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Xenopus γ-Crystallin Gene Expression: Evidence that the γ-Crystallin Gene Family Is Transcribed in Lens and Nonlens Tissues

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Crystallins, the major gene products of the lens, accumulate to high levels during the differentiation of the vertebrate lens. Although crystallins were traditionally thought to be lens specific, it has recently been shown that some are also expressed at very low levels in nonlens tissues. We have examined the embryonic expression pattern of γ-crystallins, the most abundant crystallins of the embryonic lens in *Xenopus laevis*. The expression profile of five *Xenopus* γ-crystallin genes mirrors the pattern of lens differentiation in *X. laevis*, exhibiting on average a 100-fold increase between tailbud and tadpole stages. Four of these genes are also ubiquitously expressed outside the lens at a very low level, the first demonstration of nonlens expression of any γ-crystallin gene; expression of the remaining gene was not detected outside the head region, thus suggesting that there may be two classes of γ-crystallin genes in *X. laevis*. Predictions regarding control mechanisms responsible for this dual mode of expression are discussed. This study raises the question of whether any crystallin, on stringent examination, will be found exclusively in the lens.

As part of a developmental analysis of lens determination in *Xenopus laevis*, we have examined the temporal and spatial control of γ-crystallin gene expression. γ-Crystallin is the most abundant crystallin in the embryonic lens. Crystallins are the major proteins of the vertebrate lens and can be broadly divided into two major classes: the ubiquitous crystallins α-, β-, and γ-crystallin, which are found in most if not all vertebrates, and the taxon-specific crystallins, such as δ-crystallin, which is restricted to birds. Crystallins have been intensively studied at the levels of gene structure and regulation, and their expression has been correlated with the extent of lens differentiation (43). However, it has recently been shown that many crystallins have been recruited from genes encoding metabolic enzymes or stress-induced proteins and that in addition to their role as structural proteins in the lens, they are present at low levels in nonlens tissues, where they may serve other functions (30). A generalized control mechanism that discriminates between these two modes of expression, if one exists, has not been identified.

The different classes of crystallins vary in their expression patterns in lens and nonlens tissue and exhibit distinct evolutionary relationships with other proteins. Among the crystallins found in most vertebrates, αA and αB have previously been shown to be expressed outside the lens, although at only a small fraction of their level in the lens (8, 22). α-Crystallins are evolutionarily related to small heat shock proteins. The β- and γ-crystallins form a superfamily and are related to protein S, a bacterial spore coat protein, and to spherulin 3a, a stress-induced protein from *Physarum* spp. (19, 41, 42). Two members of the large and heterogeneous β-crystallin family have also been found outside the lens at a fraction of their level in the lens (16). The taxon-specific crystallins are very closely related or identical to metabolic enzymes; they are generally expressed outside the lens and often retain enzymatic activity (30).

The development of the lens provides an accessible system in which to study determination and differentiation, particularly in amphibians such as *X. laevis* which are so amenable to embryological experimentation, and crystallin gene expression serves as a molecular indicator of this developmental process. To briefly review this process in *X. laevis*, lens differentiation commences during early tailbud stages, shortly after the optic vesicle comes into contact with the adjacent presumptive lens ectoderm, causing the ectoderm to thicken into the lens placode (Nieuwkoop and Faber stage 26/27 [29]; lens induction reviewed in reference 13). As the placode thickens, the primary fiber cells form a mass in the center, with the presumptive epithelial cells surrounding it. The fiber cells proceed to elongate, and by later tadpole stages (41 to 45+) a single-layered lens epithelium surrounds the central fiber mass, with many cells in the core already having lost their nuclei, a characteristic of fully differentiated fiber cells (26). During lens development crystallin genes are differentially regulated, and the order and level of expression of the different classes in the embryonic lens varies somewhat among species. In birds δ-crystallins appear first, in the lens placode (44). In mammals α-crystallins are detected first (40), and in amphibians γ-crystallins, probably simultaneously with β-crystallins, appear first. Immunofluoresence studies and Western immunoblotting analysis have shown that in *X. laevis* γ-crystallins appear initially in the lens placode (stage 26/27) (17, 26, 35).

The temporal profile of γ-crystallin gene expression during lens development in mice and rats has previously been investigated. In mice, γ-crystallin mRNA was detected between days 10 and 15 of embryonic development, when the primary lens fibers are forming (28). In situ hybridization data from rats also reveal expression first in presumptive primary fiber cells (39). This is later than γ-crystallin expression in *X. laevis*, which, as described above, is observed

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prior to fiber cell elongation in the lens placode. In all species, abundant γ-crystallin expression appears to be restricted to the fiber cells and is excluded from the epithelial cells of the lens. γ-Crystallin expression outside the lens has not been detected in rats or mice.

We have assayed γ-crystallin mRNA accumulation at various stages of development in *X. laevis*. As expected, a dramatic increase in levels of mRNA was observed as lens differentiation proceeded. Surprisingly, however, a low level of mRNA was detected in nonlens tissue both early in development, prior to any morphological signs of lens differentiation, and at later tadpole stages in nonlens tissue. This is the first demonstration of nonlens expression of γ-crystallin in any species and leads us to hypothesize that a strictly lens-specific class of crystallins may not exist. In addition, the data from the individual γ-crystallin genes described here suggest that, within our limits of detection, two classes of γ-crystallin genes exist in *X. laevis*, one of which is expressed in both lens and nonlens tissue and a separate class which is undetectable outside the head.

MATERIALS AND METHODS

Embryos. *X. laevis* embryos were obtained as described by Henry and Grainger (17) and maintained in 20% Steinberg's solution (31) containing 50 μg of gentamicin sulfate per ml until the desired stage. Embryos were staged by the method of Nieuwkoop and Faber (29).

RNA extraction. Embryos or portions of embryos were collected and frozen in liquid nitrogen. RNA was extracted by the sodium dodecyl sulfate (SDS)-proteinase K method, and poly(A)$^+$ RNA was isolated by oligo(dT) chromatography, as described by Sambrook et al. (34).

Generation of probes for RNA expression studies. The γ4-cry probe used in the Northern (RNA) blot analysis was generated from the 600-bp insert of a *Xenopus* γ-crystallin cDNA (36). Radiolabeled probes were synthesized by a random-primed labeling reaction by the method of Feinberg and Vogelstein (10). Protection assay probes for all genes were subcloned into Bluescript vectors (Stratagene). For a schematic representation of the probes, see Fig. 4. The γ1-cry 3′ coding region probe is a 128-bp *Hae*III fragment from exon 3 of γ1-cry. The γ2-cry coding probe is a 300-bp *Rsa*I fragment including a portion of intron 2 plus 180 bp of exon 3. The γ3-cry coding region probe is a 168-bp region from exon 3 generated by deleting the intron from a larger *Pvu*II-*Rsa*I fragment. The γ4-cry probe is a 132-bp *Bam*HI-*Eco*RI fragment from exon 2, and the γ5-cry probe consists of 125 bp of exon 2 followed by 80 bp of intron 2. The probe from the 5′ region of γ1-cry is a 345-bp fragment extending from 290 bp upstream of the translation start codon to 4 bp downstream of exon 1. The γ2-cry 5′ probe is a 220-bp *Hpa*II-*Hin*dIII fragment extending from 110 bp upstream of the translation start site to 100 bp downstream of exon 1. The 5′ γ3-cry probe has its 5′ end 340 bp upstream of the translation start site and extends 60 bp downstream of exon 1. The EF-1α probe is a modification of pSp64 EF-1α/Gs17 (25), kindly provided by M. Wormington.

