Transgenic *Xenopus laevis* tadpoles: a transient *in vivo* model system for the manipulation of lens function and lens development

Ruud H.Brakenhoff, Robin C.Ruuls, Eldine H.M.Jacobs, John G.G.Schoenmakers and Nicolette H.Lubsen*
Laboratory of Molecular Biology, University of Nijmegen, Toernooiveld, 6525 ED, Nijmegen, The Netherlands

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ABSTRACT

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

INTRODUCTION

Reverse genetics is one of the most powerful techniques for the study of biological function of gene sequences. Transgenic mice have been far the most popular vertebrate model system for this approach and have been used in such diverse studies as cell- and tissue-specificity of promoter sequences, effects of ectopic gene expression in development, and the consequences of cell loss by gene ablation (for reviews, see 1, 2).

Micro-injected *Xenopus laevis* embryos are a second widely used vertebrate transgenic system (3–6, for review see 7). The long generation time of *Xenopus* makes it unsuitable for the production of stable transgenic lines. Hence, only transient expression of the introduced genetic material during embryonic development can be followed. It is, however, this feature of the system that makes it attractive: as *Xenopus* embryos are free living animals, their development and the effects there on by the introduced genetic material can be readily investigated (8, 9). In addition, the ease with which *Xenopus laevis* embryos can be micro-injected makes it an attractive system to screen large numbers of foreign gene constructs for their effects. The majority of studies on the expression of foreign genetic material in *Xenopus laevis* embryos has made use of non-tissue-specific mammalian promoters. No targeting by mammalian promoter sequences of the expression to a specific tissue in *Xenopus laevis* tadpoles has been reported. We show here that lens-specific rodent γ-crystallin gene promoters can be used to target expression to the tadpole lens up to at least stage 49 (according to 10). The system described here will be useful for the study of the function of genes involved in the development and specification of the properties of the lens. Our data also offer the promise that similar targeting by mammalian promoters to other tadpole tissues will be possible.

MATERIALS AND METHODS

Materials

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

Methods

Construction of CAT plasmids. The mouse γF (formerly γ2) promoter cloned into the pSV0ATCAT vector was kindly donated by Dr. M.Breitman (11). The rat γD-pCAT construct was described previously (12). The −75 to +75 deletion clone was generated by ApaI/HindIII digestion of γD-pCAT, mung bean nuclease treatment and religation. The HSP70-pCAT construct was made by insertion of the blunt-ended 0.5 kb BamHI/HindIII fragment of pBN247 (13) into the SmaI site of pCAT (12).

* To whom correspondence should be addressed

Preparation of plasmid DNA. Plasmid DNA was isolated by the alkaline lysis method, followed by RNase A treatment. The DNA was purified further by centrifugation through a NaCl cushion (1 M NaCl, 10 mM TRIS.HCl, 1 mM EDTA, pH 8.0) at 60,000g for 16 hrs at 15°C (14). Prior to loading the DNA solution was treated with 1% sarcosyl for 15 min at room temperature, and cleared by centrifugation for 10 min. The DNA was redissolved in 10 mM TRIS.HCl, pH 8.0.

Micro-injection of Xenopus laevis embryos. Female frogs were injected with 500 IU pregnyl (Organon, Oss) 10 hrs prior to egg collection. Eggs were streaked from the females and collected in 100% MMR (Modified aMphibian Ringers' solution: 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 0.1 mM EDTA, 2 mM $CaCl_2$, pH 7.8). Eggs were fertilized in a minimal amount of fluid with part of a testis, after 5 min overlaid with 25% MMR and allowed to stand for another 15 min. Embryos were dejellied in 1% L-cysteine, 25% MMR (pH set to 7.8 with NaOH). After extensive washing with 25% MMR to remove the cysteine, the embryos were placed on a plastic tray and injected within one hour after fertilization with approximately 10 nl DNA solution (usually supercoiled plasmid DNA at a concentration of 25 ng/μl), using glass capillary needles with a diameter of 10 μm. Embryos were cultured overnight in 2% Ficoll, 25% MMR at 22°C. After 24 hrs the healthy embryos were transferred into aged tap water at 22°C and carefully aerated. Tadpoles were staged according to (10).

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

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

Reproducibility of the results. Usually the tissues pooled from between 10 to 15 animals were used for an assay. In triple assays of 15 animals derived from one injection the mean deviation was found to be 25%, mainly due to variation in the volume (and thus the number of DNA molecules) micro-injected. Differences in the quality of the eggs further contributes to the large variation in absolute values sometimes found between different injection experiments. The relative values always yielded comparable results between various experiments. All quantitative comparisons reported here are based upon measurements made within one injection of eggs from a single fertilization. Only normally developing animals were assayed. Usually about 50–80% of the embryos micro-injected with 250 pg of DNA developed normally.

