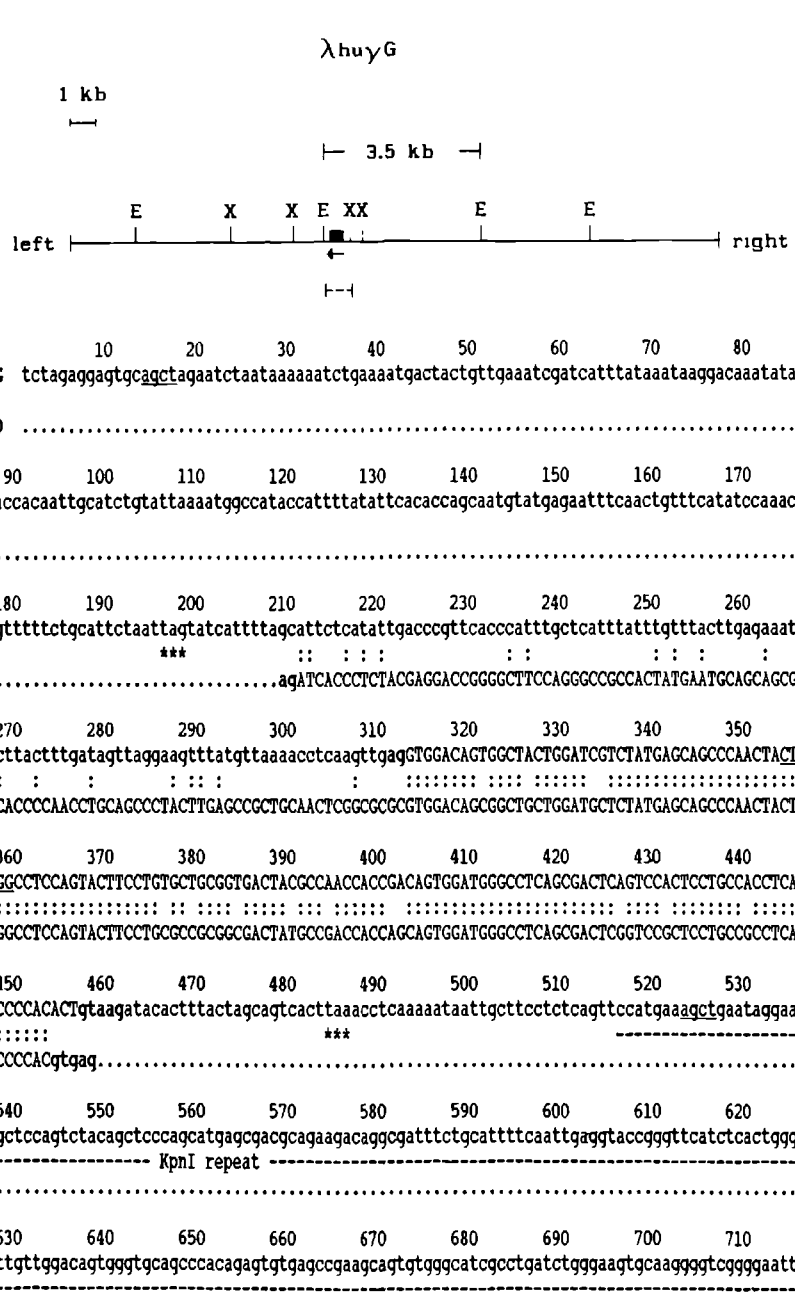
these two genomic fragments. The possibility that these fragments could be due to an EcoR I restriction fragment length polymorphism was excluded by screening Southern blots of genomic DNA digests of several individuals. In these experiments we did note a variable intensity of a 5.6 kb band which suggested that this fragment was due to partial digestion. Indeed, with an excess of restriction enzyme, the 5.6 kb fragment is split into the 4.0 kb fragment of the ψγF gene and the adjacent downstream 1.6 kb fragment (Fig. 2).

The 3.5 kb EcoR I fragment was neither polymorph nor partial, and therefore a genomic clone, which contained this fragment, was isolated (Fig. 3). The integrity of this clone was confirmed by comparing its Hind III and Pst I as well as its EcoR I sites with those in the genome. Sequence analysis of the hybridizing region of the 3.5 kb EcoR I fragment revealed that it contained a 285 bp open reading frame of which 141 bp (from nucleotide 312 to 453; Fig. 3) are closely similar to the 3' half of the second exons of the γD, ψγE and ψγF crystallin genes. This γ-crystallin like sequence was denoted the γG gene fragment. The γG gene fragment is preceded by **-ag** and followed by **-gt**, i.e. consensus splice acceptor and donor sites. Note that the putative 5' splice donor site of this gene fragment is shifted one triplet downstream relative to the second exons of the γ-crystallin genes.

Transcription of the human γG gene fragment

The sequence structure of the γG gene fragment indicated that it could be an exon of a larger gene, and the resemblance with the γD, γE and γF genes suggested transcription in lens tissue. We therefore assayed for a transcript containing this fragment in human lens RNA, using S1 nuclease protection. The only protected fragment which could be detected, however, appeared to be derived from cross-hybridization of the probe with γD RNA. A 17 mer oligonucleotide, spanning the last 17 bp of the putative γG exon, was used for differential hybridization of a Northern blot, containing 20 μg human embryonic lens RNA, but no transcripts from the γG gene fragment could be detected.

As the direct assays for a γG containing transcript failed, we designed a model system to test whether the γG gene fragment possesses the necessary sequence elements to function as an exon. To this end, a 514 bp Alu I fragment containing the γG gene fragment (flanked at the 5' end by 298 bp and at the 3' end by 68 bp) was inserted in either orientation in the large intron of the γD gene. All sequences required for splicing should be retained in this construct (see for example Wieringa *et al.*, 1984). The γD gene promoter was replaced with the SV40 promoter (see Fig. 1 and Materials and Methods), and the constructs were transfected into COS cells.


λhuγG
1 kb
3.5 kb
E X X E XX E E
left
right
10 20 30 40 50 60 70 80
γG tctagaggagtgcagctagaatctaataaaaaatctgaaaatgactactgttgaaatcgatcatttataaataaggacaaatataa
γD .....
90 100 110 120 130 140 150 160 170
aaccacaattgcatctgtattaaaatggccataccattttatattcacaccagcaatgtatgagaatttcaactgtttcatatccaaacc
.....
180 190 200 210 220 230 240 250 260
tgtttttctgcattctaattagtatcattttagcattctcatattgacccgttcacccatttgctcatttatttgtttacttgaqaaata
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CCACCCCAACCTGCAGCCCTACTTGAGCCGCTGCAACTCGGCGCGCGTGGACAGCGGCTGCTGGATGCTCTATGAGCAGCCCAACTACTC
360 370 380 390 400 410 420 430 440
GGGCCTCCAGTACTTCCTGTGCTGCGGTGACTACGCCAACCACCGACAGTGGATGGGCCTCAGCGACTCAGTCCACTCCTGCCACCTCAT
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450 460 470 480 490 500 510 520 530
CCCCCACACTgtaagatacactttactagcagtcacttaaacctcaaaaataattgcttcctctcagttccatgaaagctgaataggaac
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cttgttggacagtgggtgcagcccacagagtgtgagccgaagcagtgtgggcatcgcctgatctgggaagtgcaaggggtcggggaattc
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Figure 3. (top) Physical map of the λhuγG clone. The clone was isolated as described in Material and Methods. A schematic representation of the insert of this clone with the EcoR I (E) and Xba I (X) restriction sites are shown. Vector arms are indicated with 'left' and 'right'. The region of sequence similarity with γ-crystallin sequences is indicated with a black box. The putative direction of transcription is indicated by an arrow. The sequence of the 0.7 EcoR I/Xba I fragment is given at the bottom.
(bottom) Sequence similarity of the γG gene fragment with part of the second exon of the γD gene. The γD sequence is taken from Meakin *et al.* (1985). The sequence of the 0.7 kb EcoR I/Xba I fragment, containing the γG gene fragment, was determined by the dideoxy method on both strands. Identical residues are indicated by a colon. (Putative) Exon sequences are capitalized. The putative splice sites of the γG gene fragment, and the splice sites bordering the second exon of the γD gene, are indicated with bold letters. The Alu I and Ava I restriction sites used to construct the pSVγD/γG+ and pSVγD/γG- clones are underlined. The stopcodons bordering the open reading frame of the γG sequence are indicated with asterisks. Homology of the sequence with a Kpn I repeat is indicated by a dashed line.

RNA was isolated and analyzed by Northern blotting and hybridization with a second exon probe. If the γG gene fragment functions as an exon, then the transcript from the construct containing the γG gene fragment in the correct orientation (pSVγD/γG+) should be 144 nt longer than the transcripts from the construct pSVγD/γG- (γG gene fragment inserted in the inverted orientation) or those from the parental clone, pSVγD. However, all three constructs yielded transcripts of the same length (data not shown), suggesting that the γG sequence is not recognized as an exon.

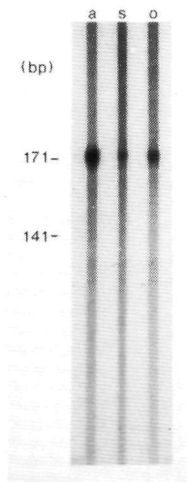


Figure 4. S1 analysis of the splicing products of the pSVγD/γG transcripts. RNA isolated from COS cells transfected with **o**: pSVγD (parental clone), **a**: pSVγD/γG- (γG fragment inserted in the **a**nti-sense orientation), **s**: pSVγD/γG+ (γG fragment inserted in the **s**ense orientation), was hybridized with a continuously labeled probe (see Material and Methods) encompassing part of the second exon and intron of the γD gene. The hybrids were treated with S1 nuclease and analyzed on a sequencing gel. A sequence ladder was used as length reference. The length of the protected fragment of the second exon of the γD gene, 171 nt, is designated with an arrow. The γG sequence, which appeared to be absent from the mature transcript, ought to yield a 141 nt protected fragment. Even when the autoradiogram was overexposed no 141 nt fragment could be detected.

To confirm the correct splicing of the γD second exon, the transcripts were characterized further by a S1 nuclease protection experiment, using a second exon fragment of the γD gene as probe. This probe should protect a 171 nt fragment from the γD transcript, and, by cross-hybridization, a 141 nt fragment of the γG sequence,

if present. In all experiments only the 171 nt fragment was found (Fig. 4), showing that the γG sequence is not spliced into the mature transcript, although the second exon of the γD gene is correctly spliced to the third exon. As a control, we have cloned βB2-1 and βB2-2 exons into the same EcoR V restriction site of the γD gene. These exons were spliced into the mature transcript with an efficiency of 100%, although the RNA yield was lowered by at least five fold as compared to the RNA yield of the original pSVγD (manuscript in preparation). Therefore we conclude that the γG gene fragment is not a functional exon in this model system, and most likely also not in its natural environment. The γG sequence is merely a silent remnant of a γ-crystallin gene. The γG fragment has remained linked to the γ-crystallin gene cluster as Den Dunnen *et al.* (1985a) have shown that all human EcoR I fragments containing γ-crystallin sequences co-segregate.

The transcriptional activity of the human γ-crystallin genes

If the human γ-crystallin genes are differentially expressed during development, as are the rodent genes (Murer-Orlando *et al.*, 1987; Van Leen *et al.*, 1987b), then the determination of the relative levels of the various γ-crystallin transcripts at any one stage of development could lead to an over- or underestimate of the activity of a particular gene.

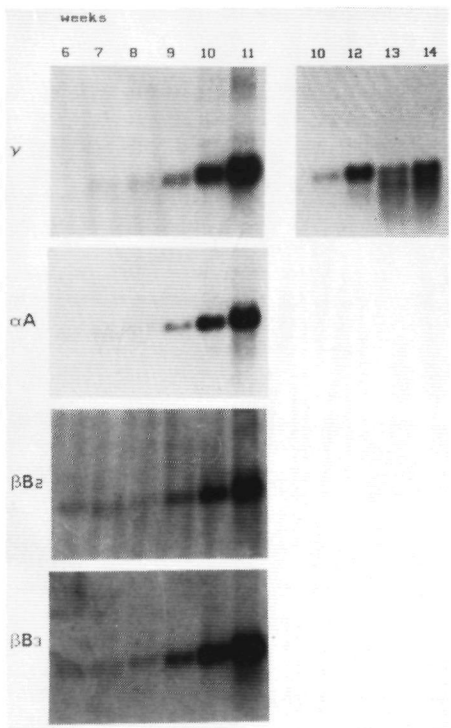
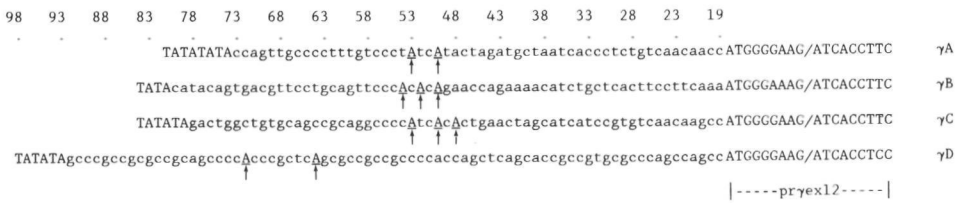


Figure 5. Northern blot analysis of crystallin transcripts in human lenses. (left panels): Autoradiograms of a Northern blot containing RNA isolated from human embryonic lenses of different ages. The blot was hybridized successively with an αA-, a βB2-, a βB3- and a γ-crystallin probe. Each lane contains one quarter of the total RNA isolated from one lens. **(right panel)**: Autoradiogram of a Northern blot containing RNA isolated from 10, 12, 13 and 14 weeks old embryonic lenses hybridized with the γ-crystallin probe. The broadening of the γ-crystallin RNA band between 12 and 13 weeks is due to the appearance of γ-crystallin transcripts with a shorter poly-A tail (unpublished data).

Hence, before the *in vivo* activity of the four possibly functional γ-crystallin genes, the γA, γB, γC and γD genes, could be measured quantitatively, it was necessary to determine the pattern of expression of the γ-crystallin genes during human lens development. Therefore, lens RNA was isolated from different developmental stages, Northern blotted and hybridized with a γ-crystallin probe as well as, for comparison, with various α- and β-crystallin probes.



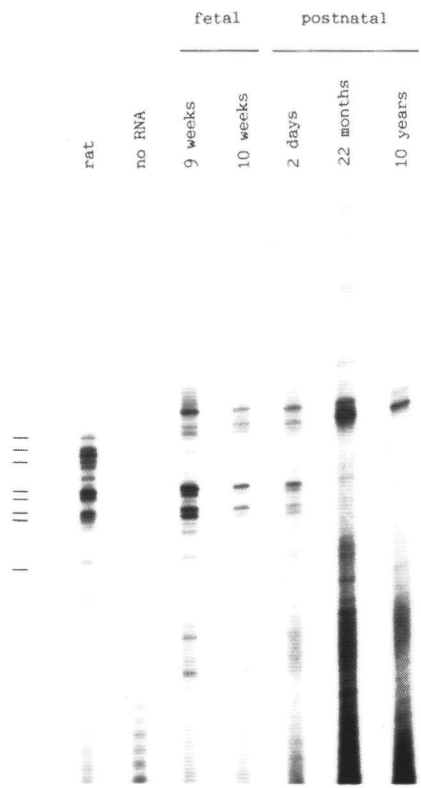


Figure 6. (top) Transcription initiation sites of four human γ-crystallin genes. The TATA box and the coding region complementary to the oligonucleotide prγex12 (see Material and Methods) are capitalized. The transcription initiation sites as determined by S1 nuclease mapping for the γC and γD genes (data not shown) or as assigned by analogy to the orthologous rat genes (Van Leen *et al.*, 1986) are indicated by arrows. The numbering indicates the lengths of the expected primer extension products shown at the bottom and starts at the 3' end of the region complementary to prγex12. **(bottom/right) γ-crystallin primer extension patterns obtained with RNA from human lenses at various developmental stages.** The source of the RNA is indicated at the top of the figure, where fetal and postnatal refer to human lenses from the corresponding developmental stage. Rat lens RNA was isolated from newborn rats. Oligonucleotide prγex12 was used as primer. The primer extension products were analyzed on a 9% polyacrylamide gel. Exposure times were 16 hrs for the part containing the rat and human fetal lens RNA primer extension products, and 72 hrs for the part containing the human post-natal lens RNA primer extension products. The bars at the left represent the rat extension products, from top to bottom: 63, 60, 58, 54, 53, 51, 49 and 43 nucleotides long (Van Leen *et al.*, 1986).