Northern blotting. RNA was electrophoresed on 1.2% agarose–formaldehyde gels and blotted onto GeneScreen (DuPont/New England Nuclear). After blotting, filters were UV cross-linked at 1,200 μW/cm^2 for 2 min per side. Hybridizations were performed for 12 to 16 h at 44°C in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–5× Denhardt's solution–1% SDS–100 μg of salmon sperm DNA per ml. Filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS up to 65°C for 1 to 2 h and then subjected to autoradiography with a Lightning Plus intensifying screen.

RNase protection assays. Assays were performed essentially as described by Melton et al. (27), with some modifications as described by Saha and Grainger (33). Hybridizations, with up to 25 μg of RNA, were carried out at 57 to 62°C overnight with 2×10^5 to 2.5×10^5 cpm of probe, in 20 μl. The specificity of the protection assay was examined, using the coding-region probes from γ1-, γ2- and γ3-cry, by the observation that pairwise hybridizations between in vitro transcribed RNA from one gene and an antisense RNA probe from another gene did not yield full-size protected fragments (data not shown). To determine the lower limit of sensitivity of the protection assays, known amounts of in vitro transcribed sense RNA from the individual γ-crystallin genes were hybridized with the homologous probes. Using this method, we were consistently able to detect 0.1 pg of sense transcript.

Quantitation of transcripts. To quantitate the amount of γ-crystallin mRNA present during development, hybridization signals from the Northern blot analysis were compared with signals generated by hybridizing known amounts of sense transcript from γ4-cry with its homologous probe. Appropriately exposed autoradiograms, which demonstrated a linear increase in intensity with increasing input RNA, were scanned by using a Gilford Response spectrophotometer with a gel-scanning attachment. The standard curves generated were used to quantitate scanned experimental samples from Northern blots from two separate experiments. The quantitated values generated from each experiment were within a factor of 2, and the values referred to in the text represent the average of the two experiments. The amount of embryo RNA present in each sample was quantitated by rehybridizing the blot with EF-1α probe.

To estimate the number of copies per cell of γ-crystallin mRNA in nonlens tissues, appropriately exposed autoradiograms of protection assays were scanned and quantitated, as described above, by using standard curves generated from the coding region probes of γ1-, γ2-, and γ3-cry.

In situ hybridization. Whole-mount in situ hybridization was performed as described by Harland (14) with the probes constructed for the protection assays.

RESULTS

Northern blot analysis of γ-crystallin expression. To examine the temporal and spatial pattern of γ-crystallin mRNA levels during *Xenopus* development, a *Xenopus* γ-crystallin cDNA (γ4-cry [36]) was used as a probe in a Northern blot analysis of tadpole (stage 42) lens versus head-minus-lens RNA. The single hybridizing band (0.7 kb), corresponding to the predicted size of γ-crystallin mRNA, was highly enriched in the tadpole lens (Fig. 1A), as anticipated from the extent of differentiation of the lens at this stage. No signal was detected in the head-minus-lens fraction in these experiments. The blot was rehybridized with elongation factor 1α (EF-1α), which is ubiquitously expressed during *Xenopus* development after blastula stages (25), to control for RNA loading. Detectable EF-1α signal was not observed in the lens lane because of the small amount of RNA in that sample.

The temporal profile of γ-crystallin mRNA levels during lens development was assessed with γ4-cry as a probe to RNA isolated from whole embryos at successive stages of

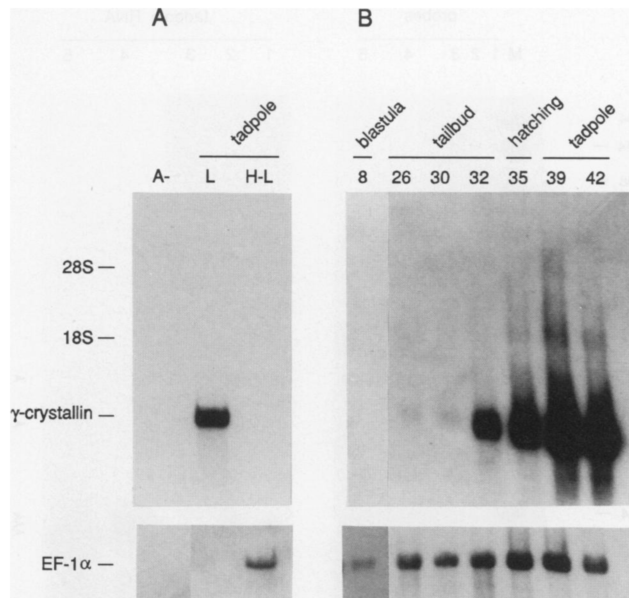

FIG. 1. RNA blot analysis of γ-crystallin expression during *Xenopus* development. The blots were hybridized with γ4-cry to detect γ-crystallin mRNA and subsequently with EF-1α to demonstrate the presence of RNA in each lane. Numbers above the lanes indicate stages from which RNA was extracted. (A) Lanes: A^-, 3 μg of poly(A)$^-$ RNA; L and H-L, one embryo equivalent of RNA from tadpole (stage 42) lens (0.2 μg) and head minus lens (2.5 μg), respectively. Exposure was for 24 h. (B) Lanes representing stages 26 to 42 contain approximately 8 μg of total RNA each. The stage 8 lane contains 0.5 μg of poly(A)$^+$ RNA. Exposure was for 5 days.

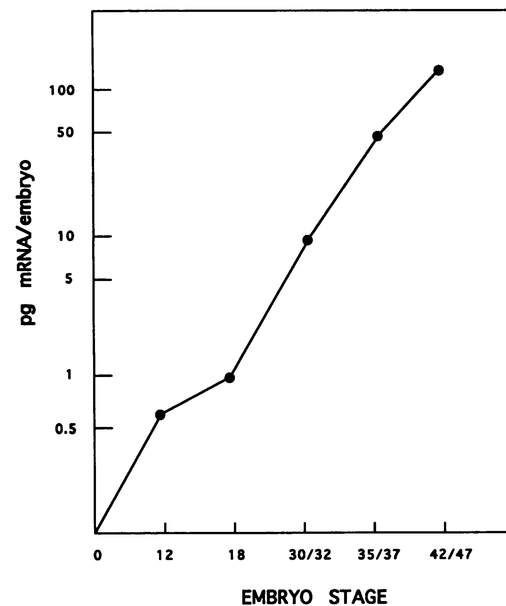

FIG. 2. Quantitation of γ-crystallin mRNA levels during development. The graph represents the steady-state levels of transcripts hybridizing to γ4-cry. The vertical axis is a log scale to allow better resolution at the earlier stages. Embryo stages are those defined by Nieuwkoop and Faber (29).

lens development. At the lens placode stage (stage 26/27), a low level of γ-crystallin mRNA, corresponding to approximately 1.2 pg per embryo, was detected (Fig. 1B). As the placode continues to thicken and the primary fiber cells begin to elongate, the level of γ-crystallin expression increases, to 12 pg per embryo by early tailbud stages (stage 30/32 [Fig. 1B]). The strong increase in mRNA levels in young tadpoles (stage 35/36) corresponds to approximately 25 pg per embryo and coincides with the recognizable formation of the lens fiber area. At later tadpole stages (stage 42), when many fiber cells in the lens nucleus have fully differentiated (26), γ-crystallin mRNA levels continue to increase. Quantitation by scanning densitometry indicated an increase of several hundred-fold in the total amount of γ-crystallin mRNA per embryo from placodal to tadpole stages (Fig. 2).