RESULTS

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

The rodent γ-crystallin gene promoters show strict tissue-specificity in transfection experiments: they are active only in (chicken or rodent) lens derived cells and inactive in all other cell types tested (12, 15). To test whether these promoters can also drive lens-specific expression in *Xenopus laevis* tadpoles, CAT fusion constructs of the two strongest γ-crystallin gene promoters, the mouse γF and the rat γD promoters, were injected into fertilized *Xenopus* eggs. Eye and tail extracts of injected tadpoles were assayed for CAT activity at various time points.

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

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

Expression of micro-injected DNA is often mosaic. We therefore measured the CAT activity in single eyes isolated from 13 day old tadpoles (stage 49) injected with the rat γD-pCAT construct. The mean deviation between the activity of the left and right eye of a single animal was 13%, and the mean deviation between the activity of the eyes of ten animals was 19% (data not shown). We thus conclude that the expression of the injected construct is about the same in each eye, but we cannot exclude mosaic expression within each lens.

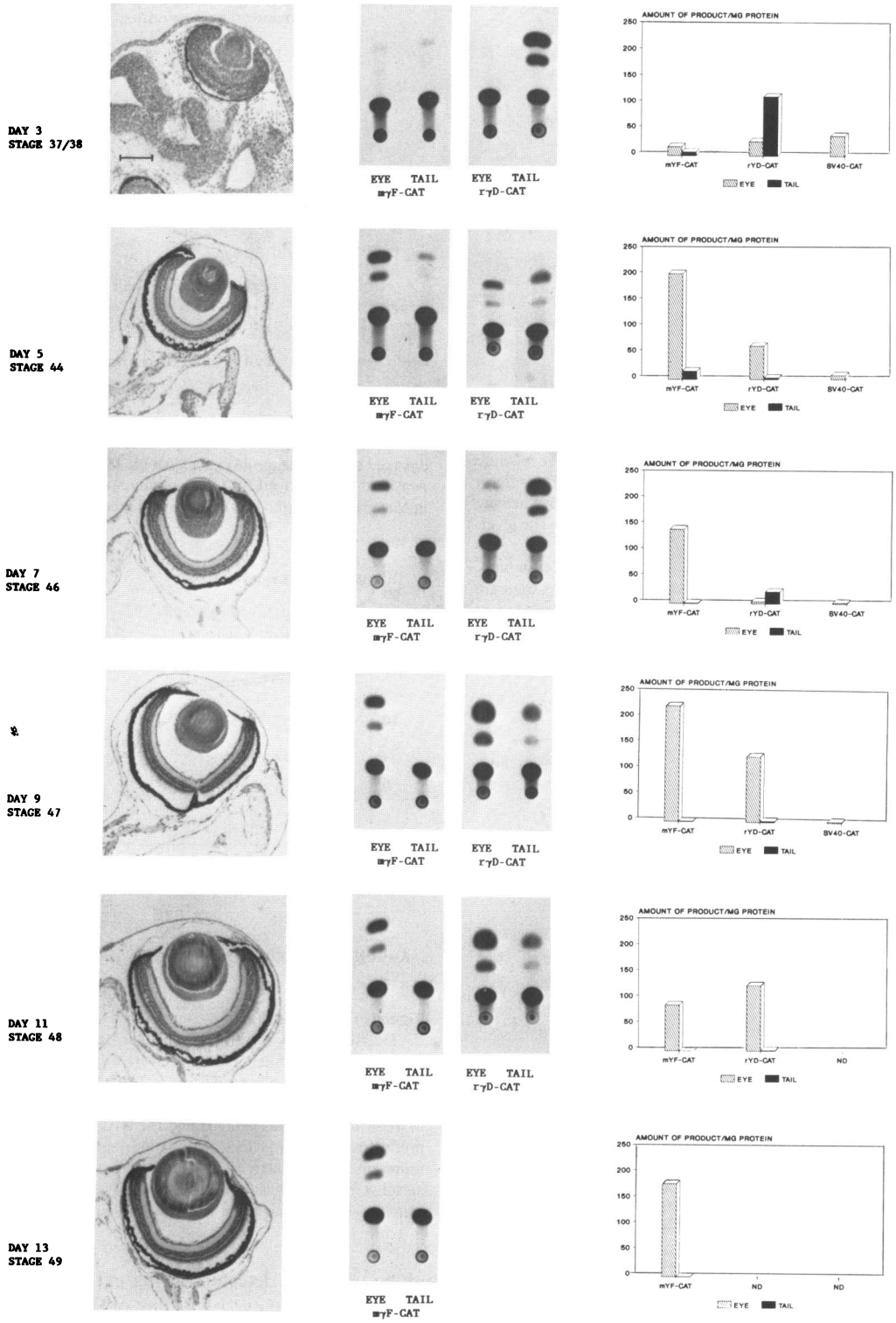

Figure 1: Tissue-specificity of expression of exogenous rodent γ-crystallin promoter-CAT constructs during lens development in *Xenopus laevis* tadpoles. In the left most column the stage, according to (10), and the actual age of the tadpoles are given. In the second column photographs of 5 μm histological sections stained with haematoxylin and eosin are shown. The bar in the photograph of 3 day animals represents 100 μm. The magnification of all histological sections is the same. Note that the high protein concentration in the lens nucleus (present from day 5) causes shatter in the histological sections. In