A novel *Xenopus laevis* larval keratin gene, *xlk2*: its gene structure and expression during regeneration and metamorphosis of limb and tail

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Abstract

A novel cytokeratin (CK) gene, *xlk2,* was cloned from a cDNA library prepared from regenerating limbs of *Xenopus* larvae. The deduced amino acid sequence indicated that its product, XLK2, is a 48 kDa type I (acidic) CK and has a high similarity to CK13, 15, and 19 with the highest homology (58%) to mouse CK15. The gene of *xlk2* exclusively expressed in basal cells of the bi-layered larval epidermis, but not in other cells in larvae and not in other periods of life. Its expression was down-regulated during spontaneous and thyroid hormone-induced metamorphosis. The basal cells of the apical epidermal cap (AEC) formed on the regenerate of larval limbs terminated the expression of *xlk2*, whereas those of the adjacent normal epidermis continued to express it. The AEC-basal cells did not re-express the gene in the regenerate. In contrast, the basal cells of the tail regenerate also once terminated the expression of *xlk2*, but was able to re-express *xlk2* later, supporting a notion that the "de-differentiated" basal cells of the tail epidermal regenerate re-differentiate into larval normal epidermal cells.

1. Introduction

The urodele of amphibia is distinguished among vertebrates in that this animal species is able to regenerate its complete limbs through life when the limbs are amputated [1]. The anuran is taxonomically close to the urodele and stands on a developmentally and phylogenically unique position in the capacity of limb regeneration. *Xenopus laevis* shows the full capacity to regenerate amputated limbs during larval development, loses it during metamorphosis [2,3], and finally becomes quite incomplete in the capacity post metamorphosis [4]. The amputation of *Xenopus* adult limb results in the formation of a spike-like tissue, a quite incomplete regenerate in morphology and anatomical structures [4]. This biological position of *Xenopus laevis* makes it a useful experimental animal to study the mechanism of the decline in regenerative capacity during metamorphosis, which has been quite poorly understood and might shed light on the causes of the regenerative failure in higher vertebrate limbs if elucidated.

In the present study a novel *Xenopus* gene of 48 kDa type I cytokeratin (CK), *xlk2*, was cloned from a cDNA library obtained from regenerating limbs of tadpoles. We characterized *xlk2* in terms of its structure and spatio-temporal expression during regeneration of limb and tail. Its expression pattern was also investigated during spontaneous and thyroid hormone (TH)-induced metamorphosis of these organs.

Relatively many studies have been reported concerning changes in the expression of CKs during anuran development and metamorphosis. However, studies on the CK expression during anuran limb regeneration have been quite limited. To our knowledge, only one type of CK has been studied hitherto during anuran limb regeneration. Estrada

et al. immunized mice with the wound epidermis of regenerating limb of the newts and obtained a monoclonal antibody called WE6 [5]. An antigen against WE6 was CK with a molecular weight of 39 kDa [6]. This keratin was up-regulated in wound epidermal cells of newts, and *Xenopus* and *Rana* froglets [5]. Investigators of anuran metamorphosis identified a variety of genes and proteins of CK, and utilized them as specific markers of embryo-, larva-, and adult-specific CK [7-19]. However, to date, there have been no CK genes whose expressions are strictly restricted to the larval period [20]. In this context, *xlk2* is unique, because our study showed that its expression is strictly confined to the larval period. In addition, this gene was exclusively expressed in the epidermal basal cells.

2. Materials and Methods

2.1. Animals

Sexually matured frogs of *Xenopus laevis* were purchased from local animal suppliers and were mated to yield embryos, tadpoles, and froglets. Developmental stages were determined according to Nieuwkoop and Faber [21]. Metamorphic stages were presented as pre-, pro-, and climax-metamorphic periods according to Benbassat [22] (also see a review [23]). Tadpoles were raised at 24°C and fed on dried powdered alfalfa, Alfalfa Meal (Oriental Yeast, Tokyo, Japan). Tadpoles were anesthetized by immersing them in 0.03% 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO, USA). To induce limb regeneration, hind limbs of tadpoles at stage 54 and 60 were amputated through the middle shank with scissors. Tails of tadpoles at stage 49-50 were amputated with a razor at their distal third to induce regeneration. The operated tadpoles were sacrificed at 3, 5, and 11 days post-amputation for analysis. Some of them were allowed to develop further to observe the progress of regeneration. Tadpoles at stage 56 were reared in water containing 10 nM 3, 3', 5-triiodothyronine (T_3, Sigma-Aldrich) to induce metamorphosis.

2.2. cDNA subtraction

Hind limbs of tadpoles at stage 54 and 60 were amputated and allowed to regenerate for 3 days. Three types of hind limbs were collected from tadpoles: whole hind limbs from normal tadpoles at stage 54 whose hind limbs had not been amputated

(normal stage 54-limbs), whole hind limbs from tadpoles whose hind limbs had been amputated at stage 54 (regenerating stage 54-limbs), and distal hind limbs from tadpoles whose hind limbs had been amputated at stage 60 (regenerating stage 60-limbs). The collected samples of regenerating limbs included the regenerates and their neighboring tissues. Total RNA was extracted with an ISOGEN RNA extraction reagent (Nippon Gene, Tokyo, Japan) from these three types of hind limbs. Synthesis and amplification of cDNA were performed using SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). Single-stranded cDNAs were synthesized from 1 μg of total RNAs and amplified 18 cycles by PCR to prepare double-stranded cDNAs. Subtraction of cDNA was performed using PCR-SELECT cDNA Subtraction Kit (Clontech). First, cDNAs of regenerating stage 54-limbs were hybridized with those of normal stage 54-limbs, which yielded "the 1st subtracted cDNAs". Next, the 1st subtracted cDNAs were hybridized with those of regenerating stage 60-limbs, which yielded "the 2nd subtracted cDNAs".

2.3. 5'- and 3'- RACE on a cDNA fragment of xlk2

The full length of *xlk2* cDNA was obtained by 5'- and 3'-RACEs [24]. PolyA(+) RNA was extracted from tissues of hind limb and tail with QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized from 1 μg polyA(+) RNA using Marathon cDNA Amplification Kit (Clontech). Primers for 5'-RACE and 3'-RACE were designed referring to the sequence of the fragment of *xlk2* cDNA and are listed in Table 1.

2.4. Northern blot analysis for xlk2 and EF-1α

Epidermis was peeled off from tails at stage 56 using forceps. The separated epidermis was associated with the collagen layer. The remaining tail tissues were used as the mesenchyme. PolyA(+) RNAs were extracted as above from each of the epidermis and the mesenchyme. Aliquots of them were loaded on formaldehyde agarose gels, electrophoresed, and blotted to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech). Digoxigenin-labeled RNA probes were prepared from the nucleotide number (nt) 661-945 of *xlk2* and from nt 1104-1372 of *EF-1α* (GenBank accession number: M25504) [25] following the manufacturer's instruction (Roche, Basel, Switzerland). Hybridization and signal detection were carried out as described previously [26].