We wished to determine whether *Xenopus* γ-crystallin mRNA is expressed during very early stages of lens determination, when the presumptive lens ectoderm acquires a lens-forming bias as a result of tissue interactions occurring during late gastrulation and early neurulation (stage 12 to 14 [17]). When RNA from early developmental stages (stage 8 blastula through stage 18 neurula) was analyzed with γ4-cry, γ-crystallin transcripts were not detected in blastula stage embryos but, unexpectedly, were detectable at all subsequent stages (Fig. 3A). (The weak EF-1α signal in the blastula lane correlates with the approximately 10-fold difference in EF-1α mRNA levels between blastula and gastrula stages [25].) In gastrula stage embryos (stage 10 to 12), considerably prior to lens determination, transcript levels were very low, approximately 0.7 pg per embryo. Levels increased slightly through neurula stage (stage 18), to about 1.1 pg per embryo (Fig. 3A). This basal level of expression is maintained until early tailbud stages, at which time the level of γ-crystallin mRNA increases dramatically in concert with the differentiation of the lens. Figure 2 graphically depicts the quantitation of γ-crystallin transcripts. Analysis of the spatial distribution of the mRNA at these early stages indicated that γ-crystallin mRNA is found at approximately equal levels in the anterior, middle, and posterior thirds of neurula embryos (Fig. 3B). It seems unlikely, therefore, that all early γ-crystallin expression is in response to initial lens induction signals occurring during gastrulation and neurulation, since these would be expected to be restricted to the presumptive lens or head ectoderm. Thus the increase in γ-crystallin transcript levels early in development (until neurula stages) is due mainly to ubiquitous nonlens expression, a surprising result in view of the lens specificity normally associated with γ-crystallin expression. In contrast, it is likely that all of the increase in γ-crystallin levels observed later in development is due to the overt differentiation of the lens between tailbud and tadpole stages. This is substantiated by the extremely high levels of γ-crystallin expression in the head region, as opposed to the middle or posterior regions, observed for individual γ-crystallin genes (or pairs of related genes) at tadpole stages (analyzed by RNase protection assays [described below]).

RNase protection assay analysis of γ-crystallin expression. We wished to examine the mRNA levels from individual γ-crystallin genes, by using RNase protection assays, to determine whether all genes exhibit the same distribution between lens and nonlens tissue. γ-Crystallins in *X. laevis* and in other species are encoded by a multigene family (43), and, on the basis of the high degree of sequence homology between the cloned *Xenopus* γ-crystallin genes, (85 to 90% at the nucleotide level [36]), it is likely that during the Northern blot analysis γ4-cry was hybridizing to all or virtually all γ-crystallin mRNAs. It was anticipated, how-

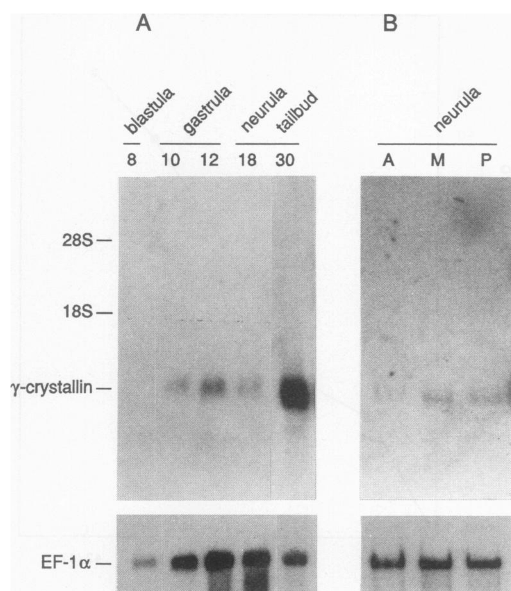

FIG. 3. Higher-sensitivity RNA blot analysis of γ-crystallin expression during early *Xenopus* development. The blots were hybridized with γ4-cry to detect γ-crystallin mRNA and subsequently with EF-1α to demonstrate the presence of RNA in each lane. Numbers above the lanes indicate stages from which RNA was extracted. (A) Stage 8 to 18 lanes contain approximately 0.5 μg of poly(A)$^+$ RNA; the tailbud (stage 30) lane contains 8 μg of total RNA. Exposure was for 5 days. (B) Neurula embryos (stage 18) were dissected into anterior (A), middle (M), and posterior (P) thirds. Approximately 1.0 μg of poly(A)$^+$ RNA was loaded into each lane, and the blot was exposed for 7 days.

ever, that transcript detection would be gene specific in the protection assays, given the stringent conditions used. Probes were generated from the coding region of four *Xenopus* γ-crystallin genes, γ1-, γ2-, γ3-, and γ5-cry, and one cDNA, γ4-cry (36) (Fig. 4). RNase protection analysis of RNA from tadpole embryos (stage 42 to 47), as depicted in

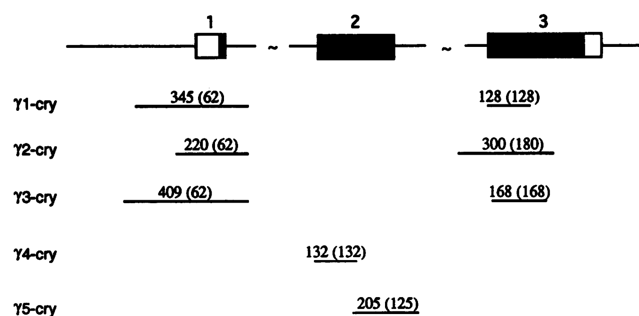

FIG. 4. Schematic representation of probes generated from γ1-, γ2-, γ3-, γ4-, and γ5-cry used in RNase protection assays. The top line represents the conserved intron-exon structure of γ-crystallin genes, and the exons are numbered 1, 2, and 3. Solid boxes represent protein-coding regions; open boxes represent 5′ and 3′ untranslated regions. The lines beneath the schematic gene structure represent the regions from each gene which were subcloned for probes. The numbers refer to the length, in nucleotides, of the crystallin sequences in the probe. The numbers in parentheses refer to the predicted length of the protected fragment. The transcribed probes seen in Fig. 5 and 7 are longer because of transcription of additional Bluescript plasmid sequences.