the central columns the results of the CAT assays of tail and eye extracts of animals of the same developmental stage, injected with 250 pg pSV0ATCAT-γF (mγF-CAT) or γD-pCAT (rγD-CAT), are shown. Autoradiography was performed for 16 hrs. Only in case of the CAT assay with tissue extracts of day 3 tadpoles injected with the mγF-CAT construct, was autoradiography performed for 64 hrs. The CAT activity was quantitated as described in Materials and Methods. In the right most column the amount of 3-acetyl-^{14}C-chloramphenicol/mg protein (see also Materials and Methods) is shown on the y-axis. On the x-axis the injected constructs are indicated. Hatched bars represent the CAT activity in eye extracts, solid bars that in tail extracts. For comparison, the CAT activity obtained in eye extracts of transgenic tadpoles injected with pSV2CAT (SV40-CAT) is shown also with hatched bars. The level of CAT activity in the eye fraction of day 3 animals injected with the rat γD-CAT construct was unusually low in this particular set. In other experiments the level of activity in eye extracts was comparable to that in tail extracts.

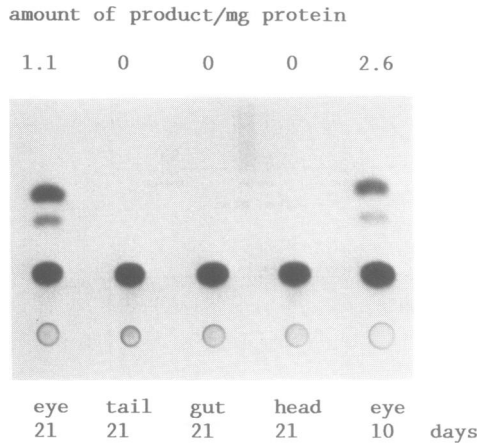

Figure 2: Tissue-specificity of the activity of a rodent γ-crystallin promoter in *Xenopus laevis*. Tissues [total head fraction (minus eyes), gut, tail and eye] were isolated from tadpoles, injected with 100 pg of the mouse γF-CAT construct (pSV0ATCAT-γF), after 21 days of development (stage 52), and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

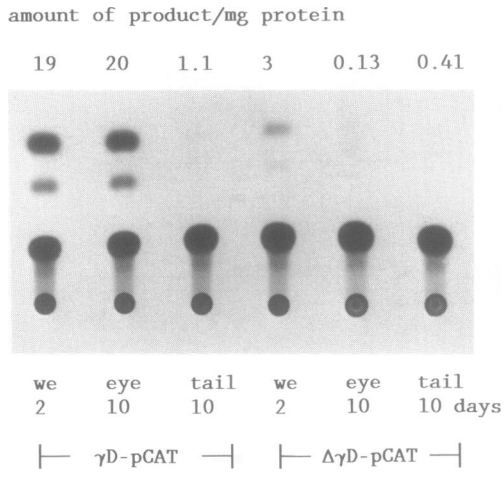

Figure 3: Dependency of the expression of the rat γD-crystallin promoter on the presence of the TATA box. Whole embryo lysates (we) or tail and eye extracts of tadpoles injected with 250 pg of the rat γD-pCAT or the rat γD-pCAT (ΔγD-pCAT) deletion constructs were prepared two (stage 32) or ten (stage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