At the earliest stage examined, 6 weeks of embryonic development, no transcripts from the γ-crystallin genes could be detected, although transcripts from the αA-, βB2-, and βB3-crystallin genes were already present (Fig. 5, left panels). One week later, γ-crystallin RNA could be detected, and the total amount of γ-crystallin RNA increased rapidly up to 14 weeks, the oldest prenatal stage examined (Fig. 5, right panel). The Northern blot experiments provide no information about the composition of the γ-crystallin RNA pool. To determine whether the composition of this pool changes during development, an 18 mer oligo-nucleotide, complementary to a common sequence in the first and second exons of all γ-crystallin genes, was hybridized with and extended on total lens RNA. Since the length of the 5' non-coding region differs between the γ-crystallin transcripts (Fig. 6), the pattern obtained is diagnostic of the composition of the γ-crystallin RNA pool. As shown in Fig. 6, no changes in the primer extension pattern with age are seen in embryonic and neonatal human lens RNA. Between 2 days and 22 months after birth, however, a loss of the smaller primer extension products is found, and from 22 months of age on only the 65 nt product, derived from the γD gene, remains. Hence, in the human lens, as in the rat lens, differential expression of the γ-crystallin genes depends not on differential gene activation (or transcript accumulation), but on differential down regulation.

Quantitation of the *in vivo* γ-crystallin transcript levels

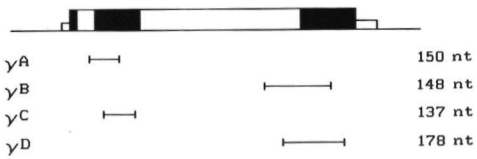
The combined Northern blot and primer extension data show that the relative contribution of the various γ-crystallin genes to the lens RNA pool can be assessed on embryonic lens RNA. Using S1 nuclease analysis, the transcripts of the γA, γB, γC and γD genes can be distinguished and their levels quantified. The probes used in these experiments are outlined in Fig. 7, left. The lengths of the protected fragments are expected to be: for the γA gene 150, for the γB gene 148, for the γC gene 137 and for the γD gene 178 nucleotides.

Using 0.5 μg of total lens RNA, transcripts of the γA, γC and γD genes but not the γB gene were detected (Fig. 7, right, left panel). Only when 2 μg of RNA was used, could a transcript of the γB gene be perceived (Fig. 7, right, right panel; Note that a trace amount of the γD probe (178 bp protected fragment) was added also in this experiment as an internal control). Measurement of the amounts of protected fragments showed that the γD transcripts accounted for 58% of the γ-crystallin RNA. The γC gene contributed about 23%, the γA gene about 14% and the γB gene a mere 5% (Table 1).

The promoter activity of the human γ-crystallin genes

The contribution of the various γ-crystallin genes to the RNA pool of human lenses

thus differs widely. These differences could be due to a variation in promoter activity or to a different stability of the transcripts.



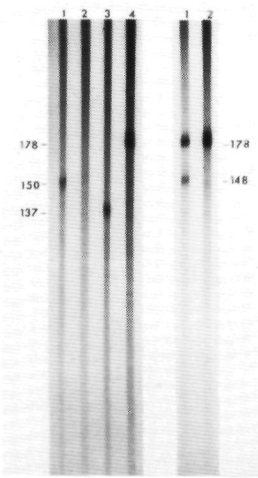


Figure 7. (left) Schematic representation of the M13mp clones used for quantitative S1 nuclease protection assays. A γ-crystallin gene is drawn schematically at the top. Black bars correspond to coding sequences, open bars to intron sequences. The small open bars correspond to the 5' and 3' non-coding regions.
The M13mp clones of the various genes are represented with a line. The expected protected fragment lengths are indicated in nucleotides.
(right) Quantitative S1 analysis of human embryonic lens RNA.
left panel: Continuously labeled M13mp probes of the γA (lane 1), γB (lane 2), γC (lane 3) and γD (lane 4) genes were hybridized with 0.5 μg of human embryonic lens RNA. The hybrids were treated with S1 nuclease and analyzed on a 6% polyacrylamide gel. The lengths of the protected fragments of the γA, γC and γD probes are indicated in nucleotides.
right panel: lane 1: S1 analysis of 2 μg of human embryonic lens RNA with the continuously labeled γB-crystallin clone as probe (148 nt protected fragment). A trace amount of labeled γD clone was added as control (178 nt protected fragment). lane 2: S1 analysis of 0.5 μg of human embryonic RNA with the continuously labeled γD probe as standard for quantitation.

A straightforward method to distinguish between these possibilities is to perform run-on assays. However, for obvious reasons, such experiments cannot be performed with human lenses. Hence, we took recourse to another approach, and assayed the promoter activity of the upstream regions of the four active γ-crystallin genes in transient expression experiments. To this end, the upstream regions were cloned in front of the CAT reporter gene, and the constructs were transfected into cells which support lens-specific gene expression (Peek *et al.*, 1990), i.e. transdifferentiating chicken neural retina cells (CNR cells). All four upstream regions promoted a significant CAT activity when inserted in the positive orientation (Fig. 8A), only the γC promoter region promoted a CAT activity in the negative orientation as well (Fig. 8C).

The tissue-specificity of the γ-crystallin promoter regions was tested by transfecting the same constructs into mouse fibroblasts, COS cells, and primary cultures of chicken embryonic heart muscle. With the exception of the γB promoter (Fig. 8B), all promoters, including the negative orientation of the γC promoter, showed an activity of 10% or less compared with their activity in the transdifferentiating chicken neural retina cells. The γB promoter was active in all tissue culture cells tested, and has apparently lost its tissue-specificity.

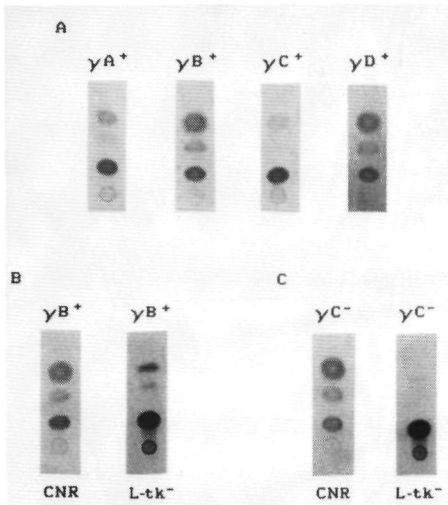


Figure 8. Representation of the promoter activity of the human γ-crystallin genes.
(A): Promoter sequences were cloned in front of the CAT reporter gene, transfected into chicken neural retina cells (CNR) and the CAT activity was assayed (see Material and Methods). The amount of acetylated [^{14}C]-chloramphenicol was quantified by densitometric scanning of autoradiograms and the values are depicted in Table 1. Only the CAT activity of the clones with positive oriented promoter sequences are shown. Autoradiography was performed for 16 hrs.
(B): Autoradiograms of CAT assays of cell extracts of chicken neural retina cells (CNR) or mouse fibroblasts (Ltk-) transfected with the γB+ CAT clone. Note the activity of the γB+ promoter in Ltk- cells, indicating the loss of tissue-specificity. Autoradiography was performed for 16 hrs (CNR cells) or 5 days (Ltk- cells).
(C): Autoradiograms of CAT assays of similar cell extracts transfected with the γC- CAT clone. Note the absence of activity of the γC- promoter in Ltk- cells, demonstrating the tissue-specificity of this aberrant promoter sequence. Autoradiography was performed for 16 hrs (CNR cells) or 5 days (Ltk- cells).

	RNA content		prom. act. (% γD)	
gene	% total	% γD	CNR	RLE
γA	14	25	26	17
γB	5	8	130	50
γC	23	40	10	45
γD	58	100	100	100

Table 1. Quantitation of the RNA composition of human lenses, and the promoter activity of the corresponding genes. The amount of γA, γB, γC and γD transcripts was determined by densitometric scanning of the autoradiograms obtained in the S1 analysis, and corrected for the amount of dAMP in the various probes. RNA levels are given relative to total RNA content (column 1) and relative to the amount of γD transcripts (column 2). Relative promoter activity (prom. act.) was assayed by transfecting CAT clones into transdifferentiating chicken neural retina cells (CNR, column 3) or rat lens epithelial cells (RLE, column 4). Quantitation of the promoter activity was performed by liquid scintillation counting of acetylated [^{14}C]chloramphenicol, or by densitometric scanning of autoradiograms. All values are corrected for the amount of protein in the cell extracts. Values are given relative to the activity of the γD-CAT construct.

The promoter activity of the various genes in transdifferentiating chicken neural retina cells, calculated relative to the γD promoter, is depicted in the third column of Table 1. These data show that the relative levels of the γA and γD transcripts found *in vivo* can be explained by the relative transcriptional activities of the cognate genes. The activity of the γB promoter, however, is too high, and that of the γC promoter too low, to explain the *in vivo* RNA levels of the corresponding genes.

Analysis of the aberrant γC promoter activity in CNR cells

The measurements of the γC promoter activity in the transdifferentiating chicken neural retina system could be influenced by the bidirectional nature of the promoter sequence, thereby causing inaccurate values. We attempted to inactivate the negatively oriented promoter by truncating the insert with 152 bp at its 5' end. Unexpectedly, this deletion did not abolish the negatively oriented promoter activity, but only lowered it by two fold. Furthermore, it did not affect the promoter activity of the plus orientation (Fig. 9A).

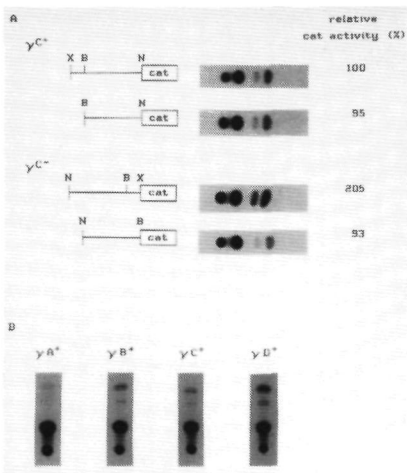


Figure 9. (A) Bidirectional promoter activity in the γC promoter. Promoter sequences are represented by a line, the CAT gene by an open box. Restriction sites are given by a X (Xba I), N (Nco I) or B (Bgl II). Promoter activities were quantified as described in the legend of Table 1, and are given relative to the original (Xba I/Nco I) γC+ CAT clone. Autoradiography was performed for 16 hrs. **(B) Representation of the promoter activity of human γ-crystallin genes in rat lens epithelial cells.** γA+, γB+, γC+ and γD+ γ-crystallin CAT clones were transfected into rat lens epithelial cells, and the CAT activity was assayed as described in Materials and Methods. Autoradiography was performed for 3 days.

We did not attempt to shorten the γC region further, as mapping of the rodent γ-crystallin promoters (Lok *et al.*, 1989; Peek *et al.*, 1990) indicates that in that case we would cut into the γ-crystallin promoter itself. An alternative explanation for the relatively low promoter activity of the γC upstream region could be that, although the transdifferentiating embryonic chicken neural retina cells support lens-specific expression, these cells do not always recognize the same sequence motifs as are recognized *in vivo*. For instance, the relative activity of the rat γC promoter region is much higher in mouse lens epithelial cells than in transdifferentiating chicken neural retina cells (Peek *et al.*, 1990). To determine the effect of cell culture system on the activity of the human γ-crystallin promoters, the human γ-crystallin promoter constructs were transfected into rat lens epithelial cells, and the CAT activity in the cell lysate was determined. The positive orientation of the γC promoter region showed a markedly higher activity in this system, being 45% relative to the γD promoter (Fig. 9B; Table 1, column 4). [The activity of the negative orientation of the γC promoter decreased to 33% relative to the γD promoter activity, data not shown.] These results thus strongly indicate that the human, as well as the rat, γC promoter is poorly recognized in CNR cells. Taken together, these data demonstrate that the relative levels of the γA, γC and γD transcripts found *in vivo* can be explained by the relative transcriptional activities of the cognate genes.

Analysis of the γB RNA level

The γB promoter is highly active in all tissue culture systems used. Either our measurements of the activity of the γB promoter bear no relevance to the *in vivo* situation, or the very low *in vivo* γB RNA level is due to rapid RNA turn-over. Scanning of the γB sequence revealed that the human γB mRNA is the only known γ-crystallin mRNA to contain multiple copies of the sequence AUUUA in its 3' non-coding region. This sequence motif is known to be involved in rapid messenger breakdown (Brawerman, 1989). To test the hypothesis that the turn-over rate of the γB transcript is much higher than that of, for example, the γD transcript, recourse was taken again to the COS cell model system. The γB gene was cloned behind the SV40 promoter (pSVγB) and, to analyse the role of the third exon sequences, the third exons of the γD and the γB gene were swapped in pSVγD (see Fig. 1, right). The RNA yield from this chimeric pSVγD/γB construct was compared with that from the parental pSVγD clone as well as that from the pSVγB construct. As illustrated by Fig. 10, the yield of γB RNA was 18% of that of γD RNA. The chimeric pSVγD/γB construct yielded only 8% of the amount of RNA yielded by pSVγD, indicating that the low yield of γB RNA is due to its third exon sequences. As the γB, γD and the chimeric γD/γB genes in these

experiments were driven by the same (SV40) promoter, and as all constructs were expressed in the same cells, the difference in RNA yields must be due to post-transcriptional processes, most likely destabilization mediated by the 3' end of the γB RNA. The discrepancy between the *in vitro* γB promoter activity and the *in vivo* γB RNA level strongly suggests that the same mechanism operates in the human lens.

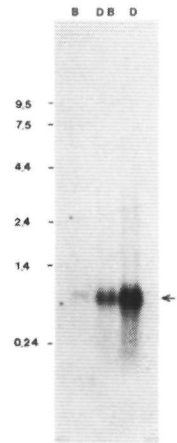


Figure 10. Yield of the γB-crystallin transcript in COS cells. The pSVγB (lane B), pSVγD/γB (lane DB), and the pSVγD (lane D) clones were transfected into COS cells. RNA was isolated, separated on a 1% agarose-formaldehyde gel, and transferred to nitrocellulose. The blot was hybridized with a second exon probe of the γD gene. Note that this probe hybridizes relatively poorly to the second exon of the γB gene. The bands were visualized through autoradiography and quantified by densitometric scanning. The strategy to quantify the RNA yields of the various constructs is described under Material and Methods. On the left, fragment lengths of an RNA reference are denoted in kilobases. The γ-crystallin transcripts are designated with an arrow.