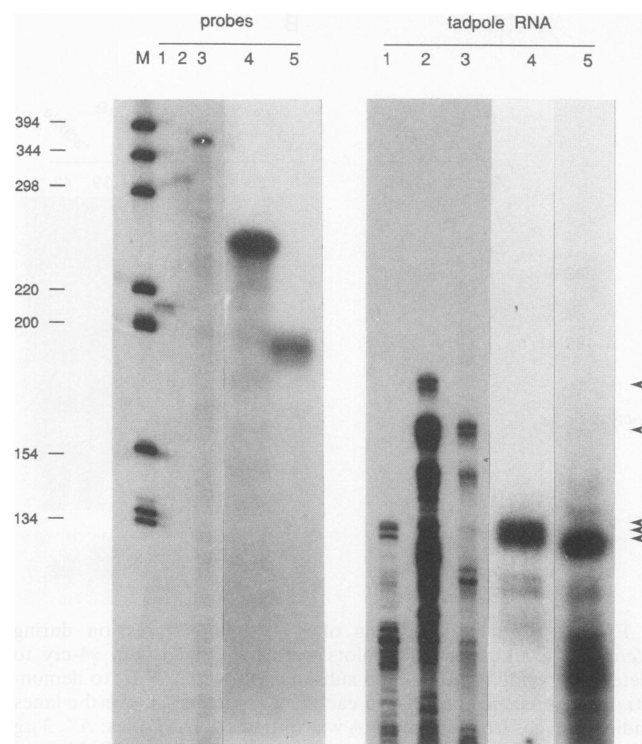

FIG. 5. RNase protection analysis of γ1-, γ2-, γ3-, γ4-, and γ5-cry expression in tadpole (stage 42) embryos. Sizes of the markers (M) are indicated on the left. Unhybridized probe is shown in probe lanes for γ1-cry (lane 1), γ2-cry (lane 2), γ3-cry (lane 3), γ4-cry (lane 4), and γ5-cry (lane 5). Tadpole RNA (7.5 μg) was hybridized with each probe, yielding the protected fragments indicated on the right. The arrowheads on the right mark the positions of the protected fragments as follows (from top to bottom arrowhead): γ2-cry, 180 bases; γ3-cry, 168 bases; γ4-cry, 132 bases; γ1-cry, 128 bases; and γ5-cry, 125 bases. The difference in bandwidths evident in some lanes is due to use of different gel systems; however, the markers on the left reflect accurate sizes for all lanes. An EF-1α probe was included in each sample and confirmed the recovery of RNA in each lane (data not shown). Exposure was for 18 h.

Fig. 5, revealed fragments of the predicted size with each of the five probes (the top band in each lane). Although the intensity of the top band did not vary between experiments, smaller fragments, which we believe are generated from closely related γ-crystallin genes (36), did vary in intensity, presumably owing to minor variations in assay conditions.

Analysis of γ-crystallin mRNAs through lens development indicates that transcripts hybridizing with each probe mirror the expression pattern of the total γ-crystallin mRNA pool assessed by Northern analysis. mRNAs complementary to γ1-, γ2-, γ3-, γ4-, and γ5-cry were each abundant at tadpole stages (stage 42 to 47), as expected at this late stage in lens differentiation (Fig. 6A). Analysis of γ-crystallin transcript levels during earlier stages of lens development indicated that the level of all five mRNAs was quite low during placodal and early tailbud stages (stage 26 to 32) but increased dramatically as differentiation proceeds (Fig. 6A). EF-1α was included as a control in all protection assays to assess RNA loading. There is some variation in the levels of mRNAs detected at different stages (confirmed by quantitation of the protection assays as described for the Northern blots [data not shown]); however, the overall pattern was the

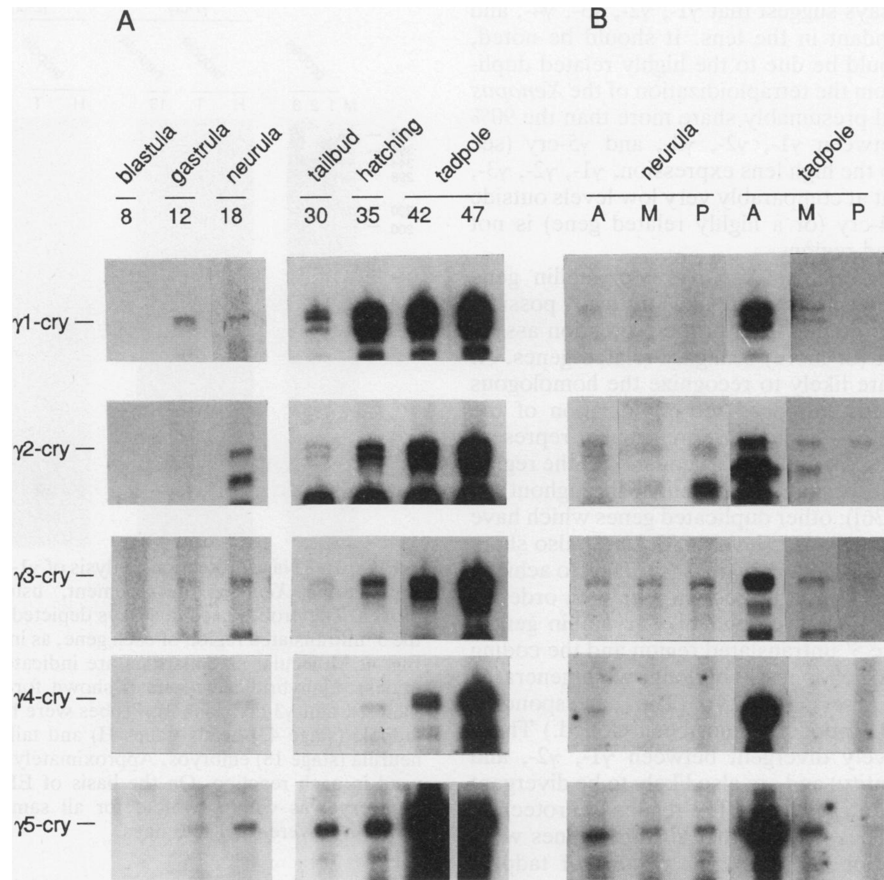

FIG. 6. RNase protection analysis of γ1-, γ2-, γ3-, γ4-, and γ5-cry expression during *Xenopus* development. Stages from which the RNA was isolated are indicated by the numbers above the lanes. The probe used in each assay is indicated on the left. An EF-1α probe was included in each sample to confirm the recovery of RNA in each lane (data not shown), as indicated below. (A) Total RNA (25 μg) was used for the hybridization to stage 8 to 18. RNA (12.5 μg) was used for stage 30 to 47. On the basis of EF-1α quantitation, RNA recovery was within twofold for all samples (data not shown), except for the stage 30 sample hybridized with the γ3-cry probe, in which approximately one-third of the RNA was recovered, and the stage 42 sample hybridized with the γ4-cry probe, in which approximately one-quarter of the RNA was recovered. Exposures for stage 8 to 18 were for 3 to 7 days; exposures for the later stages (stages 30 to 47) were for 24 h. (B) Total RNA (25 μg) from anterior (A), middle (M), and posterior (P) sections of stage 18 neurula embryos was hybridized with each probe. Exposures were for 5 days. Hybridizations with tadpole (stage 42) middle (M) and posterior (P) sections contained 25 μg total RNA each, whereas 7.5 μg of anterior (A) RNA was used. Exposures were for 5 days for lanes M and P, and 24 h for lanes A. On the basis of EF-1α quantitation, RNA recovery was within twofold for all samples (data not shown), except for the tadpole posterior sample hybridized with the γ5-cry probe, in which approximately one-fourth of the RNA was recovered.

same for all five probes and was consistent with the accumulation profile detected with γ4-cry on Northern blots, presumed to represent the total γ-crystallin family.

