The Ontogeny of γ-crystallin mRNAs in Cat$^{\text{Fraser}}$ Mice

MACIEJ KULISZEWSKI, JIM RUPERT AND R. GOLD*

Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Summary

Mice which are either homozygous or heterozygous for the Cat$^{\text{Fraser}}$ mutation have ocular cataracts accompanied by selective reduction of the γ-crystallins, a homologous family of proteins present in the lens and encoded by a family of tightly linked genes. We measured the concentrations of four different mRNAs, each encoding a different γ-crystallin, in the lenses of homozygous Cat$^{\text{Fraser}}$ mice and in normal controls at various stages of development by preparing Northern blots from lens RNA, probing with RNAs complementary to each of the four messages and densitometry of the bands thus generated. The results show that, for each of these messages, the ontogenetic patterns observed in normal mice are retained in the mutant, but at much lower concentrations.

1. Introduction

The crystallins are specialized proteins found exclusively in the vertebrate lens and thought to be necessary for its transparency (Delaye & Tardieu, 1983). In mammals, the crystallins account for 90% of the soluble lens proteins and comprise three antigenically distinct classes (α, β and γ) (Harding & Dilley 1976). Each of these classes itself comprises several homologous proteins encoded by families of homologous genes (Piatigorsky, 1984; Lok *et al.* 1985).

The three classes of crystallin make their first appearance at different times during the development of the lens. For example, in the murine lens, the α-crystallins make their first appearance on the twelfth day of intrauterine life, the β-crystallins a half a day later, and the γ-crystallins shortly thereafter.

Moreover, the three classes of crystallins first appear, and are subsequently distributed, at different sites within the lens. The α-crystallins are first found in the lens epithelium but are subsequently present in both epithelium and fibres. The first appearances of β- and γ-crystallins respectively coincide with the early and terminal differentiation of fibres and both of these classes are subsequently confined to the fibres (Piatigorsky, 1981; Zigman, 1985).

The fact that all the members of a given class of crystallins have similar ontogenies, both spatial and temporal, suggests that the family of genes which encode the class is provided with mechanisms that coordinately regulate it. Conversely, the fact that each

* Corresponding author.

class of crystallin exhibits a unique ontogeny, different from that of the others, suggests that each class is provided with regulatory mechanisms that are specific to it.

Since the crystallins occur in a relatively simple tissue in which they comprise 90% of the proteins present; and since they appear to be under the control of regulatory mechanisms which are complex and elegant, they provide an attractive system for investigating the genetics of development.

Among the strategies that have been employed to exploit this system is the investigation of mutants which perturb it. Prominent among the mutants which have been used for this purpose is the Cat$^{\text{Fraser}}$ mouse, which has co-dominantly inherited ocular cataracts accompanied by reduced amounts of all the γ-crystallins and of their mRNAs, while the α- and β-crystallins and their mRNAs appear to be unaffected (Garber *et al.* 1985). This observation, while amenable to many explanations, raises the possibility that the Cat$^{\text{Fr}}$ mutation interacts with the hypothetical mechanisms which specifically regulate the γ-crystallin gene family. The investigation of the ontogeny of individual γ-crystallins in the Cat$^{\text{Fr}}$ mouse reported here represents a continuation of our attempt to investigate this possibility.

The mouse lens contains seven different γ-crystallins (Garber *et al.* 1983; Garber & Gold, 1982), each of which contain approximately 175 amino-acid residues. Their primary structures exhibit 90% homology with one other and all of them are present in the lens as monomers (Harding & Dilley, 1976; Breitman *et al.* 1984). The seven murine γ-crystallins are produced by

at least six genes which are tightly clustered, probably within a 50 kb region, on chromosome 1 (Quinlan *et al.* 1987).

Detailed analysis of four of these genes has revealed the expected high degree of homology between them, and also similarities of structure. All have 3 exons, a small 9 bp exon followed by 2 larger exons each of which contain approximately 250 bp and exactly corresponds to one of the two globular domains of the protein. The 5′ and 3′ introns comprise 80 to 100 bp and 800 to several thousand bp, respectively. There is an average 80–90 % homology between the exons. The 3′ half of the third exon exhibits relatively low (52–71 %) homology and it is from this region that the probes, specific for individual genes, used in this study, were prepared. The introns and 3′ flanking region reveal no homology. In the 5′ flanking region, however, there is a highly conserved region, of approximately 43 bases, immediately 5′ to the TATA base (Lok *et al.* 1984; Breitman *et al.* 1984).

