Extralenticular Expression of *Xenopus laevis* α-, β-, and γ-Crystallin Genes

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**Purpose.** Extralenticular expression of α- and β-crystallin genes has been demonstrated in mammals and expression of γ-crystallin genes has been shown in *Xenopus laevis*. To determine a possible correlation between lens determination and crystallin gene expression, the site of expression of (a member of) the α-, β-, and γ-crystallin gene families was observed before and during lens formation in *X. laevis*.

**Methods.** The partial complementary DNAs (cDNAs) of αA- and βA4-crystallin and a γ-crystallin were cloned from an *X. laevis* lens cDNA library. The corresponding antisense RNAs were used to analyze the expression of these genes during *X. laevis* development by wholemount in situ hybridization.

**Results.** Expression of the βA4- and γ-crystallin (but not α-crystallin) genes could first be detected in the animal cap of the *X. laevis* gastrula. The βA4- and γ-crystallin messengers were also found in the first stage of lens development, when the ectodermal tissue overlying the optic vesicle thickens to form the lens placode. αA-crystallin messenger RNAs were only detectable when the lens epithelial cells were formed.

**Conclusions.** In contrast to observations in most vertebrates, expression of the βA4- and γ-crystallin genes was observed to precede that of the αA-crystallin gene during lens development of *X. laevis*, reflecting the determination that in amphibians, the (presumptive) fiber cells are formed before the epithelial cells, whereas in vertebrates, the order is reversed. Expression of βA4- and γ-crystallin genes in the ectodermal tissue of the *X. laevis* gastrula shows that these genes are expressed when this tissue gains competence for lens formation. Invest Ophthalmol Vis Sci. 1997;38:2764-2771.

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The optical properties of the lens are determined by the high concentration and close-range order of abundant structural proteins, the so-called crystallins. On the basis of the distribution of these proteins in different species, the crystallins can be divided into two groups: ubiquitous and taxon-specific crystallins. The group of ubiquitous crystallins, which have been found in every vertebrate examined so far, consists of the α-, β-, and γ-crystallins. Until now, approximately 12 taxon-specific crystallins, each present in a restricted set of species, have been identified.

High-level expression of the crystallins is limited to the lens; in other tissues low-level expression of at least some crystallins has been detected, and it has become clear that crystallins are not merely structural proteins of the lens but may have other roles as well. The α-crystallins, for instance, which are an evolutionary relative of small heat-shock proteins, have been shown to act as molecular chaperons and are able to convey thermotolerance. Expression of αA-crystallin is found in spleen and thymus tissues and expression of αB-crystallin in heart, muscle, kidney, and brain tissues. The taxon-specific crystallins have a close resemblance or are identical to metabolic enzymes and often have retained their enzymatic activity in the lens. Expression of β-crystallins outside the lens has also been reported, namely in chicken and mouse retinas, whereas extralenticular expression of γ-crystallins was found in *Xenopus laevis*. For these proteins no function other than a structural one has been established.

During lens development, the crystallins are dif-
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Differentially expressed. In mammals, in the embryonic stage α-crystallin is found in the lens placode, whereas β- and γ-crystallin expression is not detectable until primary fiber cell differentiation occurs. In chicken, β-crystallin is predominant in the embryonic lens, whereas the β-crystallins become the major proteins in the lens after hatching. In the duck, ε-crystallin expression is preceded by the expression of αβ- and τ-crystallin. In *X. laevis*, γ-crystallin messenger RNAs (mRNAs) are detectable at the early stages of lens development.

In this report, we describe the temporal and spatial expression of a member of each of the three classes of ubiquitous crystallin genes during *X. laevis* development, using wholemount in situ hybridization. The βA4- and γ-crystallin mRNAs are detectable from the early stages of lens development onward. αA-crystallin messengers could not be detected until between stages 33 and 34, when in the young tadpole the lens ectoderm invaginates, and the first cells start to differentiate. We also detected expression of the γ- and the βA4-crystallin genes in gastrulas (at stage 10). This demonstrates that members of these classes of crystallins are present at a time that part of the ectoderm of the gastrula has gained competence to form lens tissue.