To determine which of the γ-crystallin genes are detectable outside the lens or presumptive lens area, both early-stage blastula through neurula embryos (stage 8 to 18) and nonlens portions of neurula (stage 18) and tadpole (stage 42) embryos were analyzed with these probes by RNase protection assays. All five probes indicated the presence of γ-crystallin mRNA in a late neurula stage embryo but at levels considerably lower than at tadpole stages (Fig. 6A). Prior to neurula stages, γ-crystallin mRNAs were less consistently detectable, although late gastrula/early neurula stages were positive for γ1-, γ2-, γ3-, and γ5-cry but negative for γ4-cry. Blastula stages were negative with all probes (Fig. 6A). Thus probes derived from γ1-, γ2-, γ3-, and γ5-cry each detected mRNAs in a pattern that was consistent with the accumulation of γ-crystallin mRNA based on the Northern analysis, whereas γ4-cry was undetectable until neurula stages. Transcripts complementary to γ1-, γ2-, γ3-, and γ5-cry were also detected in anterior, middle, and posterior thirds of neurula embryos, at very low but relatively equal levels, in accord with the Northern blot results (Fig. 6B). Surprisingly, γ4-cry expression was detectable only in the anterior third of neurula embryos, also at low levels, indicating that under these assay conditions when we can detect as little as 0.1 pg of mRNA (see Materials and Methods), early expression of this gene is limited to the head region (Fig. 6B). In addition, γ4-cry expression seems to decrease between neurula and tailbud stages, before increasing up to tadpole stages. Following the pattern observed at neurula stages, middle and posterior thirds from tadpole embryos were also positive for γ-crystallin mRNA with γ1-, γ2-, γ3-, and γ5-cry probes, whereas γ4-cry was negative. The levels of expression in middle and posterior regions are less than 1% of that seen in the anterior third (Fig. 6B). Thus γ1-, γ2-, γ3-, and γ5-cry nonlens expression is not confined to early developmental stages but persists through later stages. The results of the

RNase protection assays suggest that γ1-, γ2-, γ3-, γ4-, and γ5-cry are each abundant in the lens. It should be noted, however, that this could be due to the highly related duplicated genes arising from the tetraploidization of the *Xenopus* genome, which would presumably share more than the 90% identity observed between γ1-, γ2-, γ3-, and γ5-cry (see below). In addition to the high lens expression, γ1-, γ2-, γ3-, and γ5-cry are present at comparably very low levels outside the lens, whereas γ4-cry (or a highly related gene) is not found outside the head region.

Since only a subset of the *Xenopus* γ-crystallin gene family members have been cloned, it is theoretically possible that the coding region probes used in the protection assays are recognizing a pair (or more) of highly related genes. At the very least, they are likely to recognize the homologous genes generated by the proposed tetraploidization of the *Xenopus* genome (24). γ3- and γ5-cry presumably represent duplicated genes, given their identical sequence in the region of the coding region probes (and 98% identity throughout the entire coding region [36]); other duplicated genes which have been analyzed at the nucleotide level in *X. laevis* also share 96 to 98% identity in their coding regions (4). Thus to achieve even further specificity in the protection assays in order to determine the distribution of individual γ-crystallin genes, probes which span the 5′ untranslated region and the coding sequence of exon 1 which is just 9 bp long, were generated from γ1-, γ2-, and γ3-cry (Fig. 4). (The corresponding sequences in γ4- and γ5-cry have not been cloned.) These sequences are relatively divergent between γ1-, γ2-, and γ3-cry (60 to 65% identity) and are also likely to be divergent even between duplicated genes (4). The results of protection assays with these probes showed that all three genes were detected in the anterior and posterior regions of tadpole embryos (stage 42), as well as in whole neurula embryos (stage 18) (Fig. 7). Although γ2- and γ3-cry are slightly enriched in the head sample, in general the lens enrichment observed with these probes is not as readily apparent as that seen previously with coding-region probes. This may be because the individual gene detected by the untranslated probe is not as lens enriched as the duplicated gene (or the sum of the two genes) detected with the coding-region probe. To investigate this further, we performed RNase protection assays with the untranslated probes by using lens versus head-minus-lens RNA, as opposed to head versus tail RNA, to discern whether there is even a slight enrichment in the lens, which might not have been observed when whole-head tissues were examined. This analysis confirmed the lens enrichment of these genes (data not shown), although the enrichment was not as extensive as with the coding-region probes.

In situ hybridization. To determine more precisely the spatial distribution of γ-crystallin mRNA and to substantiate the protection assay results, whole-mount in situ hybridizations to albino embryos were performed with the coding region and 5′ untranslated probes from γ1- and γ3-cry, as well as the entire γ4-cry cDNA. In tadpole embryos, the coding-region probes and the cDNA all detected high levels of γ-crystallin expression in the lens, in agreement with the RNase protection results. Hybridization of the γ4-cry probe to a stage 32 embryo is shown in Fig. 8A. As noted above, in protection assays the 5′ probes detected clear lens expression, although at lower levels. To substantiate this observation the 5′ probe from γ1-cry was used in in situ hybridizations as shown in Fig. 8B. A clear lens signal is apparent, although at consistently lower levels than with the coding region probe. Expression was not detected outside the lens

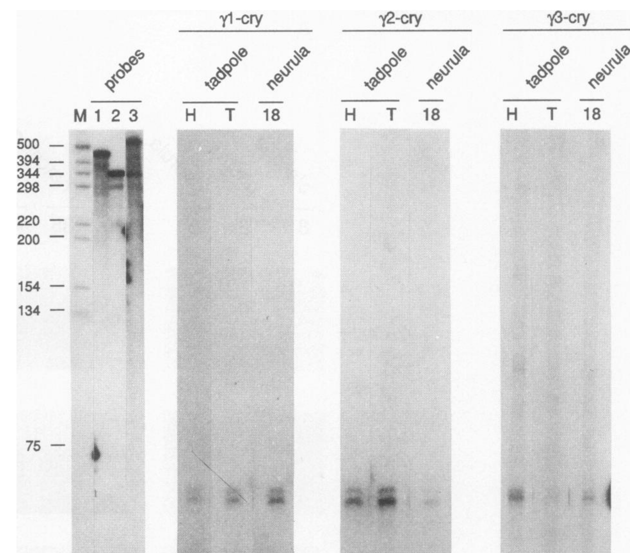

FIG. 7. RNase protection analysis of γ1-, γ2-, and γ3-cry expression during *Xenopus* development, using untranslated region probes. The probes used in assays depicted here were derived from the 5′ untranslated region of each gene, as indicated schematically in Fig. 4. Molecular size markers are indicated on the left (in nucleotides). Unhybridized probe is shown for γ1-cry (lane 1), γ2-cry (lane 2), and γ3-cry (lane 3). Probes were hybridized to RNA from tadpole (stage 42) heads (lanes H) and tails (lanes T), and whole-neurula (stage 18) embryos. Approximately 20 μg of total RNA was used in each reaction. On the basis of EF-1α quantitation, RNA recovery was within twofold for all samples (data not shown). Exposures were for 1 to 3 days.

with either set of probes, presumably because low levels of a ubiquitously expressed gene would be difficult to detect above the background level inherent in this technique. Probes generated from the sense strand consistently yielded no detectable signal (Fig. 8C).