DISCUSSION

The contention that the common phylogenetic ancestor of man and rat had six active γ-crystallin genes rests upon the finding that there is no detectable sequence similarity between the large second introns of the rat genes, while the sequence similarity between orthologous human and rat introns is about 65% (Den Dunnen *et al.*, 1989). Hence, the evolutionary distance between the rat genes must be larger than the distance between man and rat, and the genesis of the six membered gene family must have preceded the separation of the primates and rodents. The orthologous relationship between the rat and human γ-crystallin genes was established from comparison of gene and gene flanking sequences (Aarts *et al.*, 1988). For five of the six genes, viz. the γA through γE genes, the common evolutionary ancestry was unambiguous. A problem arose, however, with the assignment of the γF genes: both in man and in rat the exons of the γE and γF genes are nearly identical in sequence, but only in man does the sequence identity extend into the second intron (Meakin *et al.*, 1985), suggesting that the human γE/γF pair is more closely related than the rat γE/γF pair. On the basis of our present results, we now argue that the real orthologue

of the rat γF gene in the human genome was the γG gene. As we have shown here, the γG gene fragment preserved in the genome is closely related in sequence to the 3' part of the second exons of the γD (90%), ψγE (88%) and ψγF (88%) genes [The second exons of these genes evolve in concert by extensive gene conversions, and are nearly identical in sequence (Lubsen *et al.*, 1988)], which is in agreement with the proposed orthology. The assumption that the γG is the orthologue of the rat γF gene would also explain the sequence similarity between the introns of the human ψγE and ψγF genes, since these two genes would then have resulted from a recent gene duplication in the primate lineage. The alternative would be to assume continued gene conversions across the introns, a region that in the rat genes is clearly not contained within the gene conversion unit (Den Dunnen *et al.*, 1986). Furthermore, we have traced the γG gene fragment in primates as far distant from man as the African Green monkey, showing that the γG gene was present during early primate evolution. Thus, we suggest the following evolutionary scenario for the human γ-crystallin gene family: the common progenitor of man and rat had six active γ-crystallin genes. During subsequent evolution of the primate lineage, one of these genes, the proto-γF gene, was lost, leaving only the inactive one-quarter gene fragment, the γG fragment. A second gene, the γE gene, was inactivated by a nonsense mutation, and subsequently duplicated to yield the equally inactive ψγF gene. Four active γ-crystallin genes then remained.

In the human lens only two of these genes, the γC and γD genes, yield abundant protein (Siezen *et al.*, 1987). By comparing the *in vivo* RNA level with the *in vitro* promoter activity, we have attempted to determine at what level the activities of the γA and the γB genes have been suppressed. The results of the promoter studies must be interpreted with caution, as *in vitro* promoter activity is not necessarily a correct reflection of *In vivo* promoter activity. The 5' flanking sequences tested may not contain all sequence elements required for maximal promoter activity. In addition, the difference in the relative promoter activity of the γC gene in rat lens epithelial cells and chicken transdifferentiating neural retina cells demonstrates that the promoter activity can be dependent upon the cell system used. The transdifferentiating chicken cells express the endogenous crystallins (Okada *et al.*, 1975; De Pomerai *et al.*, 1977) and specifically recognize the rat γ-crystallin promoters (Peek *et al.*, 1990), but chickens lack γ-crystallin. Hence, in the case of the human γ-crystallin promoters, we would tend to rely more on the data obtained using the rat lens epithelial cell cultures, since these cells do express the orthologous endogenous genes (Kraft *et al.*, manuscript in preparation). Our data then support the hypothesis that the difference in levels of γA,

γC and γD RNA is primarily determined by a difference in the rate of transcription of the cognate genes.

The promoter studies show further that the γB promoter has lost its tissue-specificity. One explanation for this observation could be that the γB region has gained a binding site for an ubiquitous transcription factor. We have scanned the promoter sequence for such a site but could detect only a minimal NF1 consensus sequence (GGCAA). Alternatively, the γB gene may have lost a silencer, active in non-lens tissues. Evidence for such an element has been found in studies of the rat γD promoter (Peek *et al.*, 1990). Finally, this result could be an artifact of the promoter constructs or cell systems used. The *in vivo* activity of the γB gene in tissues other than lens will depend, of course, not only on the specificity of the promoter but on the accessibility of its chromosomal domain as well.

The promoter studies do predict that γB RNA would be abundant in the human lens. However, only trace amounts of this transcript were found to be present. The expression of the γB sequence in COS cells was also poor, even though the gene was driven by the SV40 promoter. As the presence of the 3d exon of the γB gene was sufficient to cause a low RNA yield, it is likely that this region of the γB transcript destabilizes the γB RNA, probably mediated by the AUUUA motifs. A high rate of turn-over would then account for the low *in vivo* levels of γB RNA.

A second unexpected finding was the tissue-specific bidirectional activity of the γC promoter region. We were unable to silence the upstream anti-sense promoter by removing 152 bp of upstream sequences, although the deleted region contained a putative TATA box. However, in the remaining part two putative TATA boxes are still present. As these two boxes are located only 127 and 245 bp upstream from the authentic γC TATA box, it is quite possible that the tissue-specificity of the anti-sense promoter is steered by the same elements that steer the γC promoter itself. Previously, the promoters of the human γA, γC and γD genes have shown to be active in chicken lens explants (Meakin *et al.*, 1987). Using this system the authors also noted the bidirectional activity of the γC promoter, but found, in contrast to our results, that the activity of the negative orientation was not tissue-specific. We have no explanation for this discrepancy. Our results predict that an anti-sense transcript of the γC upstream region is present in the human lens. In Northern blot experiments we were unable to detect transcripts using sequences proximal to the γC promoter as probe. We did detect a transcript from sequences further upstream. At this time we are trying to define this transcription unit.

The mode of developmental regulation of the γ-crystallin genes in the human lens

is similar to that in the rat lens. All genes are activated simultaneously during early development but are differentially down regulated during later stages. There are two subtle differences, however, between the human and rat γ-crystallin gene expression pattern. The onset of the expression of the human γ-crystallin genes is somewhat delayed, which would result in a lower γ-crystallin concentration in the lens nucleus, and the last γ-crystallin gene to be active in the human lens is the γD gene, while the γB gene is the last one that remains active in the rat lens. This change in expression pattern is surprising, as the γD protein is predicted to have a high phase-transition temperature. The γ-crystallin cryoproteins are thought to be particularly adapted to a low hydration, and are expected to be prominent in the lens nucleus (Siezen *et al.*, 1988). It is possible that the choice between γB- or γD-crystallin is influenced by the spatial distribution of the other classes of crystallins, the α- and β-crystallins. Unfortunately, extensive studies of the expression pattern of the α- and β-crystallins are available for the rat lens only.

The studies on the activity of the human γ-crystallin genes presented here, together with previous studies on the rat γ-crystallin genes, reveal that complex changes at different steps in the flow of genetic information have taken place in this family during the evolution of man, most likely favoured by a diminished need for γ-crystallins in the human lens. The genes have been inactivated by simple deletion (the γG gene) or, more subtly, by a non-sense mutation (the ψγE and ψγF genes). The overall accumulation of the γ-crystallins has been lessened by delaying the activation of the genes during development, while the contribution of the individual genes has been altered by a down mutation in the γA promoter, and by a high turn-over rate of the γB messenger. The human γ-crystallin gene family thus provides an unique example of the many ways in which the activity of a gene family is modulated during evolution.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. R. Peek for the assistance with the rat lens epithelial cell cultures and the construction of the γB supercat clone, and C. van Alebeek for technical assistance. This work has been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (NWO).

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CHAPTER 4

part B

CHIMERIC cDNA CLONES: A NOVEL PCR ARTIFACT

The results described in chapter 4, part B, were published in
Nucl. Acids Res. **19**, 1949 (1991).

RESULTS AND DISCUSSION

During the cloning of a transcript of one member of a closely related gene family, the human γ-crystallin gene family (Brakenhoff *et al.*, 1990), we encountered a novel artifact of the polymerase chain reaction: the formation of chimeric cDNA molecules. Our experimental strategy in cloning the human γE-crystallin transcript was a common one (Sambrook *et al.*, 1989): first strand cDNA synthesis on human lens RNA using AMV reverse transcriptase and a γE specific primer followed by PCR with the same γE specific primer as reverse primer and a common γ-crystallin forward primer (see Fig. 1; the sequence similarity between the γ-crystallin genes precludes the synthesis of a gene-specific 1st exon primer). The resulting fragment was purified and cloned into M13 mp vectors. Sequencing of three of these clones, however, revealed that two were chimeric, switching from either the γC or γD sequence to the γE sequence in the 3d exon. The third one contained a correct γE transcript (Fig. 1). These chimeric sequences could have resulted from somatic recombination or trans-splicing but are more likely an experimental artifact. Since the chimeric clones end with γE sequence, the initial reverse transcription reaction must have been specific for the γE transcript. However, we had noted that reverse transcription often yielded prematurely terminated γE cDNAs. We reasoned that such partial γE cDNAs could have hybridized to the γC or γD transcripts (which are 10 or 25 fold, respectively, more abundant than the γE transcript; unpubl. data, Brakenhoff *et al.*, 1990) and served as primer for reverse transcription by Taq polymerase (Tse & Forget, 1990). As the 5' PCR primer fits the γC and γD sequences as well, such chimeric molecules would have been amplified in the PCR reaction. To test this hypothesis we repeated the experiment but included a RNase A treatment after first strand synthesis. Five out of five recombinant clones contained the correct γE transcript and no chimeric clones were found. We thus conclude that the synthesis of the chimeric cDNA clones is a PCR artifact caused by the reverse transcriptase activity of Taq polymerase. Hence, this reverse transcriptase activity is actually a drawback rather than an advantage during cDNA cloning.

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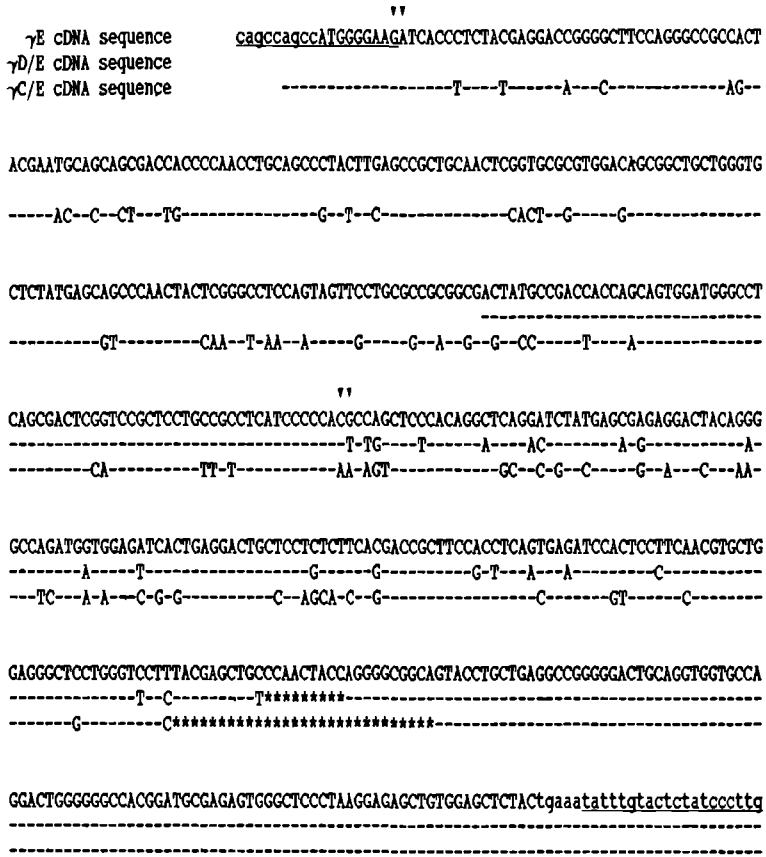

Figure 1. The sequence of the chimeric γE cDNA clones.
The γE cDNA sequence is shown in full. For the γD/E or γC/E sequences only differences are specified. The sequence of the γD/E cDNA was determined only in part. The regions where the sequences of the γD/E or γC/E cDNA clones switch from those of the γD or γC genes to that of the γE gene are indicated with asterisks (note that due to the sequence identity the exact 'cross-over' point cannot be determined). Coding sequences are capitalized. The primers used for cDNA synthesis and PCR are underlined.

CHAPTER 5

ACTIVATION OF A γ-CRYSTALLIN PSEUDOGENE IN A HUMAN HEREDITARY CATARACT

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submitted for publication.

ABSTRACT

The locus for the hereditary human "Coppock-like" cataract is characterized by an unique combination of polymorphic Taq I sites within the human γ-crystallin gene cluster. Mapping of these sites shows that the recombination event that created this novel assortment must have occurred in a 15 kd region encompassing the γD and ψγE gene. The γD and ψγE genes from the mutant "Coppock-like" cataract chromosome were cloned and characterized. The γD gene was functionally equivalent to its allele cloned from a wild-type chromosome. A cluster of sequence changes was found within and around the TATA box of the ψγE gene. Together these cause a ten-fold increase in the activity of the ψγE promoter, raising the level of expression of this gene to 30% of that of the γD gene. Expression of the protein product of the ψγE gene, a 6 kD N-terminal γ-crystallin fragment, in transgenic *Xenopus laevis* tadpoles causes an increase of lens opacity. These data suggest that reactivation of a pseudogene is the cause of the "Coppock-like" cataract.

INTRODUCTION

Transparency of the eye lens is dependent upon the spatial order of the abundant water-soluble proteins in the differentiated fibre cells, the crystallins (Delaye & Tardieu, 1983). The three dimensional structure of the crystallins is highly conserved, suggesting that the structure of these proteins is a crucial parameter of lens function (reviewed by Lubsen *et al.*, 1988). Nevertheless, there is only limited evidence that cataract can be caused by a change in the structure or expression of a crystallin gene. Two rodent cataract mutations are known in which a crystallin is affected: βB2-crystallin in the Philly mouse and ζ-crystallin in the guinea pig (Russell & Chambers, 1990; Borras *et al.*, 1990). Although it is very likely that these crystallin mutations are indeed the cause of the cataract, formal proof, i.e. genetic linkage of the crystallin gene with the cataract locus, is still lacking. Strong linkage between a crystallin gene and a cataract locus is found in man: the locus for the "Coppock-like" cataract co-segregates with the human γ-crystallin gene cluster. The "Coppock-like" cataract affects only the part of the lens formed during embryogenesis, at the time in which the γ-crystallin genes are active. The phenotype of this cataract thus supports the suggestion that it is caused by a lesion in the γ-crystallin genes (Lubsen *et al.*, 1987).

The human γ-crystallin genes are clustered on chromosome 2. The gene family consists of six genes (γA through γF, γE and γF are pseudogenes; Den Dunnen *et al.*, 1985; Meakin *et al.*, 1985) and a quarter gene fragment (γG; Brakenhoff *et al.*, 1990), of which only two, the γC and γD genes, encode abundant protein (Siezen *et al.*,

1987). A γ-crystallin gene probe detects three polymorphic Taq I fragments, the A, B, and C fragments, on Southern blots of human DNA (Lubsen *et al.*, 1987). As each of these three fragments has two alleles, eight haplotypes are possible within the γ-crystallin gene cluster. Only three of these have been detected in normal individuals: Q (A1/B2/C2), R (A2/B1/C1) and S (A2/B2/C1). A fourth haplotype, P (A1/B2/C1), marks the "Coppock-like" cataract locus. We have previously suggested that the recombination or gene conversion event which created haplotype P might be responsible for the genetic lesion in the chromosome carrying the "Coppock-like" cataract locus (Lubsen *et al.*, 1987). We show here that the site of recombination is most likely located directly upstream from the ψγE gene. The sequence changes at this site have resulted in a ten-fold increase in the activity of the ψγE promoter. Over-expression of the truncated γ-crystallin fragment encoded by the ψγE gene causes increased lens opacity in a model system.