2.5. Whole-mount in situ hybridization and immunohistochemistry

Tissues of hind limb and tail were subjected to whole-mount in situ hybridization as previously reported [27]. The antisense and sense RNA probes of *xlk-2* and *xak-c* were prepared as above and as previously reported [18], respectively. Pre-hybridization and hybridization were done at 55 ˚C instead of 60 ˚C. After the color detection, tissues were treated with 30% sucrose, embedded in Tissue-Tek O. C. T. Compound (Sakura Finetek, Torrance, CA, USA), and sectioned with a cryostat, Minotome (Damon/IEC Division, Needham Heights, MA, USA). Tissues were subjected to immunohistochemistry. Mouse monoclonal antibodies, AE1 and AE3 (both from Dako, Glostrup, Denmark) at a 1:1000 dilution, and anti-mouse IgG Alexa 594

(Molecular Probes, Eugene, OR, USA) were used as primary and secondary antibodies, respectively. The other conditions were the same as those reported previously [17].

2.6. RT-PCR of xlk2 and EF-1α

Oligo(dT)-primed first-strand cDNA was synthesized from 200 ng of total RNA at 42 ˚C using TaKaRa RNA PCR Kit (AMV) Ver. 2.1 (Takara, Tokyo, Japan) according to the manufacturer's instruction. The employed primers are listed in Table 1. The PCR produced a fragment corresponding to the sequence from nt 72 to 465 for *xlk2* and a fragment from nt 1096 to 1364 for *EF-1α*. PCR for *xlk2* was done with 30 cycles under the following conditions: denaturation at 94 ˚C for 30 sec, annealing at 60 ˚C for 30 sec, and extension at 72 ˚C for 30 sec. PCR for *EF-1α* was done with 25 cycles under the following conditions: denaturation at 94 ˚C for 30 sec, annealing at 55 ˚C for 30 sec, and extension at 72 ˚C for 30 sec.

3. Results

3.1. Cloning and characterization of xlk2 cDNA

cDNA subtraction was performed among cDNAs prepared from normal stage 54-limbs, regenerating stage 54-limbs, and regenerating stage 60-limbs as described in Materials and Methods. Twenty-five cDNA fragments were randomly picked up from the 2nd subtracted cDNA library and were sequenced. One of them, clone A14, was unknown, showed a high homology to CK genes, and was further characterized in the present study. This CK gene was designated *xlk2*, because, as described below, it was exclusively expressed in the larvae as in the case of *xlk*, a *Xenopus* larval keratin gene [16].

Its 1,843 nt-long full-length cDNA was obtained by 5'- and 3'-RACE. A cDNA of *Silurana tropicalis* (GenBank accession number: BC081376) showed a remarkable similarity (75.2%) to *xlk2*. The sequence of *xlk2* contained an open reading frame with a favorable context, [ACCATCACA (nt 41-49) and A (nt 53)], for the translational start site on the flanking regions of the putative initiator ATG (nt 50-52) [28]. There was a stop codon (nt 29-31) in frame before the initiation site. Therefore, the ATG codon (nt 50-52) seems to encode the first methionine. The coding region was comprised of 1,323 nt that encoded the polypeptide (XLK2) of 441 amino acids (molecular size of about 48 kDa), consisting of head, coils, linkers, and tail domains of keratin, its pI being 4.93. The amino acid sequence of XLK2 showed a high identity to type I (acidic) keratins, 57.7, 57.0, 56.0, and 55.4% against mouse CK15 [29], human CK15 [30], mouse CK13 [31], and bovine CK19 [32], respectively (Fig. 1A). Among

the known keratins of *Xenopus laevis*, 47 kDa type I keratin [33] was most similar (54.1%) to XLK2. The genetic distances among XLK2, and mammalian CK13, 15, and 19 were estimated by the amino acid sequences, and shown in Fig. 1B. Northern blotting of *xlk2* mRNA was performed using total RNAs prepared from the epidermis and the mesenchyme of tadpole tail at stage 56 (Fig. 1C). An approximately 2 kb single band was detected only for the epidermis. The nucleotide sequence of *xlk2* was deposited in DDBJ under an accession number AB218824.

3.2. *Expression of xlk2 during development*

The expression of *xlk2* was examined during embryonic and larval development, and metamorphosis by RT-PCR (Fig. 2). *xlk2* mRNA was exclusively expressed in the larval period (Fig. 2A) from stage 35/36 (hatching stage) to stage 55 (late premetamorphic stage). Any transcript was undetectable in all the tested organs of adult frog, esophagus, stomach, small intestine, rectum, lung, heart, liver, gall bladder, kidney, oviduct, ovary, testis, urinary bladder, brain, skeletal muscle, and skin (data not shown). The expression level of *xlk2* was determined by RT-PCR for the epidermis of body, hind limb, and tail of tadpoles from stage 50, a stage before the premetamorphic stage, to stage 60, the starting stage of climax metamorphosis (Fig. 2B). At stage 50 and 56, strong expression was seen both in the body and tail. The gene was also expressed in the epidermis of hind limb at stage 56, although the signal was much weaker. At stage 58, the expression was drastically decreased in all the tested samples. At stage 60, *xlk2* became undetectable in the body and the limb, and very faint in the tail.

3.3. Spatial expression patterns of xlk2 in the developing hind limb

Whole-mount in situ hybridization of *xlk2* was performed for developing hind limbs (Fig. 3). The gene was expressed during stage 52-55, and down-regulated drastically to a background level by stage 56 (Fig. 3A and B). *xlk2* was expressed only in the epidermis (Fig. 3C, see also Fig. 1C). The transcripts in the epidermis were not evenly distributed, more abundant in the dorsal side than in the ventral side, and not detected in the tips of the digits and a part of the shank (Fig. 3C, arrowheads). Close-up examinations identified the expressing *xlk2* cells as basal cells (Fig. 3D and E).

3.4. Regulation of xlk2 expression by T_3

Tadpoles at stage 56 were reared in water with and without T_3 for 3 days. Developmental stage of the T_3-untreated larvae did not proceed during the period of experiments. The T_3-treated animals did not show any morphological changes at the first day of treatment and showed precocious metamorphic changes in limb growth and tail regression at 3 days after the start of T_3-administration. The expression of *xlk2* was determined by RT-PCR in the epidermis of body and tail (Fig. 4). T_3 caused a down-regulation of *xlk2* expression in both the body and tail at day 1 of the treatment and completely abolished the expression in both the tissues at day 3.

3.5. Spatial expression patterns of xlk2 mRNA during regeneration of limb bud and tail

Hind limbs of tadpoles at stage 54 were amputated and allowed to regenerate.