Recent studies have shown that certain segments of the 5′ flanking region of γ-crystallin genes, when fused to a bacterial chloramphenicol acetyltransferase gene, function as a promoter in chicken lens explants but not in other tissues (Lok *et al.* 1985). It is conceivable that this lens-specific promoter activity in the 5′ flanking region has some role in the coordinated regulation of the γ-crystallin genes, possibly mediated by some transacting factor present in the lens.

Recent evidence suggests that, in addition to the putative mechanisms responsible for the coordinate regulation of all the γ-crystallin genes, there may also be mechanisms which regulate subsets of this gene family. Murer-Orlando *et al.* (1987) have shown, using specific probes, that γ1 and γ2 transcripts start at a low level at birth, rise sharply until the 20th day and then fall again, while γ3 and γ4 transcripts remain at a low level throughout the development of the lens.

The purpose of the work reported here is to acquire a more detailed understanding of the effect of the Cat^{Fr} mutation on the expression of γ-crystallin genes by determining how the amounts of these individual γ-crystallin mRNAs change during the development of the Cat^{Fr} lens and by comparing their ontogenetic patterns with those found in normal lenses.

2. Materials and methods

(i) *Animals*

The mice used were either homozygous normal or homozygous mutant at the Cat^{Fr} locus. In both cases, they were of the inbred Swiss Webster strain. Eyes were collected from 1-, 20-, 40- and 60-day-old mice and from embryos in their 15th day of intrauterine life. The lenses were surgically separated from the eyes and, when not immediately used, stored at −80 °C.

(ii) *RNA extraction*

For each preparation, at least 10 adult lenses were added to 8 ml of 4·0 M guanidinium chloride buffer and homogenized with a Kinematica polytron for 30 s at maximum setting. For embryos the same procedure was used except that whole heads were substituted for lenses. RNA was prepared from the resulting homogenate by the guanidine/chloride method. An aliquot of this preparation, containing 5 or 20 μg of total RNA in aqueous solution for adult lenses and embryonic heads respectively, was used for subsequent electrophoresis in 2·2 M formaldehyde gel (Maniatis *et al.* 1982).

(iii) *Preparation of probes for Northern hybridization*

The preparation of probes from a region of the cDNA corresponding to the 5′ half of the third exon, the most variable region, which specifically hybridize to γ1, γ2, γ3 and γ4 messages has previously been described (Murer-Orlando *et al.* 1987). Specimens of each of these probes cloned in pUC12 were obtained from Dr Breitman's laboratory and subsequently subcloned into the multiple cloning site (MCS) of the transcription vector pGem-2 (Pro-Omega). The resulting constructs were called pGem-2γ1, pGem-2γ2, pGem-2γ3 and pGem-2γ4. The pGem plasmids have T7 and Sp6 promoters flanking the MCS, allowing synthesis of RNA probes in either the sense or the anti-sense orientation.

Probes to be used in the hybridization experiments were synthesized using the T7 promoter to initiate *in vitro* transcription of the crystallin sequences. The template plasmid was first linearized with *Eco*R1 and 70 units of Pharmacia T7 polymerase were used to transcribe the probe. Probes were labelled with 5 μl of [α-^{32}P]GTP (3000 Ci/mol) obtained from New England Nuclear. The transcription reactions were performed in 15 mM cold rNTPs (ATP, UTP, CTP), 6·25 mM DTT and 1 μl (31·5 units) RNAse inhibitor (Boehringer Mannheim) at 37° for one hour. Following incubation, the plasmid template was removed by digesting with 1 μl of 0·1 M DNAse I for 10 min at 37 °C. An additional 31·5 units of RNAsin was added during this reaction to protect the RNA probe from any contaminating RNAse. The probe was then purified on a Sephadex G 150 column (6–8 ml bed volume). Examination of the probes on gels revealed that they were full-length transcripts. Since the same nucleotide of the same specific activity was always used in preparation of the probes, it can be assumed that different preparations of the same probe had similar specific activities.