MATERIALS AND METHODS

Isolation and sequencing of the alleles of the γD and ψγE gene

Using standard methods (Sambrook *et al.*, 1989) genomic libraries in λEMBL3 (Promega) were prepared from patient DNA. The libraries were screened either with a [^{32}P]-labeled 3.5 kb Hind III fragment containing the human ψγF gene or with the [^{32}P]-labeled 0.3 kb EcoR I/Pst I fragment containing part of the second intron of the ψγF gene. Positive clones were plaque purified and characterized by restriction mapping. Appropriate restriction fragments were subcloned in M13 mp vectors and sequenced by the dideoxy chain termination method.

RNA isolation, Northern blotting

Isolation of human total lens RNA or COS cell RNA, Northern blotting and hybridization was performed as previously described (Brakenhoff *et al.*, 1990). To specifically detect the transcript of the ψγE gene, the 25 mer oligonucleotide GGATCCAAGGGATAGAGTACAAATA, prhγE, was used. Of this sequence, 20 bases are complementary to a sequence in the 3' non-coding region of the third exons of the ψγE and ψγF genes, the 5 additional bases at the 5' end create a BamH I restriction site. The number of mismatches in the 20 bp sequence with the four active γ-crystallin genes, the γA, γB, γC and γD genes (Brakenhoff *et al.*, 1990), are 14, 10, 12 and 12 respectively (Den Dunnen *et al.*, 1985; Meakin *et al.*, 1985; Meakin *et al.*, 1987). Hybridization of a 20 mer oligonucleotide ACTCTATCATCTGGCCTCTG, prhγD, complementary to a sequence in the third exon of the human γD gene, was used as standard for quantification. This oligonucleotide contains 8 mismatches with the γA

gene, 8 mismatches with the γB gene, and 5 mismatches with the γC gene (Den Dunnen *et al.*, 1985; Meakin *et al.*, 1985; Meakin *et al.*, 1987). Hybridizations were performed in 6x SSC, 2.5x Denhardt's solution, 0.1% SDS, 10 μg/ml denatured salmon sperm DNA and 10 μg/ml yeast ribosomal RNA, at 42 °C for 16 hours. The blots were washed three times in 6x SSC at room temperature, and once in prewarmed 6x SSC at 42.0 °C. Autoradiography was performed on Kodak X-AR 5 film using intensifying screens.

Production of anti-ψγE antiserum

The 0.7 kb Nco I (blunt)/BamH I ψγE cDNA fragment was ligated into a Sma I/BamH I pGEX-2T vector, and transformed to *E. coli* HB101. Fusion proteins were isolated by binding to glutathione-agarose beads (Sigma) as recommended by the supplier. Two New Zealand rabbits were immunized with 600 μg fusion protein emulsified in Freund's complete adjuvant, and boosted three times at three weeks intervals with 250 μg fusion protein emulsified in Freund's incomplete adjuvant. Reactivity of the antiserum was tested on Western blots using standard methods (Sambrook *et al.*, 1989).

CAT constructs, cell culture, transfection and CAT assay

The γD promoter/CAT fusion constructs were made by ligating 0.6 kb Taq I/Nco I fragments into the Acc I/blunt-ended Hind III pCAT vector as described (Brakenhoff *et al.*, 1990). The ψγE promoters were cloned as 1.5 kb Kpn I/Nco I fragments into pCAT. Nco I sites, which contain the translation initiation codon, were removed by mung bean nuclease digestion. Cells were cultured, transfected and assayed for CAT activities as described (Brakenhoff *et al.*, 1990). All transfection experiments using CAT fusion genes were done in duplo and repeated at least once with a different batch of DNA.

Construction and analysis of the pSVγD and pSVψγE clones

The P and Q alleles of the γD gene were placed behind the SV40 promoter as previous described (Brakenhoff *et al.*, 1990). To clone the ψγE genes behind the SV40 promoter, the 4.4 kb Hind III/EcoR I fragment of pCH110 (Pharmacia) was first replaced by an mp10 polylinker. 6.5 kb Nco I (filled in with Klenow)/Sal I fragments containing the mutant (P) or wild type (S) allelic ψγE genes were inserted into the Hind III (blunt-ended)/Sal I vector. The pSVγD and pSVψγE clones were transfected into COS cells as described (Brakenhoff *et al.*, 1990).

Over-expression of the ψγE product in *Xenopus* eye lenses

A 0.4 EcoR I/Nco I fragment containing the mouse γF promoter was ligated into the EcoR I/Nco I digested ψγE/pUC19 clone (P allele), thereby replacing the ψγE promoter

by the mouse γF promoter. 250 pg of supercoiled plasmid DNA was injected into fertilized Xenopus eggs according to Brakenhoff *et al.* (1991a). Four weeks after fertilization (stage 52; Nieuwkoop & Faber, 1956) the tadpoles were sacrificed and the amount of light backscattered by the isolated eyes was measured. Details of the lens opacity meter used will be presented elsewhere (Brakenhoff *et al.*, submitted).

RESULTS

Mapping of the polymorphic Taq I sites

The locus for the "Coppock-like" cataract is characterized by a novel combination (haplotype P) of polymorphic Taq I sites within the γ-crystallin gene cluster. If there is a causal relationship between the genetic event that gave rise to the variant haplotype P and the occurrence of a lesion in a γ-crystallin gene, then the affected γ-crystallin gene must be located between the polymorphic Taq I restriction sites. To map these sites, the polymorphic Taq I fragments were first assigned to specific γ-crystallin genes by probing Southern blots of genomic DNA with gene-specific and exon-specific probes.

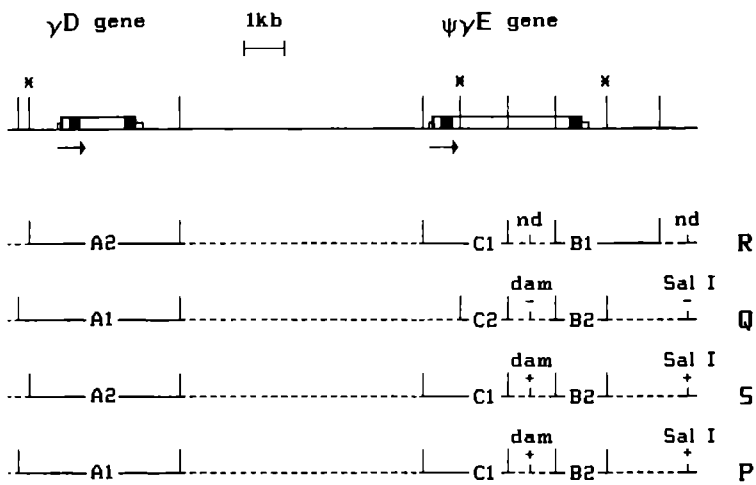

Figure 1. Physical map of part of the human γ-crystallin gene cluster.
The region containing the γD and ψγE genes is shown schematically. The heavy bars indicate the (putative) coding sequences, open bars denote introns, small open bars indicate non-coding exon sequences. The location of the Taq I sites yielding the polymorphic fragments is indicated by vertical bars, asterisks denote polymorphic Taq I sites. The haplotypes are indicated as defined in Lubsen *et al.* (1987). Other polymorphisms are indicated as: dam-/dam+, the absence or presence of a dam methylation sensitive Taq I restriction site; Sal I-/Sal I+, the absence or presence of a Sal I restriction site; nd indicates that the state of a particular site was not determined.

The polymorphic Taq I sites were then localized by restriction mapping of the corresponding genomic clones. These experiments showed that the A1/A2 fragments contain the γD gene, with the A2 polymorphic Taq I site located upstream of the gene.The B1/B2 and the C1/C2 fragments both derive from the gene located directly downstream from the γD gene, the ψγE gene. The C2 polymorphic site flanks the second exon of this gene, while the third exon is flanked by the B2 polymorphic site (Fig. 1). During the course of these experiments we noted two additional, haplotype-specific, sequence polymorphisms, i.e. a 3' polymorphic Sal I site and a variation in the *dam* methylation of an intronic Taq I site (Fig. 1). From the distribution of sequence polymorphisms in the various alleles we conclude that the P haplotype resulted from a recombination between the Q and S haplotypes, which must have occurred in the region between the polymorphic 5' A2 Taq I site and the polymorphic 5' C2 Taq I site.

Analysis of the γD gene of the P haplotype

The region in which the recombination must have occurred contains the γD gene and the first half of the ψγE gene. As the γD gene is the most active γ-crystallin gene in man (Brakenhoff *et al.*, 1990), it was the prime candidate for the cataract locus. We cloned the γD gene from the P haplotype chromosome and compared its properties to those of the γD gene cloned from a wild type (Q) chromosome. The sequence of the coding region of the γD(P) gene differed at two sites from that of the γD(Q) gene, both located at the third codon position and yielding synonymous codons. Hence, the encoded amino acid sequence of the γD(P) gene is identical to that of the γD(Q) gene.

Seemingly innocuous sequence changes can cause aberrant splicing (Collins & Weismann, 1984). To determine whether such sequence changes are present in the γD(P) gene, the splicing of the transcript was examined. As the diseased tissue is not available, recourse had to be taken to a model system. The γD(P) or, as control, the γD(Q) promoter was replaced by the SV40 promoter and the fusion constructs were transfected into COS cells. The mature transcripts of both SV40/γD constructs were of the predicted size. In addition, Western blotting showed that both SV40/γD constructs encoded a protein with the same properties as γ-crystallin (data not shown). Thus there is no cause to suspect that a mutation has affected the splicing of the γD(P) transcript.

Finally, we tested whether the γD(P) promoter was active. The promoter was fused to the chloramphenicol acetyltransferase gene and the activity of this fusion gene was assayed in transdifferentiating chicken neural retina cells and rat lens epithelial cells, cell systems known to recognize lens-specific promoters. The γD(P) promoter was as

active as the γD(Q) promoter (data not shown).

As all assayed properties of the γD(P) gene are identical to those of the γD(Q) gene, we conclude that the "Coppock-like" cataract is not caused by a lesion in the γD gene.

Analysis of the ψγE gene

The second gene contained within the region of recombination is the ψγE gene. This gene is known as a pseudogene because of the presence of an in-frame stop codon in the second exon (Meakin *et al.*, 1985). However, inspection of the sequence shows that the ψγE gene still has all necessary sequence elements for transcription and transcript processing (in contrast, the ψγF gene lacks a proper promoter region and contains a mutated splice site as well; Meakin *et al.*, 1985). If the ψγE gene is indeed transcriptionally active in the lens, then a change in this gene could conceivably be the cause of the cataract phenotype. Hence, we examined the expression of the ψγE gene in normal embryonic human lenses by Northern blotting. Oligonucleotide prhγE, which is complementary to a sequence in the 3' non-coding region of the ψγE gene, was used to detect the ψγE transcript specifically. For comparison, a Northern blot of human total lens RNA was hybridized with the γD specific oligonucleotide prhγD (Fig. 2, left).Both oligonucleotides hybridized with RNA molecules of about 0.8 kb long. From the difference in hybridization intensity we estimated that the ψγE transcript is 30 times less abundant than the γD transcript in the normal human embryonic lens. To show that these ψγE transcripts contain the predicted sequence, cDNA was prepared by the polymerase chain reaction, cloned and sequenced (Brakenhoff *et al.*, 1991b). All clones obtained had the coding sequence predicted from the genomic clone. We thus conclude that the ψγE gene is active and yields a properly spliced transcript.

Presence of the ψγE protein *in vivo*

A properly spliced ψγE mRNA should encode a protein of approximately 6 kD, containing the N-terminal region of a γ-crystallin. In heavily overloaded Coomassie Blue stained SDS gels of human lens extracts several small proteins are detectable (data not shown). To demonstrate that these proteins are γ-crystallin fragments, we raised antibodies to the ψγE γ-crystallin fragment by immunizing rabbits with a ψγE(P)/glutathione-S-transferase fusion protein (see Materials and Methods). This antiserum reacted with two proteins (of 12 and 8 kD) in the water soluble fraction of the embryonic human lens (Fig. 2, right). Although these data are suggestive of the presence of a ψγE protein in the normal lens, we cannot formally exclude the possibility that we are detecting degradation products of the γC and γD proteins, in spite of their reported absence from the embryonic lens (Zigler *et al.*, 1981).

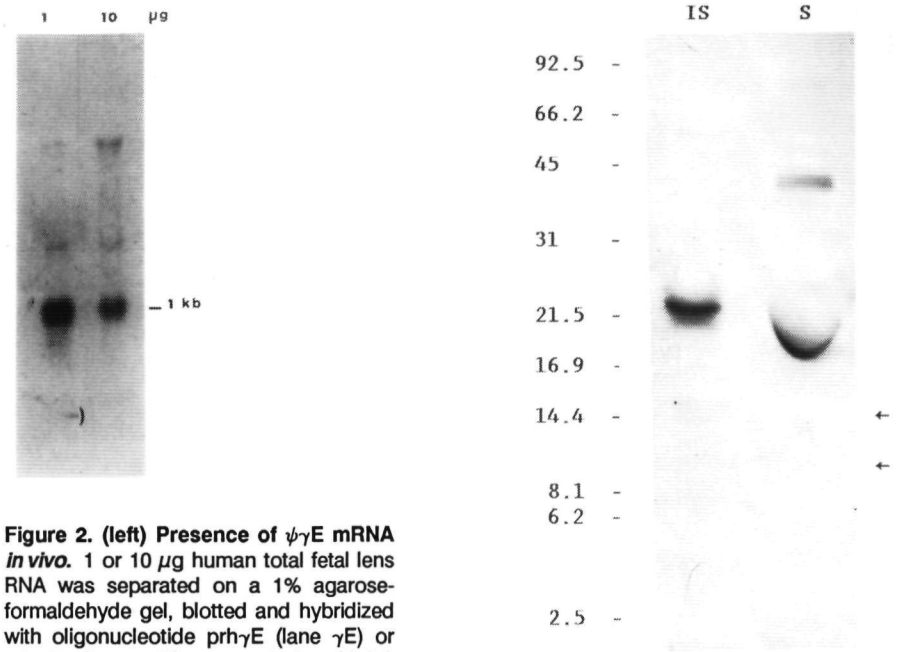

Figure 2. (left) Presence of ψγE mRNA *in vivo*. 1 or 10 µg human total fetal lens RNA was separated on a 1% agarose-formaldehyde gel, blotted and hybridized with oligonucleotide prhγE (lane γE) or prhγD (lane γD) respectively. **(right) Presence of the ψγE protein *in vivo*.** 20 µg of water-soluble (S) or water-insoluble (IS) proteins from fetal human lenses were boiled for 5 min. in cracking buffer, loaded on a tricin SDS-PAGE gel (Schägger & Jagow, 1987), electrophoresed and blotted to nitrocellulose (Sambrook *et al.*, 1989). The blot was stained with anti-ψγE antiserum, raised against the pGEX/γE-cDNA fusion protein, using standard procedures. The antiserum reacts with the human γ-crystallins as well as (faintly) with 12 and 8 kD polypeptides (arrows). The molecular weight of the protein standard is given on the left in kilodaltons.

The ψγE gene of the P haplotype

To investigate the involvement of the ψγE gene in the "Coppock-like" cataract phenotype, the gene was cloned from the P and S haplotype chromosomes and functionally characterized in the same manner as the γD(P) gene (see above). The predicted sequence of the ψγE(P) polypeptide differs at two positions from that of the S allele (pos. 35 Ala -> Val; pos. 43 Val -> Met; note that the Ala -> Val change is also found in the cDNA sequence). The in-frame stop codon is preserved in the P allele. The sequence changes are unlikely to affect the processing of the transcript of the ψγE(P) as the SV40/ψγE(P) fusion gene yielded a properly sized transcript upon transfection to COS cells. Hence we conclude that the transcript of the ψγE(P) gene is functionally equivalent to that of the ψγE wild type gene.