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The spatial expression of *xlk2* mRNA in the regenerating hind limb was examined by whole-mount in situ hybridization at day 3 post-amputation (Fig. 5[I]A and B). The hybridized tissues were micro-sectioned to histologically locate the in situ hybridization signal (Fig. 5[I]C) and their serial sections were stained with hematoxylin (Fig. 5[I]D). The amputated site was covered with the wound epidermis and the apical epidermal cap (AEC) was formed by 3 days post-amputation (Fig. 5[I]D). The epidermis of regenerating limb buds, including the thicker epidermis of AEC, did not express the gene at all, whereas the stump epidermis expressed *xlk2* (Fig. 5[I]A and C) as the normal limbs at the corresponding stage (Fig. 3A). The epidermis of limb buds at 11 days post-amputation also did not express *xlk2* mRNA (data not shown). In situ hybridization also showed that *xak-c*, a marker gene of epidermal basal cells of adult *Xenopus* [18], was not expressed in the regenerates at 3 days post-amputation (data not shown). The epidermis of the regenerates was stainable with AE1 (Fig. 6) and AE3 (data not shown), both multi-specific anti-keratin antibodies that recognize a broad spectrum of CK members.

Similarly, spatial expression of *xlk2* mRNA was investigated during regeneration of tail. Tails of tadpoles at stage 49-50 were amputated and the animals were kept for up to 5 days. *xlk2* mRNA was undetectable in the normal tail (Fig. 5[II]A) though it was detectable by RT-PCR (Fig. 2B). A small flattened cone-shaped regenerate was formed on the plane of the amputated tail at 3 days post-amputation (Fig. 5 [II]B and C). AEC-like epidermis was not formed in the tail regenerates (Fig. 5[II]E), which contrasts with the tail regenerates of newts that form AEC [34]. As in the case of limbs the epidermis of tail regenerates did not express *xlk2*, contrasting with the expression in the stump where *xlk2* was up-regulated (Fig. 5[II]B and D). The regenerates developed to

the tissues like a small tail at 5 days post-amputation (Fig. 5[II]F and G). Contrary to the epidermis of regenerating limbs, the epidermis in the proximal to middle regions of the regenerate re-expressed *xlk2* to an extent higher than that of the stump (Fig. 5[II]F and G). The distal regions of the regenerate did not express the gene (Fig. 5[II]F and G). These findings were confirmed on the sections (Fig. 5[II]H-K).

4. Discussion

Keratins are expressed in different epithelia in different combinations of keratin polypeptides [35,36]. Thus, keratins have generally been employed as useful markers for differentiation and transformation of epithelial cells [37], and also for metamorphosis of anuran skin [11-14,17,19] and for regeneration of urodele limb [38]. However, little information has been available about the expression of keratins during anuran limb regeneration. Hoffmann and Frankz [7] categorized *Xenopus* major keratins into four groups, type I to IV: type I-a, -b, and -c (64 kDa each), type II (53 kDa), type III (51 kDa), and type IV (49 kDa). Type III keratin gene is expressed at an almost constant level during metamorphosis in the skin, while type I gene is up-regulated and expressed strongly in the adult skin [10], and, thus, is used as a marker of adult epidermis [14]. XK70 and XK81 genes are expressed in the epidermis from embryos to larvae [8,9]. Watanabe et al. [16,18] isolated marker genes of *Xenopus* larval and adult epidermal cells, dubbed *xlk,* and *xak*, respectively, the latter containing subtypes of *xak-a*, *-b* and *-c*. Epidermal keratin genes were also cloned from *Rana catesbeiana*, *rlk*, *rak*, and *rk8*, which are utilized as marker genes for larval, adult, and larval to adult epidermal cells, respectively [19]. During metamorphosis, *rk8* is expressed at a constant level, whereas *rlk* and *rak* are down- and up-regulated, respectively [15].

Ferretti et al. studied the expression of CKs in regenerating limbs of the newt, *Notophthalmus viridescens* [39], and identified NvKII, NvK8 (the newt homologue of CK8), and NvK18 (the newt homologue of CK18) [40,41]. NvKII gene is expressed not in the normal limb epidermis, but in the wound epidermis of limb regenerate [40]. The transcripts of NvK8 and 18 genes increased in the blastemal mesenchymal cells of limb

regenerates [41].

We isolated from *Xenopus laevis* a novel keratin gene, *xlk2*, which encoded a type I keratin and was similar to the mammalian genes of CK13, 15, and 19. Genes of CK13, 15, and 19 are closely located in the phylogenetic gene tree and in the physical map of the human and mouse genomes [42,43], suggesting their phylogenetical kindred. The genetic distances between mammalian CK13, 15, and 19 estimated by the amino acid sequences are closer than those from *xlk2* to these mammalian keratin genes. Therefore, the genes of K13, 15, and 19 would have diverged from each other after an evolutional branching point between the ancestor of *xlk2* and the common ancestor gene of K13, 15, and 19.

We originally performed the present study with the purpose to isolate genes that are involved in the decline of regeneration capacity of tadpole limbs during larval development. cDNAs of normal stage 54-limbs, and then regenerating stage 60-limbs were subtracted from those of regenerating stage 54-limbs. This experimental procedure was considered to permit us to screen a cDNA(s) whose expression is high in regenerating limb buds amputated at stage 54, and is low or undetectable in normal limbs at stage 54. However, in fact, the presently observed expression pattern of *xlk2* was against the prediction. The regenerating stage 54-limb tissues used in the present study were composed of not only the regenerate, but also the neighboring stump tissues whose epidermis expressed *xlk2*. Both of the normal stage 54- and regenerating stage 54-limbs expressed *xlk2*. Thus, if the 1st hybridization would have worked as expected, *xlk2* cDNA fragments should not be present in the cDNA library obtained at the 2nd hybridization. But, *xlk2* was actually isolated from the library. This fact might be explainable by assuming that *xlk2* cDNAs of the neighboring stump epidermis remained

through the hybridization procedure.

To date several CK genes have been known that are expressed in the embryonic, larval, and adult epidermis as discussed above. However, Fouquet et al. [20] doubted the existence of strictly "embryo- or larva-specific" keratin, because the cited authors found that the gene of a so-called "embryo-specific keratin", XK81, is also expressed in adult oesophageal epithelium. No transcripts of *xlk2* were detected in embryos and in all the tested adult tissues. Thus, we concluded that *xlk2* is the first keratin gene that can be categorized as "strictly larva-specific keratin". Expression of *xlk2* was temporally and spatially regulated, and was similar to that of *xlk* in the trunk and tail [16]. During the early larval stages, *xlk2* was detected in the trunk, tail, and hind limbs, and decreased its expression at later stages first in the hind limbs, then, in the trunk, and finally in the tail. This *xlk2* down-regulation was inducible by exogenous T_3, which regulates the expression of metamorphosis-related genes and promotes precocious metamorphosis in pre-prometamorphic larvae [44-46].