(iv) *Northern Blotting*

Immediately after electrophoresis, the RNA was blotted to a nylon membrane (ICN Biotrans) and

hybridized by the methods recommended by the manufacturer. The amount of probe used for hybridization contained 4×10^6 counts per minute in every case. Following hybridization, the membrane was washed to a final stringency of $0.1 \times SSC$ and 0.1% SDS at 65 °C for 30 min, then exposed overnight at -70 °C using a Dupont intensifying screen. It has been shown (Murer-Orlando, 1987) that hybridization with these probes under the stated conditions results in a unique signal. In other words, each probe hybridizes only to the mRNA with which it corresponds.

(v) Quantitation of the results from northern blotting

Film was exposed to the blot for 19 h and developed for 5 min in every experiment. The resulting autoradiograms were scanned using a Bio-Rad video densitometer, model 620, connected with a Bio-Rad integrator, model 3392A. The relative size of each peak was determined by cutting it out and weighing it. The weight of the peak thus obtained from a blot of mRNA, prepared as described from 1-day-old control mice and hybridized with a $\gamma 2$ specific probe, was arbitrarily assigned the value 100. Other peaks were assigned values proportional to their weight. All experiments were repeated three times using independent isolates of RNA. The values presented are the mean of three.

3. Results

Figure 1 shows Northern blots of total RNA taken from normal and mutant mice at various ages, probed with complementary RNAs specific for each of four γ-crystallin mRNAs. Figure 2 shows the densitometric measurements of the bands thus generated.

Looking first (Fig. 2) at the data for normal mice, it will be seen that the ontogenetic pattern of $\gamma 1$ and $\gamma 2$ are extremely similar and that the same is true of $\gamma 3$ and $\gamma 4$. That is to say, while at birth all four are present at relatively low and similar levels, the amounts of $\gamma 1$ and $\gamma 2$ increase to a peak value at 20 days and subsequently decline; while $\gamma 3$ and $\gamma 4$ retain approximately their birth values. These results are in very good agreement with those obtained by Murer-Orlando *et al.* (1987) by similar methods. Some observations made on 60-day-old lenses showed no significant differences from levels observed at 40 days.

Inspection of the blots shown in Fig. 1 reveals that in some cases they do not appear to correspond to the densitometric data. For instance, the data in Fig. 1 *B* do not give the impression that $\gamma 1$ and $\gamma 2$ mRNA are increasing as indicated by the densitogram in Fig. 2. Discrepancies of this kind are often found since visual impressions of the intensity of a signal on an autoradiogram are unreliable particularly when, as in the example cited, the signal is intense. The auto-
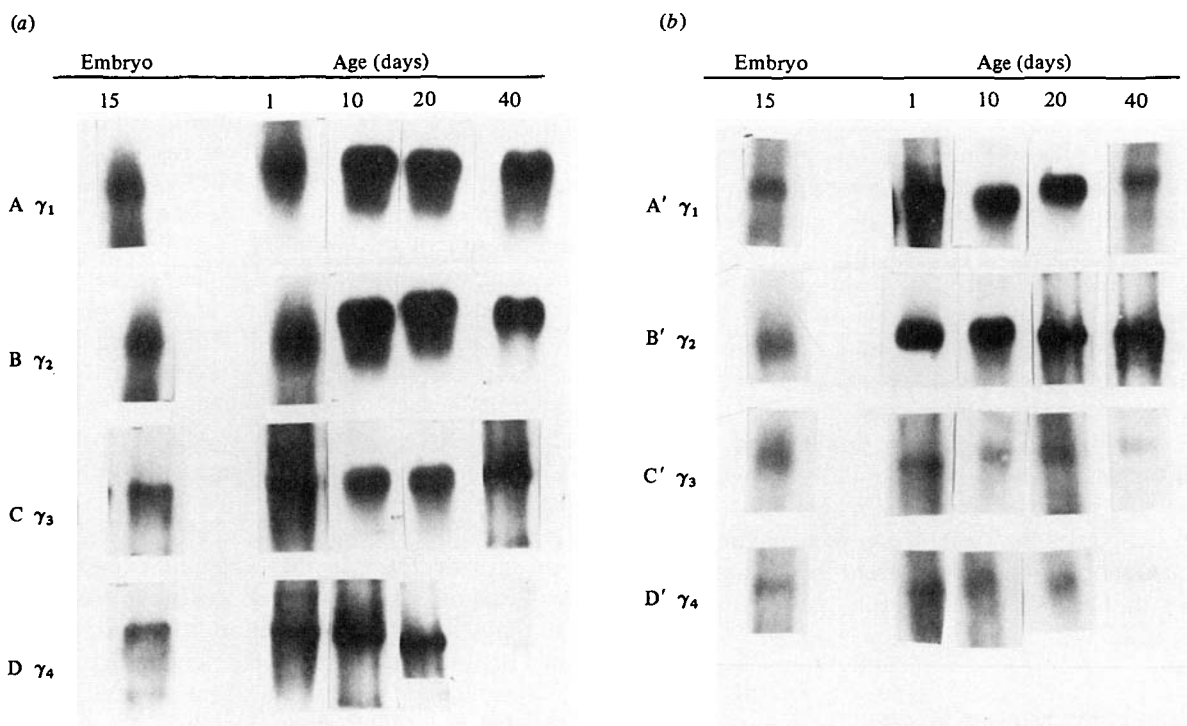