During sequence analysis of the promoter region of the ψγE(P) gene, we noticed a striking cluster of sequence changes. The promoter sequence of the ψγE(P) gene has an altered TATA box: in this gene the characteristic TATATA of the γ-crystallin genes has been restored while the ψγE(S) as well as the published ψγE(Q) sequence contain a TATACA sequence.

```
        S allele       tcgaaagct ttagagagtc taaaagaaaa catacaataa acctcccacc cccaccccta cagtcattat
        P allele       --------- ---------- ---------- ---------- ---------- ---------- ----------
        γE cDNA

atggaacaga gagagagaga aaaaaaaatg cccttgctcc cctccgggggg ccccttttgt gcggttcttg ccaacacagc agccctcctg
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                                 ▼         ▼
cTATACAgcc ccgccgcgcc gccgcccCac ccgctCagcc ccgccttgcg cccagcc agc cATGGGGAAG gtgagcccag cccgcgcccc
-----T---- --------tg *--------- ---------- -----g---- -------g--- ---------- ---------- ----------
                                                       ----- --- ----------

ccggtagctc cctgctaggc gggggccgga gactggggca ggggcaggcc tgtgagacct cgccttgcct cgccttgcct tgcagATCAC
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                                                                                           -----

CCTCTACGAG GACCGGGGCT TCCAGGGCCG CCACTACGAA TGCAGCAGCG ACCACCCCAA CCTGCAGCCC TACTTGAGCC GCTGCAACTC
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GGCGCGCGTG GACAGCGGCT GCTGGGTGCT CTATGAGCAG CCCAACTACT CGGGCCTCCA GTAGTTCCTG CGCCGCGGCG ACTATGCCGA
--T------- ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ----------
--T------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

CCACCAGCAG TGGATGGGCC TCAGCGACTC GGTCCGCTCC TGCCGCCTCA TCCCCCACgt gagtgcagtc ccgccggtcc cttccgtgcc
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cgcgagtcgc atcagtgatc cacgtggatg attcacacga caggcgatgg gatgcgatgg caggcttctt
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ttttctggct ctaactatac tctcttttcc tttattaaca tgcgtaaggt ttcctcccac tgaaaaagta
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Figure 3. Sequence of the P allelic ψγE gene. The sequence of the S allele from the 5' Taq I site to the polymorphic 2nd intron Taq I site (see Fig. 1) is shown completely, for the mutant P allele and the cDNA sequence (taken from Brakenhoff *et al.*, 1991b) nucleotide identity is indicated by dashes and only sequence differences are specified. The single bp deletion within the promoter of the P allelic ψγE gene is indicated with an asterisk. Putative coding sequences and the TATA box are capitalized. The TAG stopcodon in the second exon is underlined. The predicted transcription initiation sites are capitalized and indicated with arrows.

In addition, the ψγE(P) promoter region has a 2 bp substitution/1 bp deletion directly downstream from the TATA box (see Fig. 3). To determine whether these sequence

changes affect the activity of the ψγE promoter, the promoter of either the P or the S allele was used to drive expression of the chloramphenicol acetyltransferase gene in rat lens epithelial cells. The γD/CAT construct was transfected in a parallel experiment for comparison. The result of the CAT assays is shown in Figure 4. Quantification of the results indicated that the promoter of the mutant allele is 10 times more active than the promoter of the wild type gene, reaching about half the level of the γD gene. Such a high promoter activity would turn the ψγE transcript level from low abundant into high abundant. The level of the ψγE polypeptide would be expected to increase accordingly.

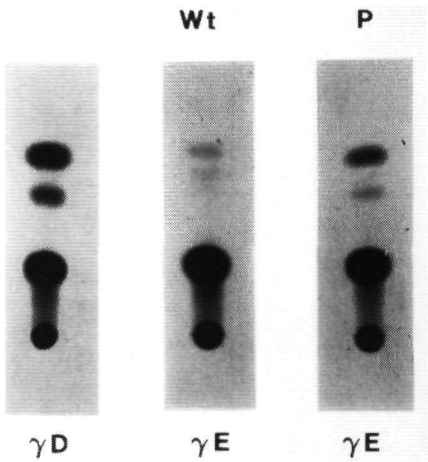

Figure 4. Promoter activity of the P allelic ψγE gene.
Promoter/CAT fusion constructs of the P allele (lane γE, P) or the S allele (lane γE, Wt) of the ψγE gene were transfected into rat lens epithelial cells, and the amount of CAT activity in the cell lysates was determined as described in Brakenhoff *et al.* (1990). A γD/CAT fusion construct (γD) was transfected in a parallel experiment as a reference.

Effect of ψγE expression on transgenic *Xenopus laevis* tadpoles lenses

If the up mutation of the ψγE gene promoter is causally related to the cataract phenotype, then over-expression of the ψγE product must affect eye lens transparency. To test whether this is the case, *Xenopus laevis* was chosen as a model system. We have shown recently that rodent γ-crystallin promoters can target expression to the eye lens of *Xenopus laevis* (Brakenhoff *et al.*, 1991a). To express the ψγE polypeptide in tadpole lenses, we linked the human ψγE gene to the mouse γF promoter and injected the fusion construct into fertilized *Xenopus* eggs. The tadpoles were sacrificed after 4-5 weeks of development (stage 52, according to Nieuwkoop & Faber, 1956). The eyes were isolated and lens transparency was measured with a high sensitivity lens opacity meter (Brakenhoff *et al.*, submitted). The results of one such experiment are depicted in Fig. 5. The amount of light backscattered is markedly increased (30%) in all assayed transgenic tadpoles. We tentatively conclude from these data that the presence of the ψγE product can indeed cause changes in eye lens opacity.

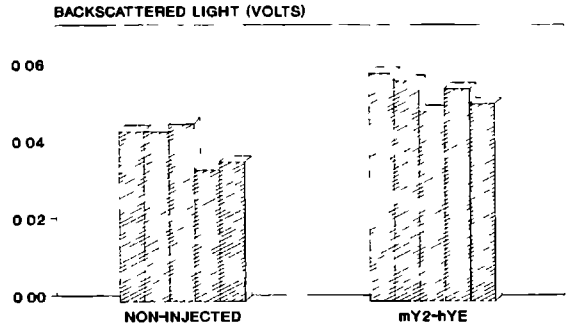

Figure 5. Effect of ψγE expression on eye lens opacity in *Xenopus* tadpoles. 250 pg of supercoiled plasmid DNA of the mouse γF promoter/human ψγE (P allele) fusion construct was injected into fertilized *Xenopus* eggs. The lenses of stage 52 (Nieuwkoop & Faber, 1956) animals were isolated and their transparency was measured by the lens opacity meter as described by Brakenhoff *et al.* (in prep.). The amount of backscattered light is expressed in Volts.

DISCUSSION

Our strategy in the search for the molecular lesion of the "Coppock-like" cataract was based upon the hypothesis that the recombination event which led to the characteristic P haplotype, was also the cause of the phenotypic effects. The cluster of sequence changes within the ψγE promoter could well be the footprint left by the chromatin breakage and reunion which accompanies the exchange of genetic information between chromosomes (Szostak *et al.*, 1983). Why these sequence changes should cause the promoter to become more active is not clear. Considering the range of sequence variation within human TATA boxes (Penotti, 1990), the change from TATACA to TATATA is unlikely to be significant unless it causes a change in response to regulatory elements. Mammalian TATA boxes have been shown to be heterogeneous in this respect (Wefald *et al.*, 1990). The sequence changes directly downstream of the TATA box border a putative Sp1 binding site and could affect the affinity of Sp1 for this site. Addition of a Sp1 site has been shown to increase the activity of a mouse γ-crystallin promoter (Lok *et al.*, 1989).

The ψγE gene was denoted as a pseudogene merely on the basis of the presence of an in-frame stop codon. Our data show that the gene is not transcriptionally inactive and that it yields a properly processed and translatable mRNA. Transcriptionally active pseudogenes have been described in other gene families as well (Moschonas *et al.*, 1981). To our knowledge, however, this is the first example of a reactivated

pseudogene associated with a phenotypic disease.

Crystallographic studies of the γ-crystallin structure have shown that protein is folded into four 'Greek key' motifs which are organized into two domains. The protein strands are intercalated such that the fourth strand of the 'Greek key' of the first motif actually derives from the second motif and vice versa (Summers *et al.*, 1986). The ψγE protein product lacks the fourth chain to complete the motif structure, and is therefore unlikely to be folded correctly. Our scenario for the cause of the "Coppock-like" cataract is then that an up mutation in the promoter of the ψγE gene results in over-expression of the ψγE protein. The high levels of this improperly folded, incomplete single motif protein would disturb the supramolecular organization of structural proteins in the lens.

ACKNOWLEDGEMENTS

The authors would like to thank E. Jacobs for technical assistance. This work has been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (NWO) as well as the E.E.C. [science program, contract no. SC1-0320-C (GDF)].

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CHAPTER 6

TRANSGENIC *XENOPUS LAEVIS* TADPOLES:

A TRANSIENT *IN VIVO* MODEL SYSTEM FOR THE MANIPULATION OF

LENS FUNCTION AND LENS DEVELOPMENT

The results described in this Chapter were published in
Nucl. Acids Res. **19**, 1279-1284 (1991).

ABSTRACT

Rodent γ-crystallin promoters were recognized as lens-specific promoters in micro-injected *Xenopus laevis* tadpoles and targeted the expression of the chloramphenicol acetyl transferase (CAT) reporter gene to the tadpole lens. The onset of expression coincided with lens cell formation. The level of expression continued to increase up to 9 days of development (stage 47), stayed at that level till at least day 13 and dropped by only 57% at day 21. In contrast, the level of expression of a non-tissue-specific promoter, the SV40 early promoter, decreased rapidly in the eye during development and was only detectable up to stage 44 (day 5). The stability of the CAT activity in the lens was assessed by delivering a pulse of activity from a heat shock promoter-CAT fusion gene. The half-life of the CAT activity in the eye was the same as that in the tail. The increase in CAT activity in the lens thus depends upon continued activity of the injected γ-crystallin promoters. Our data demonstrate that mammalian promoters can be used to target gene expression to specific tissues during *Xenopus laevis* development.

INTRODUCTION

Reverse genetics is one of the most powerful techniques for the study of biological function of gene sequences. Transgenic mice have been far the most popular vertebrate model system for this approach and have been used in such diverse studies as cell- and tissue-specificity of promoter sequences, effects of ectopic gene expression in development, and the consequences of cell loss by gene ablation (reviewed by Palmiter & Brinster, 1986; Landel *et al.*, 1990).

Micro-injected *Xenopus laevis* embryos are a second widely used vertebrate transgenic system (reviewed by Gurdon & Melton, 1981; Etkin & Pearman, 1987; Andres *et al.*, 1984; Etkin *et al.*, 1984; Giebelhaus *et al.*, 1988). The long generation time of *Xenopus* makes it unsuitable for the production of stable transgenic lines. Hence, only transient expression of the introduced genetic material during embryonic development can be followed. It is, however, this feature of the system that makes it attractive: as *Xenopus* embryos are free living animals, their development and the effects there on by the introduced genetic material can be readily investigated (McMahon & Moon, 1989; Hopwood & Gurdon, 1990). In addition, the ease with which *Xenopus laevis* embryos can be micro-injected makes it an attractive system to screen large numbers of foreign gene constructs for their effects. The majority of studies on the expression of foreign genetic material in *Xenopus laevis* embryos has made use of non-tissue-specific mammalian promoters. No targeting by mammalian promoter

sequences of the expression to a specific tissue in *Xenopus laevis* tadpoles has been reported. We show here that lens-specific rodent γ-crystallin gene promoters can be used to target expression to the tadpole lens up to at least stage 49 (according to Nieuwkoop & Faber, 1956). The system described here will be useful for the study of the function of genes involved in the development and specification of the properties of the lens. Our data also offer the promise that similar targeting by mammalian promoters to other tadpole tissues will be possible.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from Boehringer Mannheim, Amersham or Pharmacia. T4 DNA ligase was from Boehringer Mannheim and mung bean nuclease from Promega or Pharmacia. The reaction conditions were chosen as recommended by the supplier. Acetyl coenzyme A was obtained from Boehringer Mannheim and [^{14}C]chloramphenicol (57 mCi/mmol) from Amersham.

Methods

Construction of CAT plasmids

The mouse γF (formerly γ2) promoter cloned into the pSV0ATCAT vector was kindly donated by Dr. M. Breitman (Meakin *et al.*, 1989). The rat γD-pCAT construct was described previously (Peek *et al.*, 1990). The -75 to +75 deletion clone was generated by Apa I/Hind III digestion of γD-pCAT, mung bean nuclease treatment and religation. The HSP70-pCAT construct was made by insertion of the blunt-ended 0.5 kb BamH I/Hind III fragment of pBN247 (Tōrōk & Karch, 1980) into the Sma I site of pCAT (Peek *et al.*, 1990).

Preparation of plasmid DNA

Plasmid DNA was isolated by the alkaline lysis method, followed by RNase A treatment. The DNA was purified further by centrifugation through a NaCl cushion (1 M NaCl, 10 mM TRIS.HCl, 1 mM EDTA, pH 8.0) at 60,000g for 16 hrs at 15 °C (Sambrook *et al.*, 1989). Prior to loading the DNA solution was treated with 1% sarcosyl for 15 min at room temperature, and cleared by centrifugation for 10 min. The DNA was redissolved in 10 mM TRIS.HCl, pH 8.0.

Micro-Injection of *Xenopus laevis* embryos

Female frogs were injected with 500 IU pregnyl (Organon, Oss) 10 hrs prior to egg collection. Eggs were streaked from the females and collected in 100% MMR (**M**odified a**M**phibian **R**ingers' solution: 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 0.1 mM EDTA, 2 mM $CaCl_2$, pH 7.8). Eggs were fertilized in a minimal amount of fluid

with part of a testis, after 5 min overlaid with 25% MMR and allowed to stand for another 15 min. Embryos were dejellied in 1% L-cysteine, 25% MMR (pH set to 7.8 with NaOH). After extensive washing with 25% MMR to remove the cysteine, the embryos were placed on a plastic tray and injected within one hour after fertilization with approximately 10 nl DNA solution (usually supercoiled plasmid DNA at a concentration of 25 ng/μl), using glass capillary needles with a diameter of 10 μm. Embryos were cultured overnight in 2% Ficoll, 25% MMR at 22 °C. After 24 hrs the healthy embryos were transferred into aged tap water at 22 °C and carefully aerated. Tadpoles were staged according to Nieuwkoop & Faber (1956).