The wound epidermis of regenerating hind limb buds markedly decreased the level of *xlk2* mRNA as compared to the stump epidermis including the epidermis adjacent to the wound epidermis, indicating the wound epidermis differs from the original (stump) epidermis that has covered the amputation site. Generally, the combination of keratins in an epithelium depends on its anatomical (histological) type and its state of differentiation [37]. The differentiation state of the wound epidermis of the limb regenerate appears to differ from the original stump epidermis, which is supported by accumulated literatures to date [2,6,47]. In this context, CK15 of mouse and human is particularly noteworthy, because, first, its amino acid sequences show a high identity to *xlk2* and, second, its expression profiles during wound healing is similar to that of *xlk2*.

CK15 of mouse and human is expressed in the basal layer of the normal adult epidermis and down-regulated in the wound epidermis [48-50].

In this study we found a unique expression pattern of *xlk2* in the epidermis covering the regenerating limb. The most inner layer of AEC is connected to the basal layer of larval bilayered epidermis of the stump. Therefore, the basal cells of AEC may originate in the basal cells of the larval normal epidermis. The basal layer of AEC terminated the *xlk2* expression, and not re-expressed later in the larval period. The expression of *xlk2* in the tail regenerate contrasts with that in the limb regenerate. The epidermis of the tail regenerate terminated the *xlk2* expression as in the case of limb regenerate, but was capable of re-expressing *xlk2*, which is quite different from the limb regenerate. These results suggest that the wound epidermis that is formed just after the wounding of the tail is in a de-differentiated state, but differentiate again into the larval epidermis. The wound epidermis of the larval limb regenerate did not express not only *xlk-2*, but also *xak-c*, suggesting that this epidermis is unique in the expression of keratin genes, different from larval and adult normal epidermis. In this study we examined *xlk2* expression in the regenerating hind limb at stage 54 and the regenerating tail at stage 49-50. The difference in stage between the two tissues might not affect our interpretation of the results. It is thought that *xlk2* would be re-expressed in the tail wound epidermis even if the tail were amputated at stage 54, the stage of amputation of the limb from the following reason. The tail epidermis manifests the larval phenotype at least during stage 50-57: it is bilayered [13], continues to express *xlk2* (Fig. 2B), and does not express an adult marker, 63 kDa keratin [13]. These indicate that the tails do not change their basic properties during stage 50-57. Thus, it is rational to consider that the regenerating tail amputated at stage 54 re-express *xlk2* as that amputated at stage 50.

The difference in *xlk2* expression in the wound epidermis between the tail and the limb regenerate might be related to the difference of their metamorphic fate: the tail epidermis is a "larva-specific" tissue, i.e., the tissue that is not able to survive the metamorphosis, and terminates its life during metamorphosis, whereas the limb epidermis is a "larva to adult" tissue, the tissue that survives the metamorphosis and transforms to adult epidermis [23].

Acknowledgments

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Figure Legends

Fig. 1. Amino acid sequences and the mRNA size of XLK2. (A) Alignment of the deduced amino acid sequences of XLK2 and known type I keratins. Blast was done to search sequences similar to XLK2 in the Swiss-Prot protein sequence database (release 47.2). The sequences of five keratins that showed a high identity to XLK2 are aligned here: mouse CK15 (m15), human CK15 (h15), mouse CK13 (m13), bovine CK19 (b19), and *Xenopus* 47 kDa keratin (x47kD). Dashes denote deletion introduced to obtain the optimal alignments. The α-helix domains are double-lined at the top of the panel and shown as coils 1A, 1B, and 2 on the respective corresponding sites. Their assignments were done according to Nozaki et al. [29]. (B) A phylogenetic tree of XLK2 and mammalian type I keratins. The tree was obtained using ClustalW program. MK and HK indicate mouse and human CK, respectively. The scale bar represents the number of amino acid substitutions. (C) Northern blot analysis for the epidermis and the mesenchyme of tadpole tail at stage 56. One microgram (*xlk2*) or 0.1 μg (*EF-1α*) polyA(+) RNA was loaded to the gel. Tissues are indicated at the top of the panel. Mobility of 18S and 28S rRNAs is shown at the right. The expression level of *EF-1α* was determined to confirm the equality of the amounts of RNAs tested.

Fig. 2. Expression of *xlk2* during development and metamorphosis. mRNAs were quantified by RT-PCR. The developmental stages are indicated at the top of the panel. (A) Expression in embryos and tadpoles. Total RNA was extracted from the whole body of embryos and tadpoles at the indicated stages. No products were observed when RT-PCR was done without reverse-transcriptase. (B) Expression in the epidermis of the

developing tadpoles. Total RNA was extracted from the epidermis of head and trunk (body), hind limb (limb), and tail of tadpole at the indicated stages.

Fig. 3. Spatial expression of *xlk2* in developing hind limbs. (A) Whole mount in situ hybridization was performed on developing hind limbs of tadpoles at the indicated stages with the antisense probe. The figures represent views of the dorsal side of the limb. (B) A control experiment of A using the sense probe. (C) A section of the limb at the stage 55 shown in A. The arrow indicates the dorsoventral direction. The arrowheads point to the regions where *xlk2* was not expressed. (D) Magnification of the region enclosed by the rectangle in C. (E) A serial section of D was stained with hematoxylin. The insets in D and E show magnified figures of the areas enclosed by rectangles. a, the apical layer; b, the basal layer; c, collagen layer; and m; mesenchyme. The magnification of B and E is identical to A and D, respectively. Scale bars = 1 mm in A, and 100 μm in C and D.

Fig. 4. Expression of *xlk2* in the epidermis during T_3-induced metamorphosis. Tadpoles were reared in water without (T_3-) or with T_3 (T_3+). RT-PCR was done using total RNA as a template extracted from the epidermis of the body and the tail. The Arabic numerals at the top of the gel indicate the periods (days) of the hormone treatment. The expression level of the hormone-untreated tadpoles did not sinificantly change for 3 days.

Fig. 5. Spatial expression of *xlk2* during regeneration of hind limbs and tails. [I] Limb regeneration. Limbs of tadpoles at stage 54 were amputated and were subjected to