Fig. 1. Northern blots of total RNA prepared from lenses of normal (*a*) and mutant (*b*) mice, probed with RNAs complementary to $\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$ mRNA at 15 days after conception and at 1, 10, 20 and 40 days after birth.
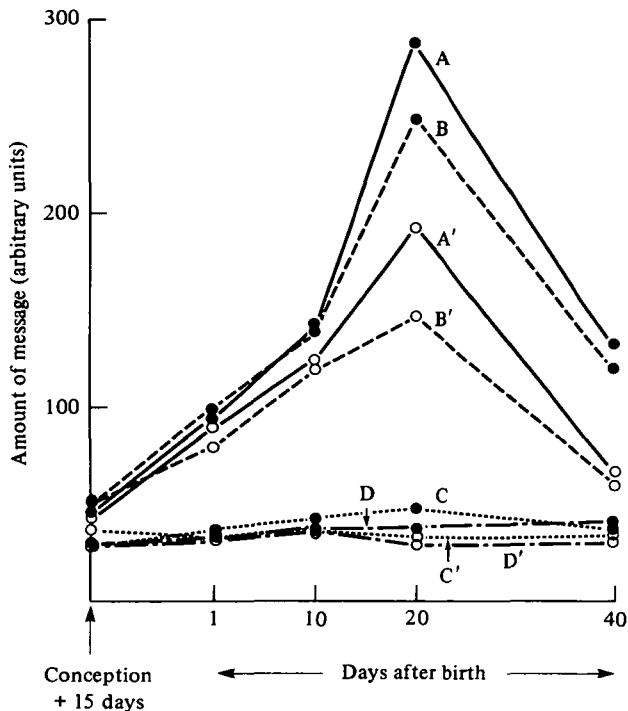
Fig. 2. Ontogenic changes in γ-crystallin mRNAs in normal and Cat^{Fr} mice. The amount of γ2-crystallin present in 5 μg of total RNA prepared from control Swiss Webster mice at birth measured in arbitrary densitometric units, is assigned the value 100. All other values are calculated proportionately to this standard using, for comparison, the weights of the densitometric peaks.

	Normal	Mutant
γ1	A	A'
γ2	B	B'
γ3	C	C'
γ4	D	D'

radiograms yielded, in each case, only a single band and hybridization was not observed in regions of the gel other than that shown in the diagram.

It will be seen that the general ontogenetic pattern observed in the normal lens is maintained in the mutant. The γ1 and γ2 mRNAs, in the mutant, as in the normal, start at a low level, rise to a maximum at 20 days and then decline while the γ3 and γ4 mRNAs, both in normal and mutant, remain at a low level throughout. However, in the mutant, the absolute amounts of all mRNAs present are, at any given age, less in the mutant than in the normal.

On the whole, the percentage decrease in the amount of message present in the mutant is roughly similar for all messages at a given age. But the percentage loss varies with age, rising to a maximum when the amount of message present is itself at a maximum.

4. Discussion

(i) *Comparison with previous results*

The results for normal mice confirm those obtained by Murer-Orlando (1987) using similar methods, namely

that the levels of γ1 and γ2 message rise from an initially low level and then fall again while the levels of γ3 and γ4 message are maintained at a low level throughout. The results for mutant mice are in good agreement with the findings of Garber *et al.* (1985) who reported that, at birth, the levels of both γ-crystallins and of their messages, as measured by *in vitro* translation and hybridization, were only slightly reduced at birth while at 20 days the decreases were much more marked.