Histological analysis

Tadpoles were fixed in Bouin (saturated picric acid: 37% formaldehyde: glacial acetic acid = 75: 15 :10 v/v/v), dehydrated and embedded in paraffin. Sections (5 μm) were cut using a Reichert-Jung microtome, stained with haematoxylin and eosin, and photographed on 100 ISO Kodak film.

CAT assays

Tissues were dissected from sacrificed tadpoles in cold tyrodes solution (137 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 12 mM $NaHC0_3$, 5.5 mM glucose, pH 7.2) under a stereo-microscope. Lenses were prepared by squeezing isolated eyes through a syringe to which a needle with a diameter of 0.5 mm was attached. Tissues were homogenized in approximately 250 μl 0.25 M TRIS.HCl, pH 7.8. Tissue debris was removed by centrifugation. The CAT activity in the supernatant was determined as described earlier (Peek *et al.*, 1990). After autoradiography the amount of acetylated chloramphenicol was determined by densitometric scanning of the signal of 3-acetyl-chloramphenicol and comparison of the intensity with a serially diluted standard. The values were corrected further for the amount of protein in the lysate (BioRad protein assay). CAT activity was expressed arbitrarily as the amount of 3-acetyl-chloramphenicol, multiplied by 5.12, produced by 1 mg of protein extract.

Reproducibility of the results

Usually the tissues pooled from between 10 to 15 animals were used for an assay. In triple assays of 15 animals derived from one injection the mean deviation was found to be 25%, mainly due to variation in the volume (and thus the number of DNA molecules) micro-injected. Differences in the quality of the eggs further contributes to the large variation in absolute values sometimes found between different injection experiments. The relative values always yielded comparable results between various experiments. All quantitative comparisons reported here are based upon

measurements made within one injection of eggs from a single fertilization. Only normally developing animals were assayed. Usually about 50-80% of the embryos micro-injected with 250 pg of DNA developed normally.

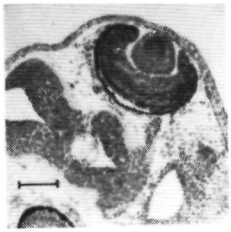
RESULTS

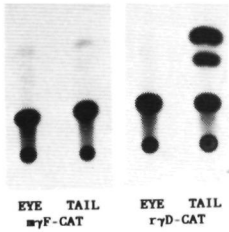
The expression of rodent γ-crystallin gene promoters during tadpole lens development

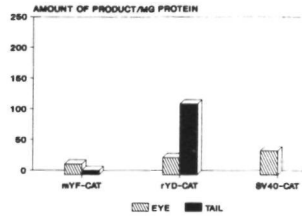
The rodent γ-crystallin gene promoters show strict tissue-specificity in transfection experiments: they are active only in (chicken or rodent) lens derived cells and inactive in all other cell types tested (Lok *et al.*, 1985; Peek *et al.*, 1990). To test whether these promoters can also drive lens-specific expression in *Xenopus laevis* tadpoles, CAT fusion constructs of the two strongest γ-crystallin gene promoters, the mouse γF and the rat γD promoters, were injected into fertilized *Xenopus* eggs. Eye and tail extracts of injected tadpoles were assayed for CAT activity at various time points.

The morphological course of lens development is shown in Figure 1. At the earliest stage examined (day 3, stage 38), the primary lens fibre cell mass has already formed. The lens fibre cells are not yet mature, however, as the cell organelles are still present in the inner cell mass. Two days later, the lens nucleus has clearly formed. The lens continues to grow (note that the lens grows by deposition of fibre cells on the outside), and the further maturation of the eye can best be seen by the morphological changes taking place in the retina. The expression of the micro-injected γ-crystallin promoters keeps pace with lens development. This is most clearly illustrated by the expression of the mouse γF promoter. As seen in Figure 1, expression from this promoter is low but detectable in eyes from 3 days old tadpoles. Two days later the expression from this promoter in the eye has increased about ten-fold. It remains at this high level till at least 13 days of development. Even at 21 days (stage 52) 43% of the activity present at day 10 still remained (Fig. 2; note the absence of activity in tail, gut or head-without-eyes in 21 day old animals). The results obtained using the rat γD promoter are somewhat less straightforward. In the eye this promoter shows an increase in activity between day 3 and day 5 followed by a reproducible dip at day 7, after which the activity increases sharply and remains high during the remainder of the assay period. The rat γD promoter appears to be very active in the tail as well during early development. We interpret the data from the γD construct as being the sum of two promoter activities: a non-specific one active during early development and a lens-specific one that becomes apparent in 7 day old tadpoles (see below).

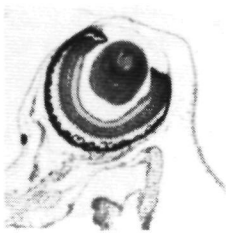
DAY 3
STAGE 37/38

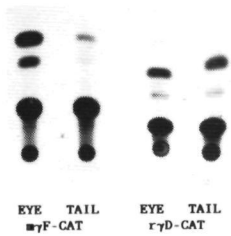


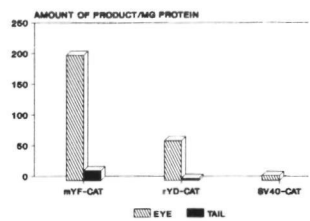




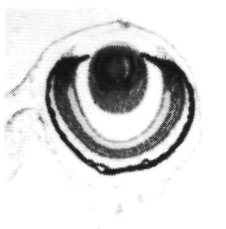
DAY 5
STAGE 44

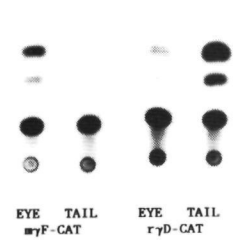


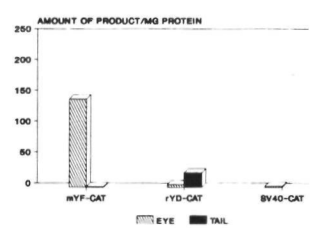




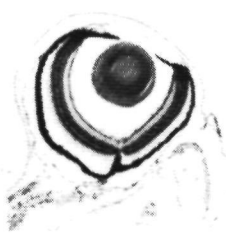
DAY 7
STAGE 46

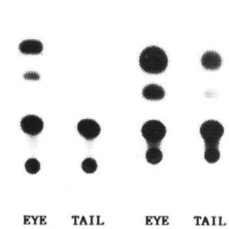


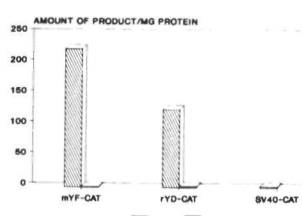




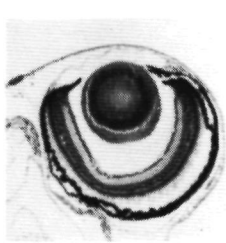
DAY 9
STAGE 47

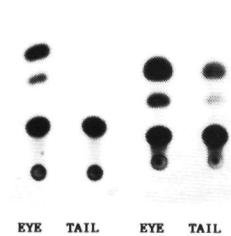


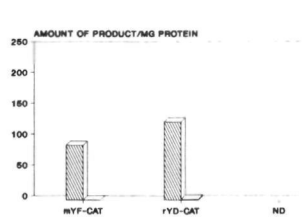




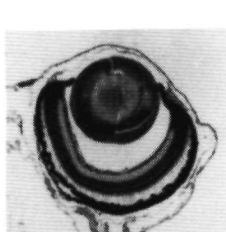
DAY 11
STAGE 48

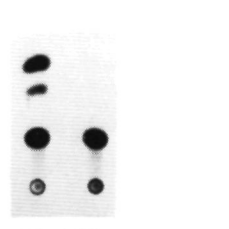






DAY 13
STAGE 49





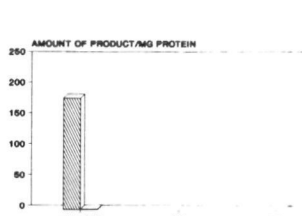
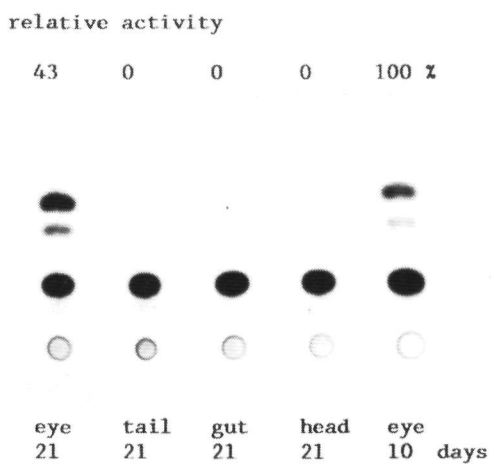


Figure 1. Tissue-specificity of expression of exogenous rodent γ-crystallin promoter-CAT constructs during lens development in *Xenopus laevis* tadpoles. In the left most column the stage, according to Nieuwkoop & Faber (1956), and the actual age of the tadpoles are given. In the second column photographs of 5 μm histological sections stained with haematoxylin and eosin are shown. The bar in the photograph of 3 day animals represents 100 μm. The magnification of all histological sections is the same. Note that the high protein concentration in the lens nucleus (present from day 5) causes shatter in the histological sections. In the central columns the results of the CAT assays of tail and eye extracts of animals of the same developmental stage, injected with 250 pg pSV0ATCAT-γF (mγF-CAT) or γD-pCAT (rγD-CAT), are shown. Autoradiography was performed for 16 hrs. Only in case of the CAT assay with tissue extracts of day 3 tadpoles injected with the mγF-CAT construct, was autoradiography performed for 64 hrs. The CAT activity was quantitated as described in Materials and Methods. In the right most column the amount of 3-acetyl-[^{14}C]-chloramphenicol/mg protein (see also Materials and Methods) is shown on the y-axis. On the x-axis the injected constructs are indicated. Hatched bars represent the CAT activity in eye extracts, solid bars that in tail extracts. For comparison, the CAT activity obtained in eye extracts of transgenic tadpoles injected with pSV2CAT (SV40-CAT) is shown also with hatched bars. The level of CAT activity in the eye fraction of day 3 animals injected with the rat γD-CAT construct was unusually low in this particular set. In other experiments the level of activity in eye extracts was comparable to that in tail extracts.

Figure 2. Tissue-specificity of the activity of a rodent γ-crystallin promoter in *Xenopus laevis*. Tissues [total head fraction (minus eyes), gut, tail and eye] were isolated from tadpoles, injected with 100 pg of the mouse γF-CAT construct (pSV0ATCAT-γF), after 21 days of development (stage 52), and the CAT activity was measured and quantitated as described in Materials and Methods.The levels of CAT activity are expressed as percentage of the activity measured in the eyes of similarly injected 10 days old tadpoles (stage 48/49), and shown at the top. The source of the tissues as well as the age of the transgenic tadpoles used are shown at the bottom.



The tissue-specificity of expression of the γ-promoters is emphasized when the expression profile of these promoters in the eye is compared with that of the SV40 promoter. The activity of this promoter shows a steady decrease in the eye with time and is virtually undetectable after day 7 (Fig. 1, histograms on the right).

Expression of micro-injected DNA is often mosaic. We therefore measured the CAT activity in single eyes isolated from 13 day old tadpoles (stage 49) injected with the rat γD-pCAT construct. The mean deviation between the activity of the left and right eye of a single animal was 13%, and the mean deviation between the activity of the eyes

of ten animals was 19% (data not shown). We thus conclude that the expression of the injected construct is about the same in each eye, but we cannot exclude mosaic expression within each lens.

The activity of the rat γD promoter is dependent on its TATA box

The data presented in Fig. 1 demonstrate that the 5' flanking region of a γ-crystallin gene directs expression to the eye. To show that it is indeed the γ-crystallin promoter that is required for lens-specific expression, we deleted the γD promoter from -75 to +75 (the CAT ATG is located at +112) from the γD-CAT construct. The pattern of expression of this deletion clone in micro-injected tadpoles was then compared with that of the parental clone. As is shown in Fig. 3, activity from the deletion clone was only detected during early development, no activity was found in the eye of 10 day old tadpoles. We thus conclude that the γ-crystallin promoter is required for lens-specific expression.

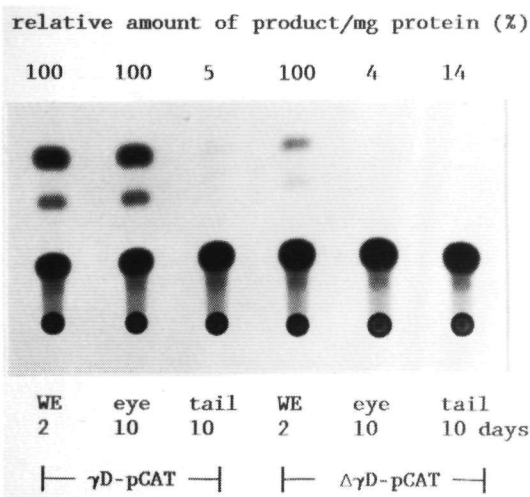


Figure 3. Dependency of the expression of the rat γD-crystallin promoter on the presence of the TATA box. Whole embryo lysates (we) or tail and eye extracts of tadpoles injected with 250 pg of the rat γD-pCAT or the rat γD-pCAT (ΔγD-pCAT) deletion constructs were prepared two (stage 32) or ten (stage 48) days after injection respectively, and assayed for CAT activity. The levels were quantitated and expressed as the percentage of the activity in the two days stage. Note that the activity of the deletion construct in the two days stage is 15% of that of the parental γD-pCAT construct.

The activity of the mouse γF promoter is restricted to the lens

For practical reasons, most assays were performed using tadpole eyes, which are

easily isolated, rather than lenses. To show that the activities measured do reflect the activity in the lens, the eyes were taken from 7 day old tadpoles micro-injected with the mouse γF construct. These eyes were further dissected to yield lens and eye-debris fractions. As shown in Fig. 4, about 90% of the CAT activity in the eye is contributed by the lens. Hence, within the eye, the mouse γF promoter activity is specific to the lens.

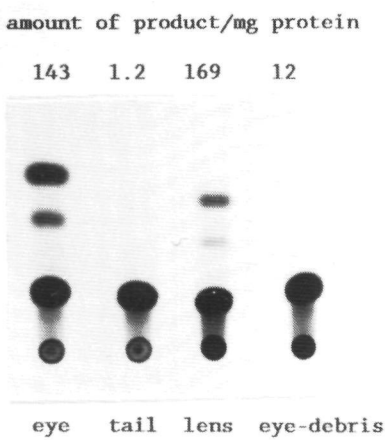


Figure 4. Lens-specificity of the activity of a rodent γ-crystallin promoter in transgenic *Xenopus laevis* tadpoles. Tail, eye, lens and eye-debris extracts of 7 days old tadpoles (stage 46) injected with 250 pg of the mouse pSV0ATCAT-γF construct were assayed for CAT activity and the levels quantitated as described in Materials and Methods. The amount of product/mg protein is indicated at the top.

The stability of the CAT activity in the eye

The level of CAT activity measured at any one stage of development is the sum of the increase in enzyme concentration by synthesis and the decrease in enzyme level by protein turnover as well as dilution due to growth. It is therefore difficult to estimate to what extent *de novo* RNA synthesis (i.e. promoter activity) is necessary to maintain the enzyme levels. To obtain an estimate of rate of decrease in enzyme levels in the absence of promoter activity, use was made of the inducible heat shock promoter. The CAT reporter gene was cloned behind this promoter, the construct was micro-injected and after 6 days the tadpoles were heat shocked for 60 min. at 30 °C. The decay of the CAT activity in time was then followed in tails as well as eyes. Although the heat shock induction was much lower in eyes than in tails, the rate of decay in both tissues was approximately the same (see Fig. 5). Since the HSP70 promoter is not lens-specific, both lens and non-lens tissues contribute to the CAT activity in the eye. However, when the activity in the lens and eye-debris fractions was determined separately, the contribution of the lens was found to be at least equal to that of the eye-debris fraction (data not shown). Therefore, we conclude that the maintenance of

the CAT activity in the lens after micro-injection of the γ-crystallin gene promoter-CAT fusion constructs is due to continued promoter activity.

The effect of the vector on the specificity of expression

We noted that deletion of the TATA box region of the γD promoter abolished CAT activity in the eye of day 10 tadpoles, whereas activity in the very young stages remained. This observation suggested that transcription starts within the pCAT vector cause CAT activity in these very young embryos. Injection of the (promoter-less) pCAT vector did indeed give rise to high CAT activity in stage 32 (day 2) but not in later stages (stage 44, day 5 and older; data not shown). If non-specific vector starts contribute significantly to the CAT activity, then differences in the vector used might also explain the apparently higher specificity of expression of the mouse γF promoter as compared to the rat γD promoter in young tadpoles.

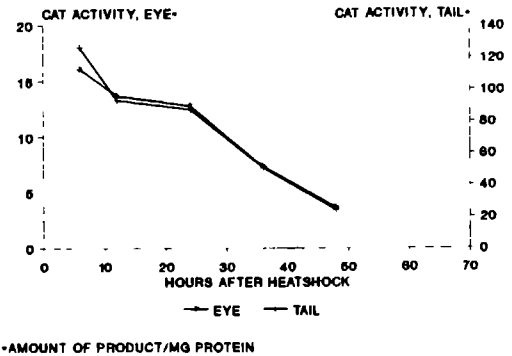


Figure 5. Determination of the stability of CAT activity in transgenic tadpoles. CAT activity in day 6 (stage 45) Xenopus tadpoles, injected with 250 pg of the HSP70-CAT construct, was induced by a heat shock from 22 °C to 30 °C for 1 hr. Animals were cultured further at 22 °C, sacrificed at various times (indicated in hrs on the x-axis) thereafter, and the eyes and tails isolated. The CAT activity in these fractions was assayed and the levels were quantitated. The amount of product/mg protein is indicated on the y-axes. The figures on the left y-axis represent the CAT activity in eye extracts (marked with *), and on the right y-axis that in tail extracts (marked with +). The CAT activity in the eyes of control animals (injected but not heat shocked) was found to be below the level of detection.