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

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

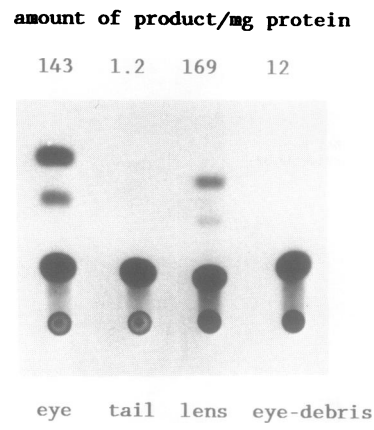

Figure 4: Lens-specificity of the activity of a rodent γ-crystallin promoter in transgenic *Xenopus laevis* tadpoles. Tail, eye, lens and eye-debris extracts of 7 days old tadpoles (stage 46) injected with 250 pg of the mouse pSV0ATCAT-γF construct were assayed for CAT activity and the levels quantitated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

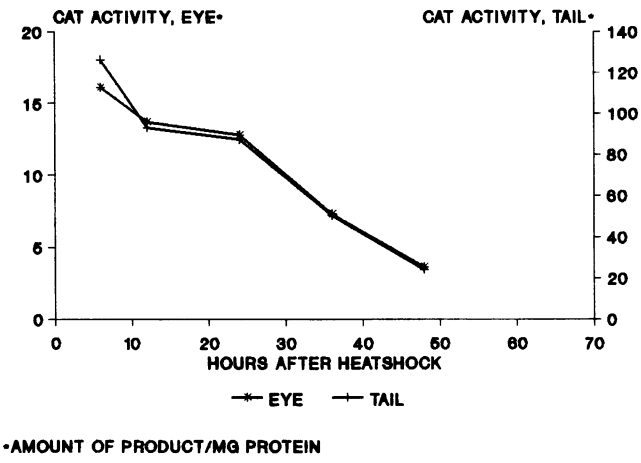

Figure 5: Determination of the stability of CAT activity in transgenic tadpoles. CAT activity in day 6 (stage 45) *Xenopus* tadpoles, injected with 250 pg of the HSP70-CAT construct, was induced by a heat shock from 22°C to 30°C for 1 hr. Animals were cultured further at 22°C, sacrificed at various times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

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

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

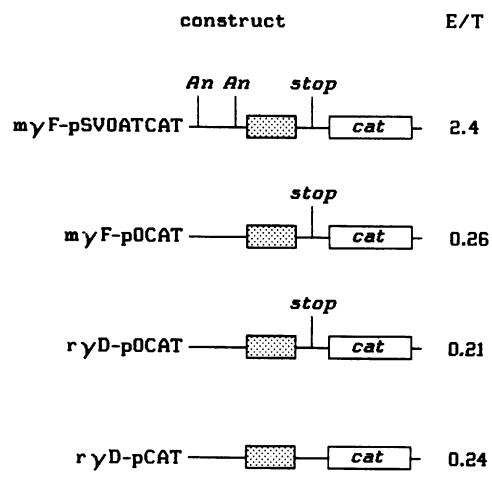

Figure 6: Non-tissue-specific expression of various γ-crystallin CAT constructs in young developmental stages. The name and schematic drawings of the micro-injected constructs are depicted on the left. The promoter regions are indicated with a stippled box, the CAT gene with an open box. Poly-adenylation sites are given by An. The translation stop region present in pSV0ATCAT (11) and in p0CAT (a derivative of pCAT, see text) is indicated with stop. The CAT activity in eye and tail extracts of day 3 tadpoles (stage 37/38 according to 10) was determined as described in Materials and Methods. The CAT activity in the extracts was quantitated and corrected for the amount of protein. The ratio of the CAT activity/mg protein in eye to the CAT activity/mg protein in tail (E/T) is given in the right column.