DISCUSSION

***Xenopus* γ-crystallin genes are expressed at high levels during lens development.** The increase in steady-state levels of γ-crystallin mRNA from each of five genes during lens development reflects the pattern of lens differentiation in *X. laevis*, although some variation was observed in the levels of the individual genes. The differences in levels of expression are already apparent at placodal and early tailbud stages (stage 26 to 30), and the relative differences in mRNA level are maintained through tadpole stages (stage 42 to 47), although sequences complementary to γ3-cry accumulate to a slightly higher level than those complementary to γ1-cry by late tadpole stage (stage 47). Differential accumulation of γ-crystallin mRNAs has previously been observed in mice and rats, in which severalfold differences in abundance were detected between the individual γ-crystallin mRNAs (28, 40). Differential spatial and temporal γ-crystallin expression may be expected if distinct crystallin polypeptides fulfill unique functional roles in the lens. Since the spatial arrangement of all classes of crystallins in the lens is believed to be critical in determining the refractive properties of the lens, the heterogeneity in γ-crystallin mRNA levels observed in *X. laevis* could be of functional significance (6).

***Xenopus* γ-crystallin genes are expressed outside the lens at low levels.** The finding that γ-crystallins are expressed out-

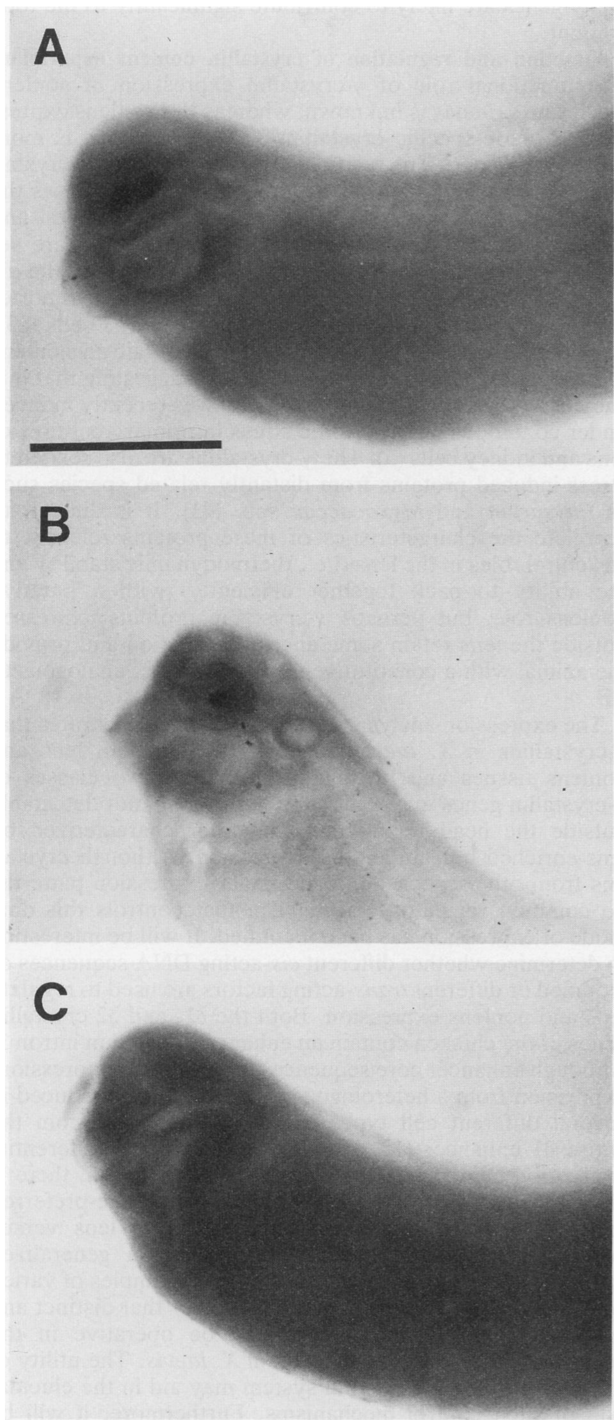

FIG. 8. Whole-mount in situ hybridization analysis. (A) Hybridization of an antisense RNA probe made from the entire γ4-cry cDNA to a stage 32 albino embryo shows strong staining in the lens. (B) A stage 35 embryo which was hybridized to a probe made from the 5′ untranslated region of γ1-cry is shown. In agreement with the RNase protection assay results, clear lens staining is apparent but is present at lower levels than that observed with probes of this gene made from the translated region. (C) As a control for monitoring background hybridization, stage 35 embryos were hybridized with a sense probe made from the untranslated region of γ1-cry. No staining is detected in the lens or elsewhere. Bar, 1 mm.

side the lens in *X. laevis* was surprising, since it has previously been argued that γ-crystallins are tissue-specific gene products (43). Although the Northern blot analysis demonstrated nonlens expression for the γ-crystallin family, it was only possible to detect the low level of *Xenopus* γ-crystallin mRNA from individual genes (or duplicated genes) in nonlens tissues and to compare this with the level of expression in the lens, because of the sensitivity of the RNase protection assays used, in which 0.1 pg of control transcript could be detected after a few days of exposure. It is likely, however, that *X. laevis* will not be the sole exception to lens-specific expression in this class of crystallin. Because of the evolutionary relationships that γ-crystallins share with other proteins and the high degree of conservation observed between γ-crystallin sequences from different species, it is possible, and perhaps probable, that an assay with sensitivity comparable to that used in this study will detect γ-crystallin expression outside the lens in other species. Another gene product presumed to be cell type specific, *Xenopus* MyoD, is expressed ubiquitously throughout the embryo early in development, although this expression is transient and MyoD is subsequently restricted to muscle precursor cells (32). It should be noted that this low level of expression is not detected for every gene product associated with a differentiated cell type. Recent studies have shown that opsin, the major gene product of the neural retina, is not detected outside the eye when assayed at the same level of sensitivity as crystallin (33).

When the distribution of individual *Xenopus* γ-crystallin gene products was assayed with respect to lens and nonlens expression, within our limits of detection (our assays are capable of detecting as little as 0.1 pg of mRNA, as noted above) two distinct patterns of expression emerged. γ4-cry is restricted to the head region from neurula stages on (stage 18) and will be discussed below. In contrast, γ1-, γ2-, γ3-, and γ5-cry were first detected during gastrula stages (stage 10 to 12), well before any lens differentiation has occurred. The continued low level of expression of these genes through neurula stages (stage 18), detectable throughout the embryo, indicates that this early expression is not a response to initial determinative events in lens induction, which would be restricted to presumptive head ectoderm (17). A functional role for γ-crystallin mRNAs outside the lens (assuming that they are translated, which is not presently known) is subject to temporal regulation, since there does not appear to be a maternal component to γ-crystallin expression. Nonlens expression of γ-crystallin mRNA is not restricted to early embryogenesis but continues in other differentiated tissues present by tadpole stages (stage 42) in middle and posterior regions. This continued expression of γ-crystallin mRNA suggests that any γ-crystallin protein that is present may play a role in general cellular processes, as opposed to having a strictly developmental function. The fact that these genes were not detected outside the lens by in situ hybridization suggests that there is not a subpopulation of cells expressing higher levels of γ-crystallin mRNA but that distribution is uniformly low in all cells. It will be of interest to determine whether nonlens expression of γ-crystallin mRNA persists in adult tissues.