(ii) *Mutant and normal*; *similarities and differences*

The results show that the ontogenetic patterns found for γ1, γ2, γ3 and γ4 messages in the normal lens are preserved in the Cat^{Fr} mutant in that the rise and fall of the γ1 and γ2 message takes place at the same periods during development as in the normal lens and that also, as in the normal, γ3 and 4 messages maintain their initial low level throughout development. But, for each of the four γ-crystallin messages, the amount present in the mutant lens is lower than that present in the normal lens at all stages of development examined.

It appears to be the case that the effects of the Cat^{Fr} mutation are distributed evenly over the whole class of γ-crystallins and are not confined to a subset of this class. It is therefore clear that the mutation does not affect the regulation of subsets of the γ-crystallin genes.

The mechanisms which can be adduced to explain the generalized effect fall into two broad categories. The mutation could cause the depression of the γ-crystallin mRNAs either as a result of the cataract itself or by interacting with those putative mechanisms which regulate the whole class.

(iii) *Possible mechanisms*

(a) *Secondary effect of cataracts.* The first possibility is that the deficiency of the γ-crystallins is merely a secondary result of the presence of a cataract. This hypothesis is consistent with the fact that other inherited cataracts, e.g. the Nakano and Philly mouse (Kador *et al.* 1980; Shinohara & Piatigorsky, 1980) are also associated with loss of γ-crystallins. But in these cases it is due to osmotic changes in the cell membrane leading to leakage of γ-crystallins from the lens. It has been shown that this does not occur in the Cat^{Fr} lens (Garber *et al.* 1985).

There is another mechanism whereby the cataract could cause a decrease in the γ-crystallin messages relative to those of the other crystallin classes, namely, that the tissue destruction it causes could occur in a region which is relatively rich in γ message. We are conducting *in situ* hybridization studies to elucidate this point. But there is a difficulty in this theory. Our previous studies showed that, in the Cat^{Fr} lens, the ratio of β-to-α message and of each of

these to a non-crystallin message is normal (Garber *et al.* 1985) and it is difficult to believe that tissue destruction could have caused the observed loss of γ message without causing some change in these other ratios.

In deciding whether the loss of γ-crystallins is a cause or an effect of the cataract, it is important to know which develops first. Ontogenetic studies are not much help in deciding this question since the first signs of cataract formation, namely, swelling and degeneration of the fibres and the initiation of γ-crystallin synthesis occur, more or less, simultaneously (Fraser & Schabtach, 1962; Zwaan & Williams, 1968; Sakurakawa *et al.* 1975; Hamai & Kuwabara, 1975; Zwaan & Williams, 1969; Day & Clayton, 1972).

(*b*) *Possible effect on regulation*. We have shown that the Cat^{Fr} mutation is not linked to the γ-crystallin gene cluster on chromosomes 1 (Rupert *et al.* 1988) and it has recently been shown that this mutation is on chromosomes 10 (Muggleton-Harris *et al.* 1987). Therefore if the mutation exercises its effect on the γ-crystallins by interacting with a mechanism that regulates them, the mechanism affected must be a transacting one. Moreover, it follows from the observations reported here that the mechanism affected must be one which regulates the class of γ-crystallins as a whole.

The existence of such transacting mechanism is implied by the discovery of lens-specific promoter regions in the 5′ flanking region of the γ-crystallin genes. One possibility therefore is that the mutation occurs in a gene encoding a transacting factor which binds to this region.

In conclusion, our study reveals that the effect of the mutation has a similar effect on all four γ-crystallin mRNAs studied. This observation is consistent either with the possibility that loss of γ-crystallins in the Cat^{Fr} mutation is a non-specific effect of the cataract or that it is caused by impairment of a mechanism which coordinately regulates the γ-crystallin gene family.

References

Breitman, M., Lok, S., Wistow, G., Piatigorsky, J., Treton, J. A., Gold, R. & Tsui, L.-C. (1984). γ-crystallin family of the mouse lens: structural and evolutionary relationships. *Proceedings of the National Academy of Science* **81**, 7762–7766.

Day, T. H. & Clayton, R. M. (1972). Multiple changes in lens protein composition associated with the Cat^{Fr} gene in the mouse. *Genetical Research* **19**, 241–249.