**METHODS**

**Embryos**

*Xenopus laevis* embryos were obtained from the Department of Zoology of the University of Nijmegen or the Hubrecht Laboratory in Utrecht and were maintained in one-third Ringer’s solution until the desired stage was reached. Embryos were staged according to Nieuwkoop and Faber. The investigation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Construction and Screening of Complementary DNA Libraries**

Total RNA was isolated from lenses obtained from 10- to 12-day-old *X. laevis* tadpoles, using the guanidinium isothiocyanate procedure, described by Sambrook et al. Complementary DNA (cDNA) was prepared from the RNA according to the manufacturer’s protocol (Boehringer-Mannheim, Almere, the Netherlands), using oligo(dT) primers, and were cloned into λgt22 or λgt11 using EcoRI linkers. The λgt22 cDNA library was screened with a calf βA3-crystallin cDNA probe under low-stringency conditions. Expression screening of the λgt11 cDNA library was performed according to standard procedures, using a rabbit polyclonal antibody directed against calf crystallins. EcoRI inserts of positive phages were cloned into pBluescript and sequenced according to standard procedures.

Computer alignments and comparisons of the sequences were performed, using the GCG package of the CAOS–CAMM Center at the University of Nijmegen.

**Wholemount In Situ Hybridization**

Wholemount in situ hybridization was essentially performed, as described by Harland. Sense and antisense RNA probes were made, using the DIG RNA labeling kit according to the manufacturer’s protocol (Boehringer Mannheim). To enhance the sensitivity of the assay, 10% polyvinyl alcohol (13 to 23 kD; Aldrich, Milwaukee, WI) was included in the color-reaction mixture. After the color reaction, the embryos were refixed in 4% formaldehyde for 4 hours. The embryos were stored in methanol at −20°C until they were embedded in paraffin.

**RESULTS**

Cloning of *Xenopus laevis* α-, β-, and γ-Crystallin Complementary DNA

Low-stringency screening of a λgt22 cDNA library with a calf βA3-crystallin probe resulted in the isolation of a β-crystallin cDNA (Fig. 1A). The cDNA insert is 706 bp long (excluding the polyA tail), which correlates well with the length of the corresponding mRNA, determined by Northern blot analysis (data not shown). It codes for a protein of 196 amino acids. Sequence comparison revealed that this protein is the *X. laevis* homologue of βA4-crystallin. The deduced amino acid sequence shows a similarity of 78.4% and 73% with the calf and chicken βA4 proteins, respectively (Fig. 1B). Because we did not map the 5′ end of the βA4 mRNA, we cannot exclude that this mRNA possesses a more upstream start codon. However, we feel that this is unlikely, because the predicted protein is of the same length as that of the calf and chicken βA4-crystallin. α- and γ-crystallin cDNAs were obtained by screening a λgt11 library with a polyclonal antibody directed against total calf crystallins. Two partial cDNAs, one encoding the 56 C-terminal amino acids of αA-crystallin (Fig. 2A), the other encoding the 106 C-terminal amino acids of a γ-crystallin, were obtained (Fig. 3A). The similarity between the deduced amino acid sequence of the *X. laevis* αA-crystallin and the previously identified αA-crystallins ranges from 62.5% to 73.1% (Fig. 2B), with the highest score that of the North American opossum. The cDNA encoding the γ-crystallin represents a new member of the γ-crystallin gene family, because it differs from the γ-crystallin sequences identified by Smolich et al.