The finding of nonlens expression of γ-crystallin mRNAs in *X. laevis* is in contrast to reports that γ-crystallin promoters from other species are lens specific. Two rodent γ-crystallin promoters have been reported to direct lens-specific expression in *Xenopus* tadpoles (2). However, if the transgene in these experiments is expressed in nonlens tissue, it may be below the level of detection for this kind of assay,

taking into account both the nonspecific activity which was observed and the fact that the distribution of the transgene DNA is probably mosaic (21). It is also possible that the promoter regions that were used lack sequences required for recognition by *Xenopus* factors to direct nonlens expression. A muscle-preferred enhancer element has been identified upstream of the murine αB-crystallin gene, suggesting that lens and nonlens expression may be controlled by previously identified overlapping common regulatory elements combined with tissue-specific enhancer elements (7). It is also possible that the rodent promoters that were tested are lens specific. In another example of tissue specificity of γ-crystallin promoters, lens-specific defects were induced in transgenic mice by fusion of the mouse γ2 promoter to the diphtheria toxin A gene. These defects could also be explained by lack of necessary control sequences or low levels of expression, especially since phenotypic heterogeneity among the affected eyes and lenses in the transgenic mice was observed, suggesting that lethal amounts of the toxin do not accumulate in all cells (3). Thus, until sensitive expression studies such as those performed with *X. laevis* are performed with other species, the possibility for nonlens expression of γ-crystallins, as is the case in *X. laevis*, remains.

Among the other classes of crystallins found in all vertebrates, nonlens expression has been demonstrated for some members of the β-crystallin gene family and for the α-crystallins. A more thorough analysis of expression patterns of the large β-crystallin family is required, although transcripts from at least two β-crystallin genes have been detected in the developing chicken retina, at levels between 1 and 0.1% of that found in the lens (16). For α-crystallins, with only two family members, the analysis is more complete, and it is clear that a somewhat different expression profile is exhibited from that observed for *Xenopus* γ-crystallins. αA accounts for most of the α-crystallin in the lens but has recently been shown to be present at low levels in other tissues in rats (22). αB, although expressed mainly in the lens, is also expressed at significant levels in several nonlens tissues (8). In general the level of nonlens expression appears higher for αB than for γ1-, γ2-, γ3-, or γ5-cry, whereas the level of expression in the lens appears to be approximately the same. However, neither α-crystallin gene has the more restricted pattern of expression seen for γ4-cry in *X. laevis*.

An example of crystallin expression which may be more comparable to γ-crystallins in *X. laevis*, and one of the only examples of nonlens crystallin expression examined during early development, is that of the δ-crystallin genes in the chicken, which are expressed at extremely low levels outside the lens (1, 37). Of the two δ-crystallin genes, δ1 mRNA accounts for 95% of the δ-crystallin in the lens, whereas δ2 accounts for 65 and 80% of the δ-crystallin mRNA in the heart and brain, respectively (38). Even though δ2 is the more prevalent species in the heart and brain, it is estimated that there is on average only one copy per cell in these tissues, similar to the levels estimated for γ1-, γ2-, and γ3-cry (see Materials and Methods for explanation of quantitation); however, there is still 100- to 1,000-fold more δ2 in the lens than in nonlens tissues. Thus the low levels of δ2 mRNA in the heart and brain and the relatively high levels of δ2 mRNA in the lens may be comparable to the patterns described here for γ1-, γ2-, γ3-, and γ5-cry. A major difference between γ- and δ-crystallins however, and also between γ- and α-crystallins, is that a single *Xenopus* γ-crystallin gene does not appear to contribute the majority of the γ-crystallin mRNA found in the lens, but, rather, many genes are likely to contribute significantly to the total amount.

Function and regulation of crystallin nonlens expression. The functional role of γ-crystallin expression in nonlens tissues in *X. laevis* is unknown, whereas the nonlens expression of taxon-specific crystallins and α-crystallins is more readily explained. The relationship of taxon-specific crystallins to metabolic enzymes, either as shared genes or as the same gene, provides a basis for both their expression and function outside the lens (30). The α-crystallins share sequence similarity with small heat shock proteins, and the αB promoter is able to confer heat inducibility on a reporter gene in transient-transfection assays of NIH 3T3 cells (23). In addition, αB has been shown to accumulate in nonlens tissues under certain disease conditions, suggesting that it is subject to stress regulation (9, 20), and was recently induced under conditions of hypertonic stress in primary cultures of lens and kidney cells (5). The γ-crystallins are also related to stress-induced proteins from distantly related species such as *Physarum* and *Myxococcus* spp. (41). It is difficult to correlate the characteristics of these proteins relevant to structural roles in the lens (i.e., thermodynamic stability and the ability to pack together efficiently) with a putative nonlens role, but perhaps γ-crystallin proteins expressed outside the lens retain some ancestral function and provide the animal with a constitutive stress response, analogous to αB.

The expression analysis described here demonstrates that γ-crystallins in *X. laevis* are expressed in both lens and nonlens tissues and further suggests that two classes of γ-crystallin genes may exist in *X. laevis*, one not detectable outside the head region and the other characterized by lens-enriched but ubiquitous expression. Although crystallins from other species exhibit similar expression patterns, no common regulatory mechanism that controls this dual mode of expression has been identified. It will be interesting to determine whether different *cis*-acting DNA sequences or modified or different *trans*-acting factors are used to regulate lens and nonlens expression. Both the δ1- and δ2-crystallin genes of the chicken contain an enhancer located in intron 3. Although enhancer core sequences promote lens expression, expression from a heterologous promoter can be induced in several different cell types by using sequences from the entire δ1 enhancer (12, 15, 38). In contrast to differential expression being specified by enhancer sequences, there is also at least one example of alternative tissue-preferred promoters directing crystallin expression in lens versus nonlens tissue (11); however, this is not a generalized crystallin control mechanism (18). These examples of varied control schemes underscore the possibility that distinct and varied regulatory mechanisms may be operative in the control of γ-crystallin expression in *X. laevis*. The utility of *X. laevis* as an experimental system may aid in the elucidation of such control mechanisms. Furthermore, it will be informative to undertake expression studies at a higher level of sensitivity in other species, to determine whether nonlens expression of γ-crystallins is a common occurrence and, if so, whether regulatory mechanisms similar to those in *X. laevis* control expression.