Delaye, M. & Tardieu, A. (1983). Short-range order of crystallin proteins accounts for eye lens transparency. *Nature* **302**, 415–417.

Fraser, C. & Schabtach, G. (1962). 'Shrivelled': a hereditary degeneration of the lens in the mouse. *Genetical Research* **19**, 383–387.

Garber, A. T. & Gold, R. (1982). Comparative two dimensional electrophoresis of water soluble proteins from bovine and murine lens. *Experimental Eye Research* **36**, 585–596.

Garber, A. T., Stirk, L. & Gold, R. (1983). Abnormalities of crystallins in the lens of the Cat^{Fraser} mouse. *Experimental Eye Research* **36**, 165–169.

Garber, A. T., Winkler, C., Shinohara, T., King, C. R., Inana, G., Piatigorsky, J. & Gold, R. (1985). Selective loss of a family of gene transcripts in a hereditary murine cataract. *Science* **227**, 74–77.

Hamai, Y. & Kuwabara, T. (1975). Early cytologic changes of the Fraser cataract. An electron microscopic study. *Investigative Opthalmology* **14**, 517–527.

Harding, J. & Dilley, K. (1975). Structural proteins of the mammalian lens: A review with emphasis on changes in development, aging and cataract. *Experimental Eye Research* **22**, 1–73.

Kador, P. F., Fukui, H., Fukusui, S., Jernigan, H. Jr. & Kinoshita, J. (1980). Philly Mouse: a new model of hereditary cataract. *Experimental Eye Research* **30**, 59–68.

Lok, S., Breitman, M., Cheplinsky, A., Piatigorsky, J., Gold, R. & Tsui, L.-C. (1985). Lens specific promoter activity of a mouse γ-crystallin gene. *Molecular and Cellular Biology* **5**, 2221–2230.

Lok, S., Tsui, L.-C., Shinohara, T., Piatigorsky, J., Gold, R. & Breitman, M. L. (1984). Analysis of the mouse γ-crystallin gene family: assignment of multiple cDNAs to discrete genomic sequences and characterization of a representative gene. *Nucleic Acids Research* **12**, 4517–4529.

Maniatis, T., Frisch, E. F. & Sambrook, J. (1982). In *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor.

Muggleton-Harris, A. L., Festing, M. F. W. & Hall, M. (1987). A gene location for the inheritance of the cataract Fraser (Cat^{Fr}) mouse congenital cataract. *Genetical Research* **49**, 235–238.

Murer-Orlando, M., Paterson, R., Lok, S., Tsui, L.-C. & Breitman, M. (1987). Differential regulation of γ-crystallin genes during mouse lens development. *Developmental Biology* **19**, 260–267.

Piatigorsky, J. (1981). Lens differentiation in vertebrates. A review of cellular and molecular features. *Differentiation* **19**, 134–153.

Piatigorsky, J. (1984). Lens crystallins and their gene families. *Cell* **38**, 620–621.

Quinlan, P., Oda, S., Breitman, M. L. & Tsui, L.-C. (1987). The mouse eye lens obsolescence (*Elo*) mutant: Studies on crystallin gene expression and linkage analysis between the mutant locus and the γ-crystallin genes. *Genes and Development* **1**, 637–644.

Rupert, J., Kuliszewski, M., Tsui, L.-C., Breitman, M. L. & Gold, R. J. M. (1988). The murine cataractogenic mutation, Cat^{Fr}, segregates independently of the γ-crystallin genes. *Genetical Research* (in press).

Sakurakawa, M., Kuwabara, T., Kinoshita, J. H. & Jukui, H. N. (1975). Swelling of the lens fibers. *Experimental Eye Research* **21**, 381–394.

Shinohara, T. & Piatigorsky, J. (1980). Persistence of crystallin messenger RNAs with reduced translation in hereditary cataracts in mice. *Science* **210**, 914–916.

Zigman, S. (1985). Selected aspects of lens differentiation. *Biological Bulletin* **168**, 189–213.

Zwann, J. & Williams, R. (1968). Morphogenesis of the eye lens in a mouse strain with hereditary cataracts. *Experimental Zoology* **169**, 407–422.

Zwann, J. & Williams, R. (1969). Cataracts and abnormal proliferation of the lens epithelium in mice carrying the Cat^{Fr} mutation. *Experimental Eye Research* **8**, 161–167.

4