The stability of the CAT activity in the eye

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

The effect of the vector on the specificity of expression

We noted that deletion of the TATA box region of the γD promoter abolished CAT activity in the eye of day 10 tadpoles, whereas activity in the very young stages remained. This observation suggested that transcription starts within the pCAT vector cause CAT activity in these very young embryos. Injection of the (promoter-less) pCAT vector did indeed give rise to high CAT activity in stage 32 (day 2) but not in later stages (stage 44, day 5 and older; data not shown). If non-specific vector starts contribute significantly to the CAT activity, then differences in the vector used might also explain the apparently higher specificity of expression of the mouse γF promoter as compared to the rat γD promoter in young tadpoles. The mouse γF promoter was cloned in pSV0ATCAT, a derivative of pBR322, while the γD promoter was inserted into pCAT, a derivative of pUC12. The pSV0ATCAT vector contains two elements designed to minimize background transcription: poly-adenylation signals upstream from the promoter insert and stopcodons in all three reading frames between the promoter insert and the CAT coding sequence (11, see also Fig. 6). To test whether these features of the pSV0ATCAT vector were responsible for the apparently higher tissue-specificity of the expression of the pSV0ATCAT-γF construct, we constructed a derivative of the pCAT vector which contains the stopcodon region, denoted p0CAT, and transferred the rat γD or the mouse γF promoter region to this vector. The activity of these constructs was assayed in tail and eye extracts of 3 day old micro-injected tadpoles. The eye/tail ratio (E/T) of the CAT activity/mg protein was taken as a measure of the tissue-specificity of the constructs. As indicated by this ratio, the use of the stopcodon region in the p0CAT vector did not increase the specificity of expression of the rat γD promoter relative to the parental pCAT vector. Moreover, use of the p0CAT vector decreased the apparent tissue-specificity of the mouse γF promoter relative to the pSV0ATCAT vector (Fig. 6). Comparison of the E/T ratios of both rodent promoters cloned in the same vector (p0CAT) revealed further that the specificity of the mouse γF and rat γD promoter appears to be about equal. These results thus indicate that vector starts are involved in the non-specific expression patterns, and that the stopcodon region is not sufficient to minimize the effect of these vector starts: the poly-adenylation sites present in pSV0ATCAT are required as well.

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

DISCUSSION

The fate of the DNA micro-injected in *Xenopus laevis* embryos has been followed in a number of experiments (3, 4, 6, 16, 17). It is generally found that the micro-injected DNA replicates only within the first few hours and is gradually lost thereafter. Although lens cells do not die, they do lose their nucleus and hence the micro-injected DNA during terminal differentiation. Taking into account the gradual loss of DNA from the lens as well as the increase in protein content due to growth, the maintenance of high CAT levels up to the latest stage measured (13 or 21 days) must mean that the γ-crystallin promoters become even more active as the lens cells mature. This is in agreement with the finding that, in rodents, γ-crystallin synthesis is confined to the mature lens fibre cell (18) and suggests that the specificity of recognition of the γ-crystallin promoter has been maintained during the long evolutionary time that separates *Xenopus laevis* from rodents. As many *Xenopus laevis* and rodent genes share a common evolutionary ancestry, promoter specificity may have been maintained for a number of other genes as well. To our knowledge, the only other tissue-specific mammalian promoter that has been tested in transgenic *Xenopus laevis* is the rabbit

β-globin promoter, but no tissue-specificity was found in micro-injected tadpoles (16). With a *Xenopus* promoter, the *Xenopus borealis* actin promoter, tissue-specific expression of exogenous DNA was obtained (19).

The γ-crystallin genes encode one of the three ubiquitous families of crystallins, the abundant structural proteins in the vertebrate eye lens (20). The crystallins are a good example of proteins of which the function needs to be studied by 'reverse genetics' techniques. The optical properties of the vertebrate eye lens are determined by the exact spatial arrangement of the crystallins (21), an arrangement that cannot be mimicked *in vitro*. To fully elucidate the role of the crystallins in the lens architecture, it is necessary to engineer lenses which lack a crystallin species or which contain an additional (mutated) crystallin. Such lenses can be generated by making transgenic animals which carry either a mutated gene or a gene which expresses an anti-sense sequence, silencing the endogenous gene. Our results show that such experiments will be possible using transgenic *Xenopus laevis* tadpoles.

The use of promoters of genes for highly abundant proteins, such as the γ-crystallins, in transgenic animals has the advantage that often high levels of expression are achieved. For instance, the CAT activity obtained here from the rat γD or mouse γF promoter is, as determined in day 5 (stage 44) transgenic tadpoles, at least 100 fold higher than that obtained from the SV40 promoter. The tissue-specificity of the γ-crystallin promoters further allows the introduction of possibly deleterious constructs as lens abberations do not affect the viability of the organism. Hence, in principle the system described here can be used to intervene in general cellular processes. In practice, the potential of this system is limited by the non-specific expression found in young embryos. Non-specific expression in early development has also been noted by others (22, 23). It is not clear why such non-specific promoter activity should be abundant in one particular stage of embryogenesis and it is thus difficult to design a direct strategy to counter this non-specific activity. Possibly, sequences in the vector bind ubiquitous transcription factors. One such site, an AP1 binding site, has already been detected in pUC derivatives (24). Careful choice of vector and transcription stop sequences is then required to limit general expression during early embryogenesis as much as possible.

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