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REFERENCES

1. **Agata, K., K. Yasuda, and T. S. Okada.** 1983. Gene coding for a lens-specific protein, δ-crystallin, is transcribed in non-lens tissues of chicken embryos. Dev. Biol. **100:**222–226.
2. **Brakenhoff, R. H., R. C. Ruuls, E. H. M. Jacobs, J. G. G. Schoenmakers, and N. H. Lubsen.** 1991. Transgenic *Xenopus laevis* tadpoles: a transient *in vivo* model system for the manipulation of lens function and lens development. Nucleic Acids Res. **19:**1279–1284.
3. **Breitman, M. L., S. Clapoff, J. Rossant, L.-C. Tsui, L. M. Glode, I. H. Maxwell, and A. Bernstein.** 1987. Genetic ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. Science **238:**1563–1565.
4. **Chien, Y.-H., and I. B. Dawid.** 1984. Isolation and characterization of calmodulin genes from *Xenopus laevis*. Mol. Cell. Biol. **4:**507–513.
5. **Dasgupta, S., T. C. Hohman, and D. Carper.** 1992. Hypertonic stress induces αB-crystallin expression. Exp. Eye Res. **54:**461–470.
6. **Delaye, M., and A. Tardieu.** 1983. Short-range order of crystallin proteins accounts for eye lens transparency. Nature (London) **302:**415–417.
7. **Dubin, R. A., R. Gopal-Srivastava, E. F. Wawrousek, and J. Piatigorsky.** 1991. Expression of the murine αB-crystallin gene in lens and skeletal muscle: identification of a muscle-preferred enhancer. Mol. Cell. Biol. **11:**4340–4349.
8. **Dubin, R. A., E. F. Wawrousek, and J. Piatigorsky.** 1989. Expression of the murine αB-crystallin gene is not restricted to the lens. Mol. Cell. Biol. **9:**1083–1091.
9. **Duguid, J. R., R. G. Rohwer, and B. Seed.** 1988. Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. Proc. Natl. Acad. Sci. USA **85:**5738–5742.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
11. **Gonzalez, P., P. V. Rao, C. Hernandez-Calzadilla, T. Borra, and J. S. Zigler.** 1992. A comparative study of zeta-crystallin gene in four species with different levels of expression in the lens. Exp. Eye Res. **55:**(Sep. Suppl. 1):199.
12. **Goto, K., T. S. Okada, and H. Kondoh.** 1990. Functional cooperation of lens-specific and nonspecific elements in the δ1-crystallin enhancer. Mol. Cell. Biol. **10:**958–964.
13. **Grainger, R. M.** 1992. Embryonic lens induction: shedding light on vertebrate tissue determination. Trends Genet. **8:**349–355.
14. **Harland, R. M.** 1991. In situ hybridization: an improved whole mount method for *Xenopus* embryos. Methods Cell Biol. **36:** 685–695.
15. **Hayashi, S., K. Goto, T. S. Okada, and H. Kondoh.** 1987. Lens-specific enhancer in the third intron regulates expression of the chicken δ1-crystallin gene. Genes Dev. **1:**818–828.
16. **Head, M. W., A. Peter, and R. M. Clayton.** 1991. Evidence for the extralenticular expression of members of the β-crystallin gene family in the chick and a comparison with δ-crystallin during differentiation and transdifferentiation. Differentiation **48:**147–156.
17. **Henry, J. J., and R. M. Grainger.** 1990. Early tissue interactions leading to embryonic lens formation in *Xenopus laevis*. Dev. Biol. **141:**149–163.
18. **Hodin, J., and G. Wistow.** 1993. 5′-RACE PCR of mRNA for three taxon-specific crystallins: for each gene one promoter controls both lens and non-lens expression. Biochem. Biophys. Res. Commun. **190:**391–396.
19. **Ingolia, T. D., and E. A. Craig.** 1982. Four small *Drosophila* heat shock proteins are related to each other and to mammalian α-crystallin. Proc. Natl. Acad. Sci. USA **79:**2360–2364.
20. **Iwaki, T., A. Kume-Iwaki, R. K. H. Liem, and J. E. Goldman.** 1989. αB crystallin is expressed in non-lenticular tissues and accumulates in Alexander's disease brain. Cell **57:**71–78.
21. **Jonas, E. A., A. M. Snape, and T. D. Sargent.** 1989. Transcriptional regulation of a *Xenopus* embryonic epidermal keratin gene. Development **106:**399–405.
22. **Kato, K., H. Shinohara, N. Kurobe, S. Goto, Y. Inaguma, and K. Ohshima.** 1991. Immunoreactive αA crystallin in rat non-lenticular tissues detected with a sensitive immunoassay method. Biochim. Biophys. Acta **1080:**173–180.
23. **Klemenz, R., E. Frohli, R. H. Steiger, R. Schafer, and A. Aoyama.** 1991. αB-crystallin is a small heat shock protein. Proc. Natl. Acad. Sci. USA **88:**3652–3656.
24. **Kobel, H. R., and L. Du Pasquier.** 1986. Genetics of polyploid *Xenopus*. Trends Genet. **2:**310–315.
25. **Kreig, P. A., S. M. Varnum, W. M. Wormington, and D. A. Melton.** 1989. The mRNA encoding elongation factor 1-α (EF-1α) is a major transcript at the midblastula transition in *Xenopus*. Dev. Biol. **133:**93–100.
26. **McDevitt, D. S., and S. K. Brahma.** 1973. Ontogeny and localization of the crystallins during embryonic lens development in *Xenopus laevis*. J. Exp. Zool. **186:**127–140.
27. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
28. **Murer-Orlando, M., R. C. Paterson, S. Lok, L.-C. Tsui, and M. L. Breitman.** 1987. Differential regulation of γ-crystallin genes during mouse lens development. Dev. Biol. **119:**260–267.
29. **Nieuwkoop, P. D., and J. Faber.** 1967. Normal table of *Xenopus laevis* (Daudin). North-Holland, Amsterdam.
30. **Piatigorsky, J.** 1992. Lens crystallins: innovation associated with changes in gene regulation. J. Biol. Chem. **267:**4277–4280.
31. **Rugh, R.** 1962. Experimental embryology. Burgess, Minneapolis.
32. **Rupp, R. A. W., and H. Weintraub.** 1991. Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of *X. laevis*. Cell **65:**927–937.
33. **Saha, M. S., and R. M. Grainger.** 1993. Early opsin expression in *Xenopus* embryos precedes photoreceptor differentiation. Mol. Brain Res. **17:**307–318.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Shastry, B. S.** 1989. Immunological studies on gamma crystallins from *Xenopus*: localization, tissue specificity and developmental expression of proteins. Exp. Eye Res. **49:**361–369.
36. **Smolich, B. D., S. K. Tarkington, M. S. Saha, D. G. Stathakis, and R. M. Grainger.** 1993. Characterization of *Xenopus laevis* γ-crystallin-encoding genes. Gene **128:**189–195.
37. **Sullivan, C. H., S. O'Farrell, and R. M. Grainger.** 1991. δ-Crystallin gene expression and patterns of hypomethylation demonstrate two levels of regulation for the δ-crystallin genes in embryonic chick tissues. Dev. Biol. **145:**40–50.
38. **Thomas, G., P. S. Zelenka, R. A. Cuthbertson, B. L. Norman, and J. Piatigorsky.** 1990. Differential expression of the two δ-crystallin/argininosuccinate lyase genes in lens, heart, and brain of chicken embryos. New Biol. **2:**903–914.
39. **van Leen, R. W., M. L. Breuer, N. H. Lubsen, and J. G. G. Schoenmakers.** 1987. Developmental expression of crystallin genes: *in situ* hybridization reveals a differential localization of specific mRNAs. Dev. Biol. **123:**338–345.
40. **van Leen, R. W., K. E. P. Van Roozendaal, N. H. Lubsen, and J. G. G. Schoenmakers.** 1987. Differential expression of crystallin genes during development of the rat eye lens. Dev. Biol. **120:**457–464.
41. **Wistow, G.** 1990. Evolution of a protein superfamily: relationships between vertebrate lens crystallins and microspecies dormancy proteins. J. Mol. Evol. **30:**140–145.
42. **Wistow, G., L. Summers, and T. Blundell.** 1985. *Myxococcus xanthus* spore coat proteins S may have a similar structure to vertebrate β-crystallins. Nature (London) **315:**771–773.
43. **Wistow, G. J., and J. Piatigorsky.** 1988. Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. Annu. Rev. Biochem. **57:**479–504.
44. **Zwaan, J., and A. Ikeda.** 1968. Macromolecular events during differentiation of the chicken lens. Exp. Eye Res. **7:**301–311.