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whole mount in situ hybridization for *xlk2* mRNA at 3 days post-amputation with the antisence (A) and the sense probe (B). Three limbs are shown. The closed arrowheads point to the amputation sites. The right limb in A is sectioned and shown in C. A serial section of C was stained with hematoxylin (D). The magnification of B and D is identical to that of A and C, respectively. Scale bars = 1 mm in A, and 200 μm in C. [II] Tail regeneration. Normal tails of tadpoles at stage 50 were subjected to whole mount in situ hybridization for *xlk2* mRNA. A representative photograph is shown in A. Tails of tadpoles at stage 49-50 were amputated and subjected to whole mount in situ hybridization for *xlk2* mRNA at 3 days post-amputation with the antisence (B) and the sense probes (C). Three tails are shown. There were non-specific signals in the notochord of tails and the mesenchyme of regenerates. The closed arrowheads point to the amputation sites and the open arrowhead in the left sample in C points to the notochord. The left tail in B is sectioned and shown in D. A serial section of D was stained with hematoxylin (E). Similarly, whole mount in situ hybridization was performed at 5 days post-amputation with the antisence (F) and the sense probes (G). The dotted line of the left sample in F indicates the regenerate. A longitudinal section was made from a proximal and distal region of the left tail shown in F and is shown in H and J, respectively. Serial sections of H and J were stained with hematoxylin and are shown in I and K, respectively. The magnification of B, C, F, and G is identical to that of A. The magnification of sections of D, E, and H-K is identical and its scale bar is shown in D. Scale bars = 1 mm in A, and 100 μm in D.
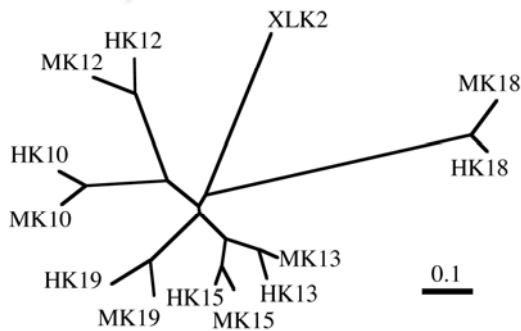
Fig. 6. Immunohistochemistry on regenerating limbs using monoclonal antibody AE1. Limbs of tadpoles at stage 54 were amputated and were subjected to

28

immunohistochemistry at 3 days post-amputation. The arrowheads point to the amputation sites. Scale bars = 200 μm.

A

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XLK2    M-SYSFSQSSS-SR-L-SS--GI---SSRL----SDG--IYRAASMH---GGY-GGS-GISMSSAKF-TS         49
m15     *ATT-*L*T**T--FGG**TR*A---*L*-AGGG*F*GG-----*LY---**--***RS**A***R*VS*         53
h15     *TTT-*L*T**-*TFGGG*TR*G---*L-*AGGGGF*GG-----*LS---*--***RS**A***R*VS*         54
m13     *-*CR*-****M*-----YGG*FGAG*CQ*-GG---*RN*SSC-*SRFVT**SA**Y-*GG**CG-*GGG         56
b19     *T***YR****T*-----*FG-*MGGG*M*FGAG--*A--F**P*I*---*S-**-R*V*V***R*VS*         55
x47kD   *-*--*R-***-*YS*Q*K--**---*----GG---*G--*G*---------*F-**--*-*G--*G*---         34

                                                                          1A
XLK2    AA----GSGM---GF----GSGFGSGFGG--GAS--FG-AS---FG----SGG--GE-GLLSG-DEKYTM         92
m15     G*GGGF*G**S-C**-----*G***G****---*----**--GG---**D---F**--*DG*****N-**V**        102
h15     GSGGGY*G**RVC**GGGA**V**G***GV*GG--**-GG---*-*------**-D-*-*****N-**I**         109
m13     -*-----*-*----**----*G***G***SY*GG--*-GG---**D---F**VD*--*****N-**I**         100
b19     -S------**----*Y----*---*-*Y**---*L-----*T---------*-----D-***A*N-**L**         83
x47kD   --GG--***A---*-*----*-*-*Y*A*FGG***SG*SLS*AGG**AAAA*SSFSNF-*---*N*-*Q**         86

                                                                          1B
XLK2    QNLNGRLASYLAKVKSLEEANTDLEAKIRDWYAKQ-G-SV--AIREQ--SYGALYT-TI-DELRGKILAA        154
m15     ****D******D**RA**Q***E**V******Q**SPA*PD---*--DY*H---*FK*ME-*I*D*****        163
h15     ****D******D**RA*****A***V**H***Q**TPA*P-----*CDY*Q---*FK**E-***D**M*T        170
m13     ****D******D**RA*A**A***V*****HL**SPA*P-----*RDY*--*-*YK**E-***I***E*        161
b19     ****D******E**RA****G**V******Q**--*P-GP-*-*--DY*H---*FK**E-**DQ**G*        143
x47kD   ****D******E**RA**A**A***L***E**E**K*-*GIG*GS-KDF*K----*FEI*S*-**N***S*        150

XLK2    TINNAKLV-LEIDNARLAADDFKLKYENEFSLRQTVENDISGLRHILDDLTMARSDLELQIESLKEELVY        223
m15     **D*SRV*-************R*****LT***G**A**N***RV*E**L**T***M***Q*N***A*        232
h15     **D*SR-*I************R*****LA***G**A**N***RV*E**L**T***M***G*N***A*        239
m13     *TD*NR-II***********LT***S**A**N***RV*E**L*KT***M*****N***A*        230
b19     **E*S*I-*Q***********RT*F*T*QA**MS*A**N***RV*E**L**T***M***G*K***A*        212
x47kD   **D*SRV*-*Q****K******R**F**LA***S**T**N***RV*E**L*G***M*****T***A*        219

                                 2
XLK2    LKANHEEEVSEKK-QHAAGTVSVELDAAPGIGLLNT--LNDLREQYEHIADKNRREAEAWFLSQV---ES        287
m15     **K*****MK*FSS*L-**Q*N**M*****VD*--RM*AEM*****A**E****DV****F*KTE--*-        296
h15     **K*****MK*FSS*L-**Q*N**M*****VD*--RV*AEM*****AM*E****DV****F*KTE--*-        303
m13     **K*****MK*FSN*VV--*Q*N**M***T***D*--RV*AEM*****AL*E****D**E**--*TKSA*        294
b19     **K*****M*VL*G*VG--*Q****V*S****D*A--KI*S*M*S***V**E***KD*****I**TE--*-        276
x47kD   **K*****M*IA*SSS--**Q*N**M******D*-*-KI*S*M*AD**TL*E****D**L**N-*-KSG*-        283

XLK2    LQSEVVTS-T--QQVQ-STKSESTELRRGLQSLELELQTLLSTKAGLEAS-LADTEGRYAAQLFQ-IQNI        351
m15     *NK**A-*N*--EMI*T*-*T*I*D***T**G**I***SQ**M*****N*-**EV*C***T**-*Q**GV        360
h15     *NK**A-*N*--EMI*T*-*T*I*D***TM*E**I***SQ**M*****N*-**V*C***T**-*Q**GL        367
m13     *NK**-S*NA--EMI*T*-*T*I******T**G**I***SQ**M****-*T**E*C***L**-*Q**GL        358
b19     *NR**AGH-*--E*L*I*-*T*V*D***T**G**I***SQ**M*A**G-T**E**A*FG***A*-**AL        340
x47kD   *KK**IQ---*GVE***T*-***IND***S*****I***SQ*AM*KS**D-T**E*D***G***-*T**FS        347