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sequence identity between the cloned region of newly identified γ-crystallin, which we call xcryf, and xcrya is 86.4%, whereas the predicted amino acid sequence identity with xcrya is 91.5%. The predicted amino acid sequence identity of xcryf with xcryb to xcrye is less, approximately 80%. Although the genome of X. laevis is tetraploid, xcryb probably does not represent the duplicated gene of xcrya, in that most duplicated genes share 96% to 98% sequence identity in their coding regions.15

Crystallin Expression During Lens Development

The expression of these α-, β-, and γ-crystallin genes during lens development of X. laevis was analyzed by wholemount in situ hybridization. The first crystallin messengers to be detected during X. laevis lens development are the γ-crystallin mRNAs, which are already present at stage 28 (Fig. 4). At this stage, the internal layer of the prospective lens ectoderm, underlain by the optic vesicle, thickens to form the lens placode. At stage 28, γ-crystallin gene expression is limited to these internal layers of the ectoderm (Fig. 4). In wholemount in situ hybridization, βA4-crystallin mRNAs were detectable somewhat later, at stage 29, when the lens ectoderm thickens further (data not shown). At this stage, βA4-crystallin mRNA could not be visualized in sections of lenses, presumably because of the low level of expression. At stages 30 and 33/34, the βA4-crystallin as well as the γ-crystallin messengers are located in the central cells, which probably correspond to the prospective primary lens fiber cells (Figs. 4, 5). The posteriorly located cells do not show expression of the βA4- or the γ-crystallin gene. These cells are thought to be precursors of the lens epithelial cells.21 The fact that γ-crystallin gene expression is detectable before βA4-crystallin gene expression does not necessarily mean that transcription of the latter gene is induced later during lens development. The signal obtained with the γ-crystallin probe is the sum

FIGURE 1. (A) Nucleotide and deduced amino acid sequence of the complementary DNA encoding βA4-crystallin of Xenopus laevis. The amino acids are shown below their respective codons. (B) Alignment of the βA4-crystallin sequences of the North American opossum,34 the frog (Rana temporaria),35 the rat,36 and human.12 The amino acid sequence of the 3' part of βA4-crystallin of Xenopus laevis is shown completely; for the other sequences, only differences are specified. Dots indicate sequence not determined. Above the sequence, the predicted limits of the structural motifs are indicated.

FIGURE 2. (A) The complementary DNA and the deduced amino acid sequence of the 5' part of αA-crystallin of Xenopus laevis. The amino acids are shown below their respective codons. (B) Sequence comparison of the deduced amino acid sequence of the C-terminal part of αA-crystallin from X. laevis with the αA-crystallin sequences of the North American opossum,34 the frog (Rana temporaria),35 the rat,36 and human.12 The deduced amino acid sequence of the X. laevis αA-crystallin complementary DNA is shown completely; for the other sequences, only differences are specified. A dash indicates a gap in the corresponding sequence.
Development whereas the βA4- and γ-crystallin messengers are located in the differentiated fiber cell zone.

Recently, it has been reported that the γ-crystallin genes are already expressed in the gastrula stages, using RNase protection and Northern blot analyses. During neurulation (stages 12 to 18), the expression levels decreased but were still detectable at the Northern blot level. To examine the site of crystallin expression in early X. laevis development and to determine whether other crystallin genes are also active at this stage, we analyzed gastrula stages of X. laevis by whole-mount in situ hybridization, using the αA-, βA4-, and γ-crystallin probes. The weak and nonreproducible signal obtained with the αA-crystallin probe (Fig. 6) did not differ from the signal obtained with the sense probe (data not shown) and therefore probably represents nonspecific hybridization. The strong and reproducible signals obtained with the γ- and β-crystallin probes show that not only are γ-crystallin mRNAs present during gastrulation, but βA4-crystallin messengers as well (Fig. 6). Expression is localized to the animal cap and marginal zone, extending to the dorsal lip (Fig. 6; note however, that the vegetal part of the gastrula is a poor substrate for in situ hybridization).

**DISCUSSION**

In this study, the spatial and temporal expression of a member of each class of the ubiquitous crystallin genes, α, β, and γ-crystallins, in X. laevis is described. The (partial) cDNAs encoding these crystallins were obtained from Xenopus lens cDNA libraries. Alignment of the deduced amino acid sequences identified these crystallin as the αA- and βA4-crystallins and as a novel member of the γ-crystallin family.