The mouse γF promoter was cloned in pSV0ATCAT, a derivative of pBR322, while the γD promoter was inserted into pCAT, a derivative of pUC12. The pSV0ATCAT vector contains two elements designed to minimize background transcription: poly-adenylation signals upstream from the promoter insert and stopcodons in all three reading frames between the promoter insert and the CAT coding sequence (Meakin *et al.*, 1989; see also Fig. 6). To test whether these features of the pSV0ATCAT vector were responsible for the apparently higher tissue-specificity of the expression of the pSV0ATCAT-γF construct, we constructed a derivative of the pCAT vector which

contains the stopcodon region, denoted p0CAT, and transferred the rat γD or the mouse γF promoter region to this vector. The activity of these constructs was assayed in tail and eye extracts of 3 day old micro-injected tadpoles. The eye/tail ratio (E/T) of the CAT activity/mg protein was taken as a measure of the tissue-specificity of the constructs. As indicated by this ratio, the use of the stopcodon region in the p0CAT vector did not increase the specificity of expression of the rat γD promoter relative to the parental pCAT vector. Moreover, use of the p0CAT vector decreased the apparent tissue-specificity of the mouse γF promoter relative to the pSV0ATCAT vector (Fig. 6). Comparison of the E/T ratios of both rodent promoters cloned in the same vector (p0CAT) revealed further that the specificity of the mouse γF and rat γD promoter appears to be about equal.

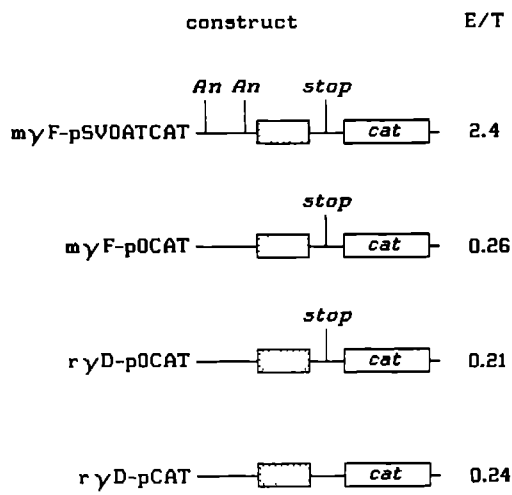


Figure 6. Non-tissue-specific expression of various γ-crystallin CAT constructs in young developmental stages. The names and schematic drawings of the micro-injected constructs are depicted on the left. The promoter regions are indicated with a stippled box, the CAT gene with an open box. Poly-adenylation sites are given by An The translation stop region present in pSV0ATCAT (Meakin *et al*, 1989) and in p0CAT (a derivative of pCAT, see text) is indicated with stop The CAT activity in eye and tail extracts of day 3 tadpoles (stage 37/38 according to Nieuwkoop & Faber, 1956) was determined as described in Materials and Methods. The CAT activity in the extracts was quantitated and corrected for the amount of protein. The ratio of the CAT activity/mg protein in the eye to the CAT activity/mg protein in the tail (E/T) is given in the right column.

These results thus indicate that vector starts are involved in the non-specific expression patterns, and that the stopcodon region is not sufficient to minimize the effect of these vector starts: the poly-adenylation sites present in pSV0ATCAT are required as well.

These data together with those presented in Figure 3 favour a model in which the general CAT expression found in the very young stages of development is the result of cryptic promoter sequences in the vector whereas the lens-specific expression in the later stages is driven by the rodent γ-crystallin promoter sequences.

DISCUSSION

The fate of the DNA micro-injected in *Xenopus laevis* embryos has been followed in a number of experiments (Rusconi & Schaffner, 1981; Etkin & Pearman, 1987; Anders *et al.*, 1984; Etkin *et al.*, 1984; Hofmann *et al.*, 1990). It is generally found that the micro-injected DNA replicates only within the first few hours and is gradually lost thereafter. Although lens cells do not die, they do lose their nucleus and hence the micro-injected DNA during terminal differentiation. Taking into account the gradual loss of DNA from the lens as well as the increase in protein content due to growth, the maintenance of high CAT levels up to the latest stage measured (13 or 21 days) must mean that the γ-crystallin promoters become even more active as the lens cells mature. This is in agreement with the finding that, in rodents, γ-crystallin synthesis is confined to the mature lens fibre cell (Van Leen *et al.*, 1987) and suggests that the specificity of recognition of the γ-crystallin promoter has been maintained during the long evolutionary time that separates *Xenopus laevis* from rodents. As many *Xenopus laevis* and rodent genes share a common evolutionary ancestry, promoter specificity may have been maintained for a number of other genes as well. To our knowledge, the only other tissue-specific mammalian promoter that has been tested in transgenic *Xenopus laevis* is the rabbit β-globin promoter, but no tissue-specificity was found in micro-injected tadpoles (Rusconi & Schaffner, 1981). With a *Xenopus* promoter, the *Xenopus borealis* actin promoter, tissue-specific expression of exogenous DNA was obtained (Wilson *et al.*, 1986).

The γ-crystallin genes encode one of the three ubiquitous families of crystallins, the abundant structural proteins in the vertebrate eye lens (Wistow & Piatigorsky, 1988). The crystallins are a good example of proteins of which the function needs to be studied by 'reverse genetics' techniques. The optical properties of the vertebrate eye lens are determined by the exact spatial arrangement of the crystallins (Delaye & Tardieu, 1983), an arrangement that cannot be mimicked *in vitro*. To fully elucidate the role of the crystallins in the lens architecture, it is necessary to engineer lenses which lack a crystallin species or which contain an additional (mutated) crystallin. Such lenses can be generated by making transgenic animals which carry either a mutated gene or a gene which expresses an anti-sense sequence, silencing the endogenous gene. Our results show that such experiments will be possible using transgenic *Xenopus laevis* tadpoles.

The use of promoters of genes for highly abundant proteins, such as the γ-crystallins, in transgenic animals has the advantage that often high levels of expression are achieved. For instance, the CAT activity obtained here from the rat γD or mouse

γF promoter is, as determined in day 5 (stage 44) transgenic tadpoles, at least 100 fold higher than that obtained from the SV40 promoter. The tissue-specificity of the γ-crystallin promoters further allows the introduction of possibly deleterious constructs as lens abberations do not affect the viability of the organism. Hence, in principle the system described here can be used to intervene in general cellular processes. In practice, the potential of this system is limited by the non-specific expression found in young embryos. Non-specific expression in early development has also been noted by others (Fu *et al.*, 1989; Krone & Heikkila, 1989). It is not clear why such non-specific promoter activity should be abundant in one particular stage of embryogenesis and it is thus difficult to design a direct strategy to counter this non-specific activity. Possibly, sequences in the vector bind ubiquitous transcription factors. One such site, an AP1 binding site, has already been detected in pUC derivatives (Yang-Yen *et al.*, 1990). Careful choice of vector and transcription stop sequences is then required to limit general expression during early embryogenesis as much as possible.

ACKNOWLEDGEMENTS

The authors are indebted to Drs P. M. T. Deen for initial help with the *in vitro* fertilization and the micro-injection, and to R. Engels for culturing the *Xenopus laevis*. We thank A. J. M. Coenen for help with the histological sections. This work has been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (NWO) as well as the E.E.C. [science program, contract no. SC1-0320-C (GDF)].

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CHAPTER 7

A DEVICE FOR QUANTITATIVE MEASUREMENT OF THE OPACITY OF SMALL ANIMAL LENSES: APPLICATION TO *XENOPUS LAEVIS*

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submitted for publication.

Experimental set up

Cataract is still the most common cause of blindness in man. Human cataract can be detected and classified with the aid of a variety instruments. Most of these instruments are not designed such that they can be directly used to measure cataract formation in small animals, often used in model systems. Yet to follow the course of induced cataract, some quantitative measurement of eye lens opacity is necessary. We have therefore adapted the system described by Flammer & Bebie (1987) for use with small lenses, in our case the lenses of *Xenopus laevis* tadpoles, which have a diameter of about 200 μm. The apparatus is shown in Fig. 1.

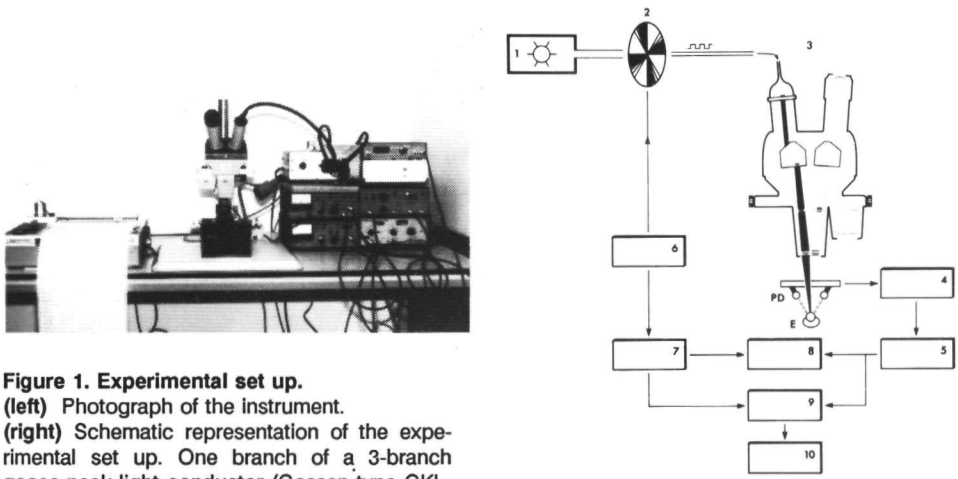


Figure 1. Experimental set up.
(left) Photograph of the instrument.
(right) Schematic representation of the experimental set up. One branch of a 3-branch goose-neck light conductor (Gossen type GKL-315), adapted with the supplied focusing lens, was used as a light source (1). The light beam was interrupted with a frequency of 600 Hz (chopper: 2), directed into one of the ocular tubes of a Zeiss binocular stereomicroscope (type DR; 3), and focused on the lens of a Xenopus eye (E). The 4X objective of the stereomicroscope, which reduces the diameter of the light beam to approximately 50 μm, was used routinely, but the system works with other objectives as well. Backscattered light was detected by four cascaded photodiodes (PD; AEG, type BPW20) in parallel, which are connected in reverse to a $\pm$15 V DC power-supply (4). The signal was immediately filtered by a highpass filter with its 3 dB point at 200 Hz. After filtration by a lowpass filter with a cut-off frequency of 1 kHz, the chopped signal was amplified by the lock-in technique, using a Brookdeal 9454 differential AC amplifier (5), two phase sensitive detectors (Brookdeal Electronics, Ltd, type 411; 8, 9), and a reference unit (Brookdeal Electronics, Ltd, type 422; 7). The chopper was regulated by a Stanford Research Systems, Inc. chopper controller (6), model SR540, which signal was also used as reference. The final signal was passed to a XY writer (10). We routinely used the 4X objective of the stereomicroscope. The 6.3X objective can be used as well but a diminished signal is obtained.

The optics of a stereomicroscope are used to focus a light beam with a diameter of about 50 μm on the lens. Light backscattered by the lens is detected by photodiodes.

The signal is then filtered and amplified by the lock-in technique. A relatively high chopper frequency, in combination with a highpass filter with its 3 dB point at 200 Hz and a lowpass filter with a cut-off frequency of 1 kHz, was chosen to minimize the amount of background signal due to artificial light sources. The instrument has a maximum electronic output at 600 Hz with these settings. The amplification was usually adjusted at a level just below the overload on the phase sensitive detectors (2.8 V), being about 50-60 dB. The difference between a white and a black surface (about 2.10 - 0.082 = 2.02 V) was used as an external control for the calibration of the device.

Preliminary experiments on isolated lenses indicated that preparation artifacts caused high levels of backscattering due to tissue damage. Furthermore, beams passing through transparent lenses scattered on the background. Measurement of whole eyes in air prevented background scattering as the retina absorbs light very efficiently, but caused artifacts due to desiccation. Therefore, all further measurements were performed on lenses *in situ*, covered by buffer solution. Under these conditions the amount of backscattered light is stable for at least 1 hour. A drawback of this method is the reflection of a significant amount of light on the surface of the buffer solution. Although this reflected beam falls into the second objective and not into one of the photodiodes, the amount of light reaching the lens is considerably reduced.

The values obtained in an actual experiment are shown in Table I. Lenses fixed *in situ* were used to mimic cataractous lenses, as fixation causes a visible non-transparent precipitate to be formed within the lens. The amount of backscattering was measured in four lenses, two fixed and two untreated, isolated from sibling tadpoles. To obtain independent measurements, the eye on the slide was moved and repositioned, and the light beam refocussed. The settings of the electronics were not changed. The relative deviation of 1-2% indicates that measurement on one lens is very reliable (Table I). The two transparent lenses yielded a mean value of 0.101 ± 0.008 (8%) Volts, while the two fixed lenses yielded a mean value of 0.373 ± 0.003 (1%) Volts.

The effect of age on eye lens transparency

Lens development in *Xenopus* is very rapid. After 5 days of development (stage 44) a lens nucleus has already been formed (Brakenhoff *et al.*, 1991) and the center of the lens should be transparent at this stage. However, measurement of the lens transparency at such an early stage could be hampered by interference from the immature cortical fiber cells. Hence, we have determined whether the measured opacity of the *Xenopus* lens varies with age. As shown in Table II, there is a small but significant decrease in the apparent opacity with age. Measurements of lenses of adult

frogs revealed no significant further increase in transparency. The data in this table also give an indication of the variability of the values obtained using lenses from different tadpoles. An important cause of variation was found to be the upwards placement of the lens. Slanting placement of the eyes prevents part of the backscattered light to reach the photodiodes, thus artifactually lowering the signal.

	Transparent		Cataractous	
measurement	lens 1	lens 2	lens 3	lens 4
1	0.111	0.092	0.365	0.370
2	0.109	0.095	0.375	0.370
3	0.112	0.093	0.385	0.360
4	0.108	0.090	0.388	0.373
5	0.104	0.095	0.365	0.378
mean	0.109	0.093	0.376	0.370
deviation	0.002	0.002	0.007	0.004

Table I. Backscattering in normal transparent and model-cataractous lenses. *Xenopus laevis* tadpoles were prepared as described earlier (Brakenhoff *et al.*, 1991) and staged according to Nieuwkoop & Faber (1956). Tadpoles were sacrificed, and the eyes were placed under tyrodes buffer (137 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 12 mM $NaHC0_3$, 5.5 mM glucose, pH 7.2) in the indentations of an aluminium slide blackened by anodization, with the lenses oriented upwards. To obtain the model cataractous lenses, the eyes were fixed in 4% formaldehyde in cacodylate buffer, pH 7.5. Measurements were performed on normal and fixed eyes of stage 52 tadpoles. Data are given in Volts. Five measurements were done on each lens. Each measurement was preceded by repositioning of the lens in the beam, and refocussing of the beam. Amplification was about 50 dB.