XLK2    I-SGMEAQFSDL--RLDLERQNQEYKALLDIKSRLEQEILKYHQLLEGEGG-LIA--AGA-DAKSGSS--        412
m15     *-T*L*T*L*E*--HSQM*A*****NM*****T******AT*RN***DQD----*KM**I-GVRE***GG        422
h15     *-G*L***L*E*--CEM*A*****M*****T******AT*RS****QD----*KM**I-GIREA**GG        429
m13     *S*-I***L*E*--SEM*C*****M*****M*****T*RS****QD----*KMT*FN---**-*G-        415
b19     *-**I***L**V--*A*T******QH*M***T******AT*RN****QD----*Y---FN*L-*L----        395
x47kD   LR*-L*E*L--*QI*S*M****M**RQ*****T***M**ET*RR*****F*S*----------**--*--        400

XLK2    --SVS-TT--TRVHAIIKEES-GGKVVSSTLL-----KTF        441
m15     GG*S*SSSNF---*-*SV***VD******R-------*REI        452
h15     GG*-*S--NF---*-NV***VD*Q****H-------*REI        456
m13     ---NN-**--*---------*N*SP--**NSGRPDFR*Y        437
b19     ---------------*--*A------------*        399
x47kD   --I*QA*EVS*SQ--SS---*-------**-------K*D        419
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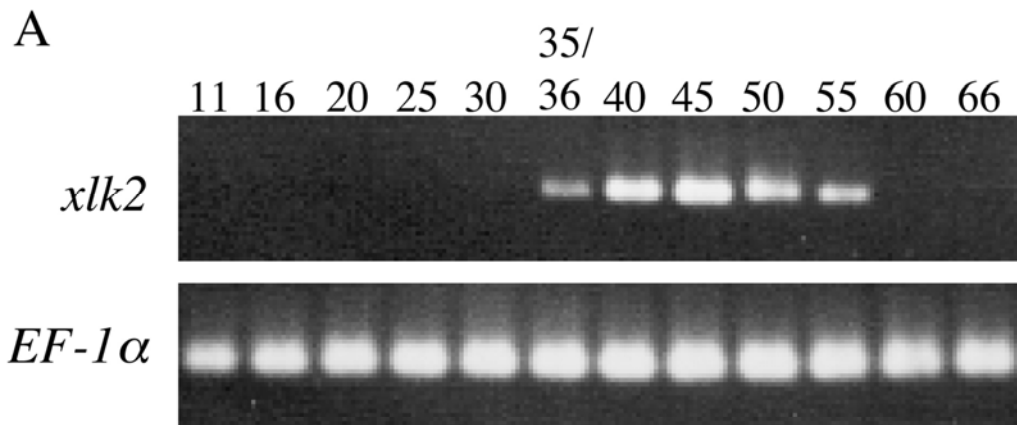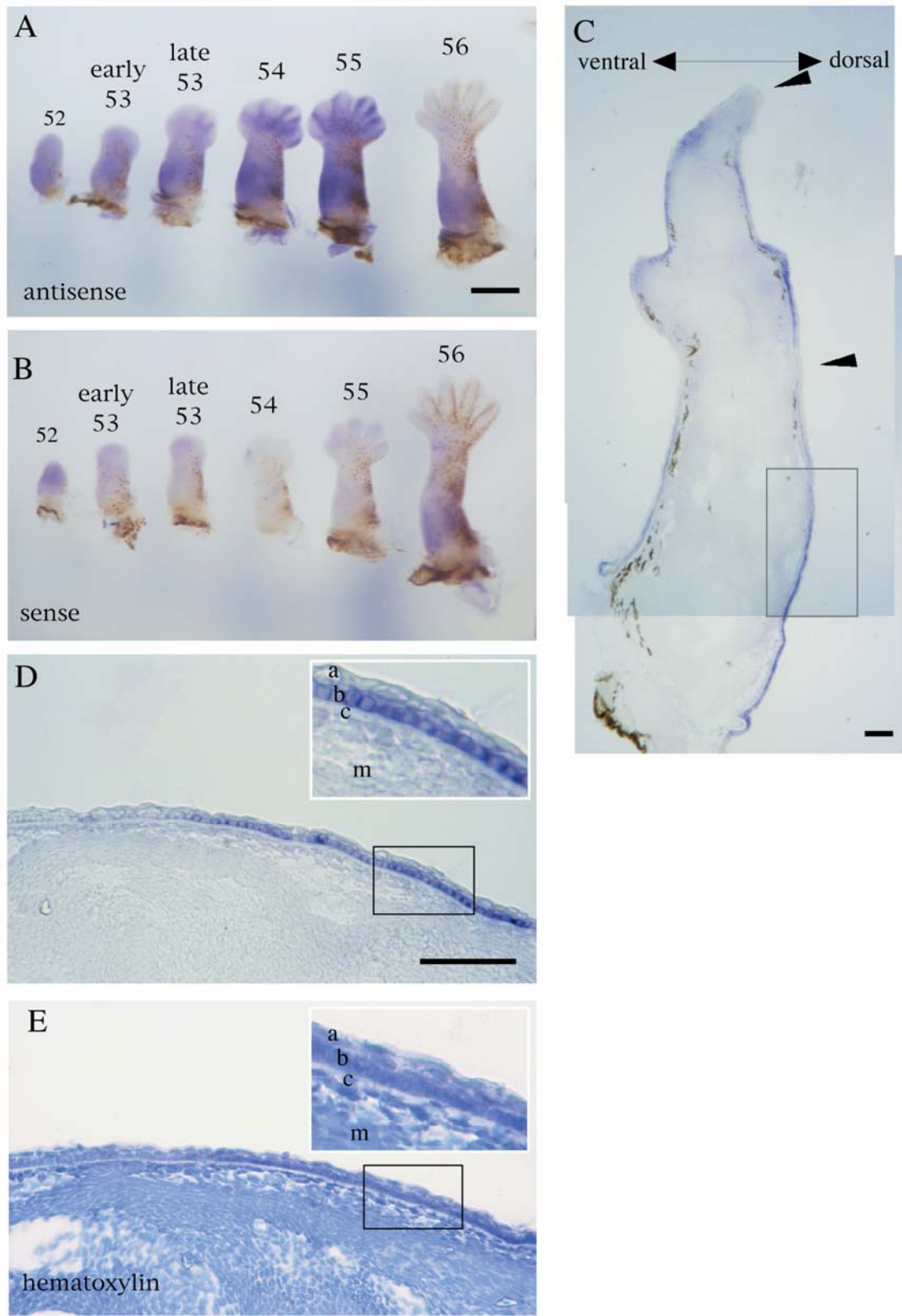
B

C

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

[I]

A 3 days

B 3 days (sense)

C 3 days regenerate dorsal ventral AEC

D 3 days (hematoxylin)

[II]

A

B 3 days

C 3 days (sense)

D 3 days regenerate

E 3 days (hematoxylin)

F 5 days

G 5 days (sense)

H 5 days regenerate

I 5 days (hematoxylin)

J 5 days

K 5 days (hematoxylin)

Fig. 5.