The βA4- and γ-crystallin transcripts are detectable at the first stage of lens development, characterized by a thickening of the presumptive lens ectoderm, which subsequently develops into the lens placode (stages 26/27 to 30). During further lens development, when the primary fiber cells are formed (stages 35 to 41), the expression of the βA4- and γ-crystallin genes is greatly enhanced. At stage 33/34, when the final shape of the lens, a central mass of differentiating fiber cells surrounded by a single layer of epithelial cells, becomes recognizable, the αA-crystallin gene expression also becomes detectable. The results obtained in this study correspond well with the observations made by others at the protein level. Using antibodies directed against total lens protein, crystallin proteins could be detected from stage 29/30 on in the area were fiber cells are forming. Later during lens development, at stage 41, fluorescence was also seen in the epithelial layer.

The developmental order of expression of the ubiquitous crystallins observed in X. laevis differs from that of crystallins in other vertebrates. In most vertebrates—for instance, in the rat—β- and γ-crystallin
gene expression is preceded by α-crystallin gene expression. This order reflects the differences in the pattern of lens development between X. laevis and the other vertebrates. Whereas in most vertebrates a lens vesicle whose cellular arrangement is similar to that of the final lens is formed after the lens placodal stage, in X. laevis, an irregular flattened mass of presumptive fiber cells is formed first. In X. laevis, a lens vesicle cannot be detected until stage 36/37, after which the typical single-layered epithelium is formed. This process coincides with the onset of αA-crystallin gene expression.

Smolich et al showed that γ-crystallin genes are also expressed during gastrulation and neurulation, long before lens formation. In the neurula stage γ-crystallin mRNAs could be detected in the anterior, middle, and posterior parts of the embryo, although most of the transcripts seemed to be localized anteriorly. Our wholemount in situ hybridization experiments confirm and extend these observations. We find that not only γ-crystallin genes but also a β-crystallin gene, the βA4 gene, is expressed in stage 10 embryos. However, in contrast to the findings of Smolich et al, we did not detect hybridization in embryos of the neural tube stage (data not shown). This is most likely because of the insensitivity of the in situ hybridization in comparison with RNase protection, because the level of the γ-crystallin mRNAs in the neurula was reported to be lower than that in gastrula stages. Alternatively, it is possible that expression of these...
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**Figure 6.** Expression of βA4- and γ-crystallin in stage 10 albino embryos. (A) Wholemount in situ hybridizations with αA-, βA4-, and γ-crystallin antisense probes to stage 10 albino embryos. Embryos were photographed in Murray's. Magnification, X50. The blastocoel has partially collapsed during the in situ hybridization procedure. Note that the signal obtained with the α-crystallin probe is weak and non-reproducible and therefore is likely to represent background hybridization. (B) Sagittal section (10 μ) of stage 10 albino embryo hybridized with antisense γ-crystallin probe showing staining of the animal cap. Magnification, X50.

A small area of the head ectoderm. Later, at the neural tube stage of development, when this region is underlaid by the optic vesicle, lens formation is induced. Finally, the primary and secondary differentiations of lens cells take place.

In view of this model, the animal cap cells of a *X. laevis* gastrula, which express βA4- and γ-crystallin mRNA, might be envisaged as the earliest precursors of lens cells. The acquisition of competence for lens formation of these cells could be coupled with a low level of expression of the βA4- and γ-crystallin genes. This would imply that the first steps toward lens formation are part of a cascade of signals that determine the ectodermal phenotype. This might also explain the relative ease with which a number of tissues of neuroectodermal origin—for instance, the retina or cornea—transdifferentiate into lens cells, given the right (culture) conditions.

Alternatively, the relatively uniform expression of these crystallin genes in the ectodermal tissue of the *X. laevis* gastrula embryo could be independent of lens induction. The presence of these crystallin mRNAs at the time when tissue is committed to lens formation may be the explanation for recruitment of these genes as crystallin genes. This would suggest an additional function of these proteins during embryogenesis.

**Key words**
crystallins, gastrula, lens development, *Xenopus laevis*

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