The effect of colour filters on the measurements

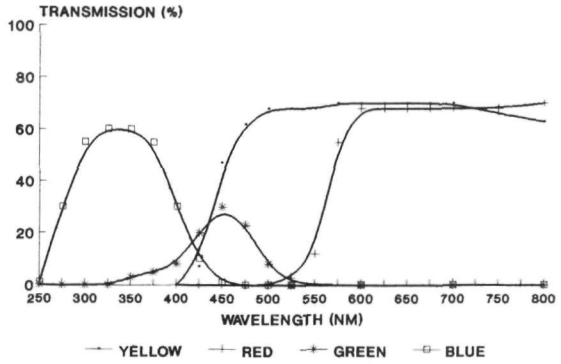
The data presented in Table I were obtained using white light. However, scattering intensity can be wave length dependent (Bettelheim, 1985). We therefore tested the effect of filtering the incident light with different colour filters (see Fig. 2, top). The largest difference between normal and cataractous lenses was obtained with unfiltered light, the smallest with blue filtered light (Fig. 2, bottom). Restricting the wavelength of the incident light thus did not increase the sensitivity of detection of lens opacity, at least not when using fixed lenses as a model system.

lens	tadpole (stage 49)	tadpole (stage 54)	froglet (stage 62)
1	0.330	0.128	0.160
2	0.330	0.220	0.218
3	0.270	0.215	0.195
4	0.290	0.320	0.163
5	0.240	0.195	0.100
6	0.240	0.245	0.100
7	0.240		0.168
8	0.210		
9	0.220		
10	0.340		
mean	0.271	0.221	0.158
deviation	0.049	0.063	0.041

Table II. Eye lens transparency of larval and frog stage *Xenopus laevis*.
Xenopus eyes were isolated as described in the legend to Table I. The animals used were either at the larval stage (tadpoles stage 49, 13 days old, or 54, 26 days old) or post-metamorphosis (froglets at stage 62, 8 weeks old). Data are given in Volts. The amplification was about 55 dB.

CONCLUSION

The amount of backscattering measured in fixed (model cataractous) lenses as compared to normal transparent lenses demonstrated that the device described here is suitable for the quantitative determination of lens opacity. Surprisingly, we found that even the apparently transparent lenses are not as clear as they seem. As mentioned above, the lenses of 5 day old tadpoles already appear to have a mature nucleus, since the central fibre cells in these lenses have lost their organelles. Nevertheless, these lenses are not totally translucent. Moreover, even lenses of young and adult frogs do backscatter light. It is not likely that this signal is due to the backscattering from the cornea or the retina, since lenses on which the light beam is focused appear to glow, a phenomenon which is increased in model cataractous lenses. We have used this apparatus to measure the transparency of newborn mice lenses also, but these are completely opaque as expected from the presence of cell organelles in the centre of the lens. Together these data show that we succeeded in developing a device for the quantitative measurement of eye lens opacities in small lenses.



M-C/T RATIO 3.1 3.1 2.6 1.6 1.4

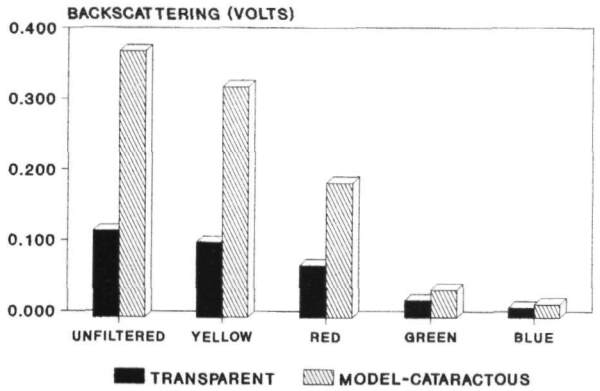


Figure 2. Backscattering intensities as a function of wave length.
(top) Schematic representation of the transmission characteristic of the various filters, measured by a Philips PU 8620 UV/VIS/NIR spectrophotometer. The transmission is indicated in percentage of the maximum on the y-axis. The wavelength is given in nm on the x-axis.
(bottom) Measurements were performed on normal eyes of stage 52 animals, fixed as well as untreated (see legend to Table I). The mean of the backscattering values of two fixed (model-cataractous) eyes or untreated (transparent) eyes are represented by hatched or solid bars respectively. The amount of backscattered light is indicated in Volts on the y-axis. The filter used is indicated on the x-axis. In the row M-C/T, the values of the model-cataractous lenses were divided by the values of the transparent lenses. Amplification was about 50 dB.

Experiments are now in progress to change the lens cell architecture by transgenesis in *Xenopus*, and to determine the effects of these changes on eye lens opacity. This kind of equipment should be of value in other fields of cataract research as well. It should be especially useful in following cataractogenesis, or measuring the effects of treatment of cataract in rat or mouse model systems (see for example Fukushi *et al.*, 1980; Hu *et al.*, 1983) as the small diameter of the beam should make it possible to detect localised changes. Quantitative determination of eye lens transparency in animal models could be an important guide in the prevention of this most frequent eye disease: lens opacity.

ACKNOWLEDGEMENTS

The authors are indebted to R. Engels for culturing the *Xenopus laevis*, and the Technical Centre of the University of Nijmegen for the construction of the set up. They also thank E. Jacobs and R. Ruuls for their technical assistance. This work has been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (NWO) as well as the E.E.C. [science program, contract no. SC1-0320-C (GDF)].

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SUMMARY

SAMENVATTING

SUMMARY

The abundant water-soluble proteins of the eye lens are known as the crystallins. The function of the crystallins is thought to be a structural one: their spatial order and interaction determine the optical properties of the lens. If crystallins are necessary for the establishment and maintenance of the transparency of the eye lens, then mutations in the crystallin genes might cause turbidity of the lens: cataract. Such mutations could be responsible for hereditary cataract in man. Indeed, one form of hereditary cataract, the "Coppock-like" cataract, was found to be closely linked to the human γ-crystallin gene family, suggesting a causal relation between the malfunction of a γ-crystallin gene and a cataract phenotype. As a first step towards the identification of the lesion in the "Coppock-like" cataract, the structure and expression of the human γ-crystallin gene family was investigated. As reported in Chapter 4, the gene family consists of six and one quarter γ-crystallin genes, of which only two genes are fully active. During the course of the primate evolution the other four genes have been (partially) inactivated at the transcriptional or post-transcriptional level. Genetic analysis placed the "Coppock-like" cataract locus in a region encompassing one active and one ψγ-crystallin gene (Chapter 5). Cloning of this region from the mutant chromosome allowed a more exact localization of the sequence changes associated with the cataract mutation to the promoter region of the ψγ-crystallin gene. These sequence changes cause a ten-fold increase in the activity of the ψγ-crystallin gene promoter, presumably resulting in a corresponding increase in the synthesis of the small N-terminal γ-crystallin fragment encoded by the pseudogene. The over-expression of this protein fragment is suggested to be the cause of the "Coppock-like" cataract phenotype (Chapter 5).

Other forms of human hereditary cataract could also be caused by lesions in crystallin genes. A systematic search for such cataract loci would be eased by the characterization and chromosomal assignment of all human crystallin genes. Two of the gaps in our knowledge of the human crystallin genes were therefore filled in: the αB-crystallin gene was mapped to chromosome 11 (Chapter 2) and the second βB2-crystallin gene was shown to be transcriptionally inactive (Chapter 3).

A search for crystallin mutations among the natural variation within the human population is not only laborious but may well fail. The desired mutation may yield a unrecognizable phenotype or may simply not exist. In contrast, with the techniques of reverse genetics, theoretically any mutation can be obtained. In Chapter 5 it is shown that transgenic *Xenopus laevis* appear to be a suitable model system for the manipulation of the crystallin genes. Introduction of genetic information under the

control of rodent γ-crystallin promoters directs expression of the exogenous DNA to the tadpole lens. To measure the effect of the expression of introduced constructs on the transparency of the lens, an instrument with which the amount of light backscattered by *Xenopus laevis* tadpole lenses can be quantitatively determined, was designed and tested (Chapter 7).

SAMENVATTING

De wateroplosbare eiwitten van de ooglens staan bekend onder de naam crystallines. Men neemt aan dat de funktie van de crystallines van structurele aard is: de ruimtelijke ordening van deze eiwitten en hun onderlinge interakties bepalen de optische eigenschappen van de ooglens. Indien de crystallines nodig zijn voor het genereren van bijvoorbeeld de lichtdoorlaatbaarheid van de ooglens, dan zouden mutaties in de genen die voor deze lenseiwitten coderen, een troebeling van de lens kunnen veroorzaken: een zg. cataract. Of anders gezegd, erfelijke vormen van cataract zouden mogelijkerwijs door mutaties in de crystalline genen kunnen worden veroorzaakt. Inderdaad is een erfelijke cataract, bekend onder de naam "Coppock-like" cataract, genetisch nauw gekoppeld aan de γ-crystalline genfamilie, hetgeen een oorzakelijke relatie suggereert tussen een gestoorde funktie van een γ-crystalline gen en het cataract fenotype. Om de genetische afwijking van het "Coppock-like" cataract locus moleculair biologisch te karakteriseren, is allereerst de structuur en de expressie van de humane γ-crystalline genfamilie nader geanalyseerd. Uit het onderzoek, zoals beschreven in Hoofdstuk 4, komt naar voren dat deze genfamilie bij de mens bestaat uit zes volledige genen. Daarnaast wordt nog een onvolledig gen, slechts een vierde deel van een normaal gen omvattend, aangetroffen. Slechts twee γ-crystalline genen zijn aktief. Gedurende de evolutie van de primaten zijn de vier andere genen geheel of gedeeltelijk uitgeschakeld op het transcriptionele of post-transcriptionele nivo.

Het "Coppock-like" cataract locus kon via moleculair genetische analyse geplaatst worden in een gebied dat twee γ-crystalline genen bevat, een aktief gen en een pseudo-gen (Hoofdstuk 5). Klonering van dit gebied, afkomstig van het mutante chromosoom, maakte een meer nauwkeurige localisatie van de mutatie mogelijk. De sequentie veranderingen die mogelijk causaal met de "Coppock-like" cataract zijn verbonden, werden aangetroffen in het promotergebied van het pseudo-γE-crystalline gen. De sequentie veranderingen bleken een tienvoudige verhoging van de aktiviteit van de pseudo-gen promoter te veroorzaken, hetgeen waarschijnlijk een tienvoudige verhoging tot gevolg heeft van de synthese van het door dit pseudo-gen gecodeerde N-terminale gedeelte van een γ-crystalline. De verhoogde expressie van dit vreemde crystalline produkt zou dan het cataract fenotype verklaren (Hoofdstuk 5).

Andere erfelijke vormen van cataract bij de mens zouden eveneens veroorzaakt kunnen worden door mutaties in een van de genen behorende tot de α-, β- of γ-crystalline genfamilies. Een systematische aanpak om dergelijke vormen van erfelijk cataract op te sporen zou ongetwijfeld vergemakkelijkt worden door een volledige karakterisatie en chromosomale localisatie van alle humane crystalline genen. Twee

nieuwe gegevens konden uit ons onderzoek hieraan worden toegevoegd : het αB-crystalline gen werd op chromosoom 11 gekarteerd (Hoofdstuk 2) en van het tweede βB2-crystalline gen van de mens werd aangetoond dat het niet aktief is en derhalve als een pseudo-gen moet worden aangemerkt (Hoofdstuk 3).

Een speurtocht naar crystalline mutaties binnen de natuurlijke variatie van de humane populatie is niet alleen arbeidsintensief, maar is ook bepaald niet zonder risico en zou kunnen falen. Immers, een mutatie zou geen herkenbaar afwijkend fenotype kunnen veroorzaken of zelfs gewoon niet voorkomen in de populatie. Met behulp van 'reverse genetics' technieken daarentegen, zou theoretisch echter iedere mutatie verkregen kunnen worden. In Hoofdstuk 6 wordt aangetoond dat transgene *Xenopus laevis* larven een geschikt model systeem kunnen zijn voor de manipulatie van de crystalline genen. Immers, wanneer genetische informatie wordt geintroduceerd in het *Xenopus* ei onder de controle van een knaagdier γ-crystalline promoter, dan blijkt het exogene DNA selectief in de lens van de larve tot expressie te komen. Om te analyseren in welke mate de expressie van geïntroduceerde DNA constructen van invloed zijn op het transparante karakter van de ooglens van dit modelsysteem, is een instrument ontwikkeld en getest, waarmee de hoeveelheid licht die door een *Xenopus laevis* lens wordt verstrooid, kwantitatief bepaald kan worden (Hoofdstuk 7).

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CURRICULUM VITAE

De schrijver dezes werd geboren op 24 januari 1959 te Arnhem. In 1977 behaalde hij het OVWO diploma aan het Thomas à Kempis College te Arnhem. Na vijf lotingen werd hij uiteindelijk via de hardheidsclausule toegelaten tot de studierichting geneeskunde aan de Katholieke Universiteit te Nijmegen. In de tussenliggende jaren heeft hij het diploma histologisch analist, afgelegd aan de OLAN te Arnhem, en het diploma biochemisch analist, afgelegd aan de HMLS te Oss, behaald.

In overleg met het bestuur van de faculteit der Geneeskunde en Tandheelkunde is een vrije doctoraalopleiding geneeskunde gevolgd onder leiding van Prof. Dr. J.J. de Pont. In het kader van deze vrije doctoraalopleiding werd het bijvak parasitologie gevolgd aan de afdeling Medische Parasitologie (Dr. A.N. Vermeulen; Prof. Dr. J.H.E.T. Meuwissen) alsmede het hoofdvak Biochemie, zowel binnen de vakgroep Biochemie te Nijmegen als aan het Nederlands Kanker Instituut te Amsterdam (Dr. P.J.A. Krimpenfort; Dr. A.M. Berns). Het doctoraalexamen geneeskunde werd afgelegd op 25 augustus 1986.

Vanaf 16 december 1986 tot 16 december 1990 is hij als AIO werkzaam geweest op het Laboratorium voor Moleculaire Biologie waar het in dit proefschrift beschreven onderzoek is uitgevoerd in de werkgroep van Dr. N.H. Lubsen. Tijdens de promotieperiode is het diploma C-deskundigheid stralingshygiëne behaald, is een bijdrage geleverd aan het practisch onderwijs aan 2e en 3e-jaars biologiestudenten en zijn een zestal hoofd- en bijvakstudenten Moleculaire Biologie begeleid.

Sinds 1 augustus 1991 is hij werkzaam binnen de afdeling K.N.O. van het Academisch Ziekenhuis van de Vrije Universiteit te Amsterdam om monoklonale antilichamen te humaniseren voor de diagnostiek en behandeling van hoofd- en halstumoren en de antigenen waartegen zij gericht zijn te kloneren en te karakteriseren.