AEC
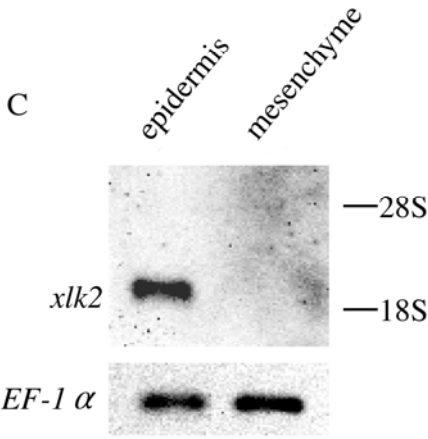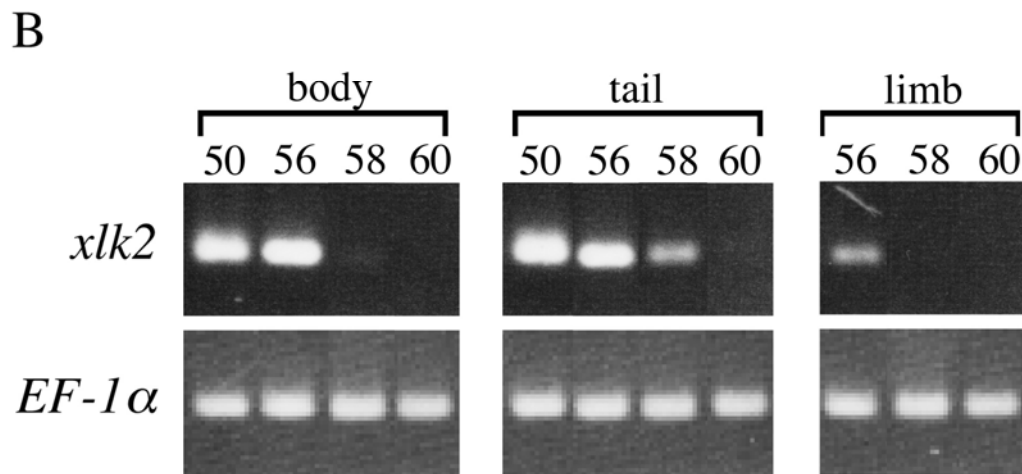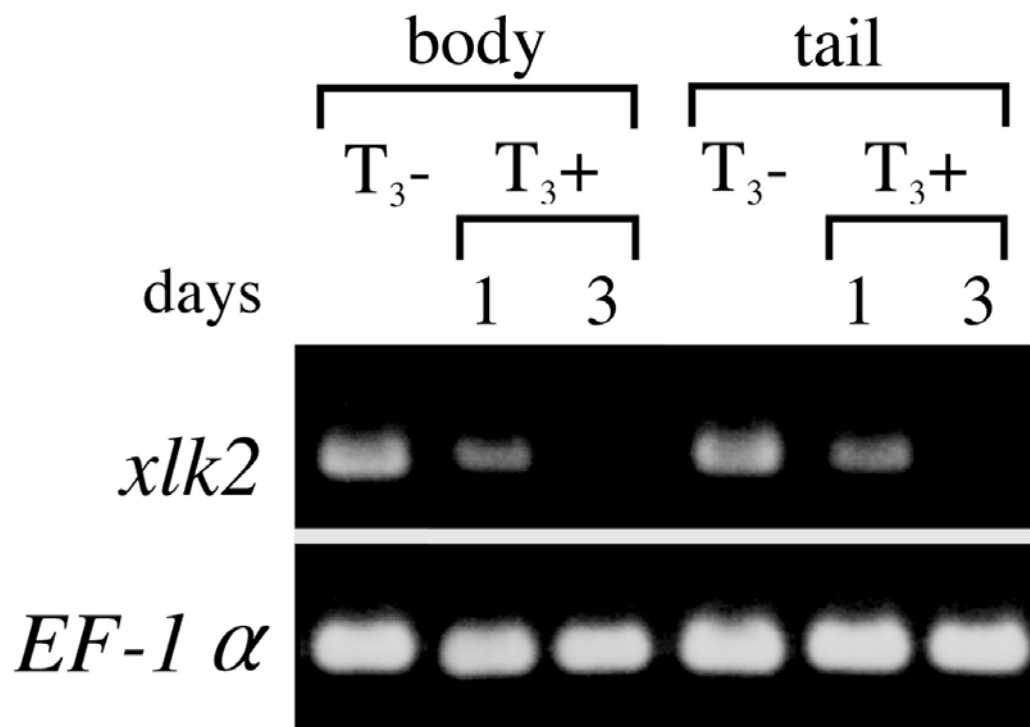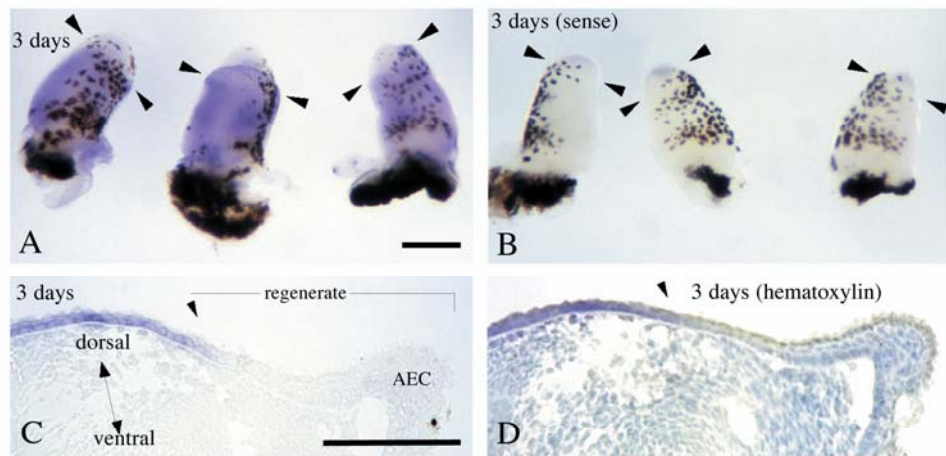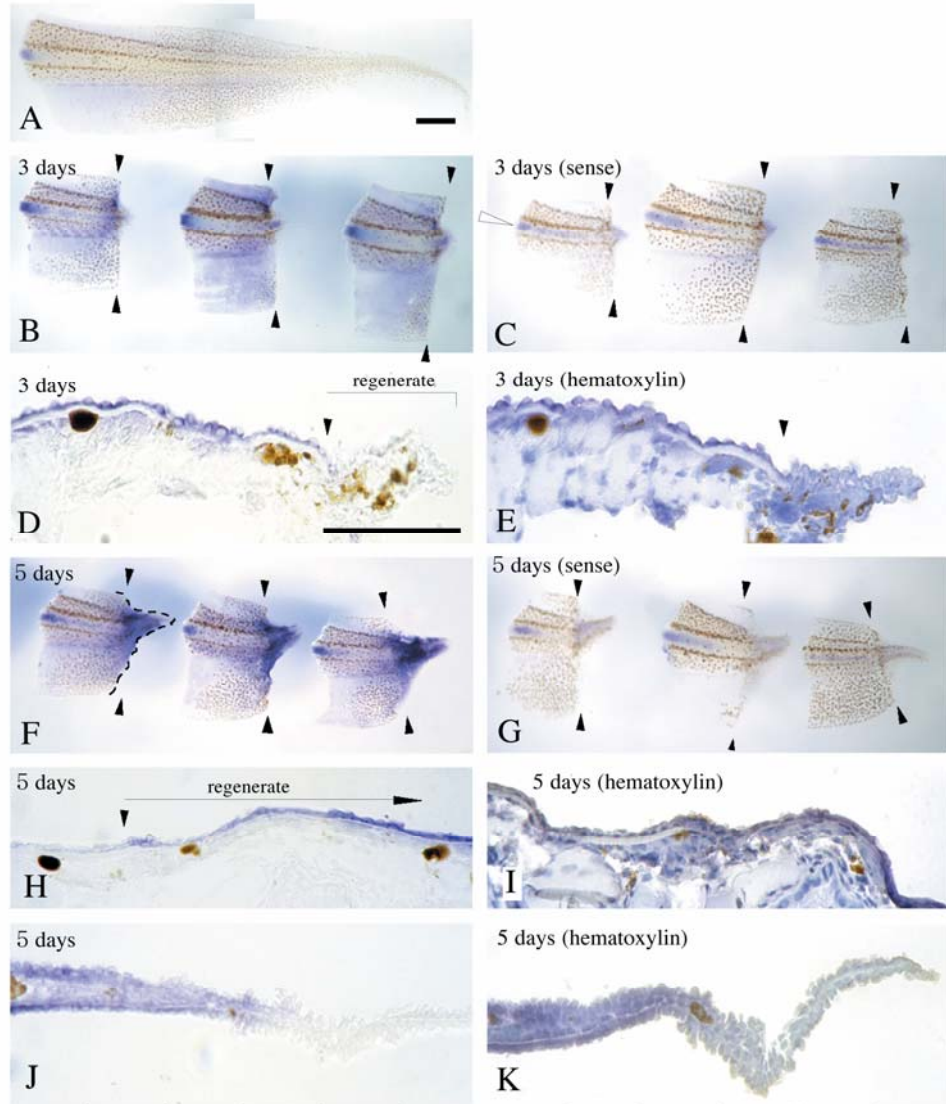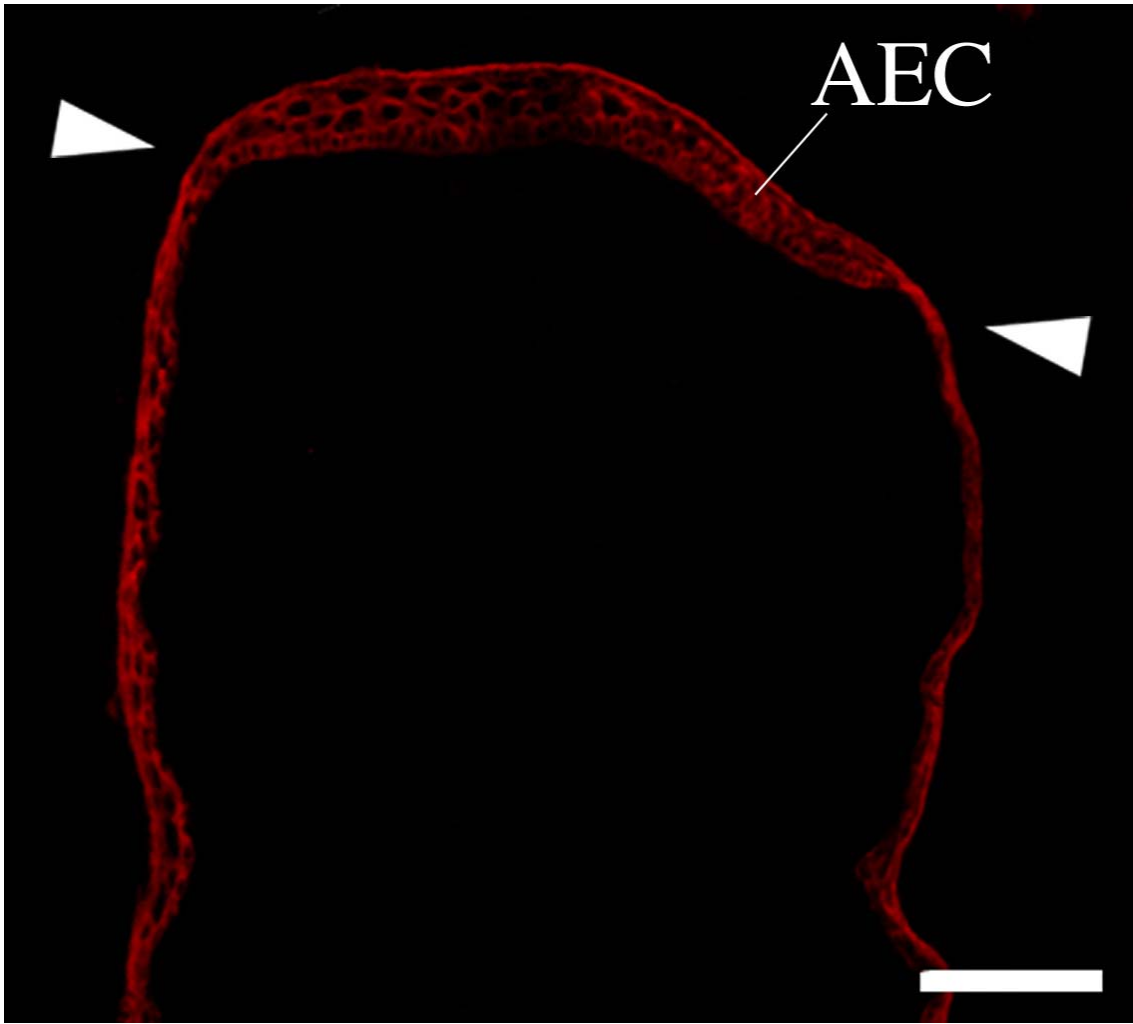
Fig. 6.

Table 1

Primers for PCR of *xlk2* and *EF-1α*

Purpose	Target	Primer sequence (5' □ 3')
RACE	*xlk2*	GCTCCATAGCTTTGTTCACGGATAGC (for 5'-RACE)
		AGCATGCAGCAGGAACAGTGAGTG (for 3'-RACE)
RT-PCR	*xlk2*	GCAGTTCATCTAGACTGAGCAGTG (sense primer)
		GCTCCATAGCTTTGTTCACGGATAGC (antisense primer)
RT-PCR	*EF-1α*	CAGATTGGTGCTGGATATGC (sense primer)
		ACTGCCTTGATGACTCCTAG (antisense primer)