Spatial and temporal expression pattern of a novel gene in the frog *Xenopus laevis*: correlations with adult intestinal epithelial differentiation during metamorphosis

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Abstract

We report the cloning of a novel gene (ID14) and its expression pattern in tadpoles and adults of *Xenopus laevis*. ID14 encodes a 315-amino acid protein that has a signal peptide and a nidogen domain. Even though several genes have a nidogen domain, ID14 is not the homolog of any known gene. ID14 is a late thyroid hormone (TH)-regulated gene in the tadpole intestine, and its expression in the intestine does not begin until the climax of metamorphosis, correlating with adult intestinal epithelial differentiation. In contrast, ID14 is expressed in tadpole skin and tail and is not regulated by TH. In situ hybridization revealed that this putative extracellular matrix protein is expressed in the epithelia of the tadpole skin and tail and in the intestinal epithelium after metamorphosis. In the adult, ID14 is found predominantly in the intestine with weak expression in the stomach, lung, and testis. Its exclusive expression in the adult intestinal epithelial cells makes it a useful marker for developmental studies and may give insights into cell/cell interactions in intestinal metamorphosis and adult intestinal stem cell maintenance.

1. Results and discussion

Frog metamorphosis is a dramatic, post-embryonic developmental process where whole organs are lost and new ones arise de novo. Most tadpole organs, however, undergo extensive remodeling during metamorphosis from the larval to adult forms (Dodd and Dodd, 1976). The metamorphic process is completely dependent upon thyroid hormone (TH) (Kaltenbach, 1996). In order to understand the molecular basis of the hormonal control of post-embryonic development, we have chosen the TH-induced remodeling of the intestine as a model. The tadpole intestine is a simple tubular organ consisting predominantly of larval epithelial cells with little surrounding connective tissue or muscles (Shi and Ishizuya-Oka, 2001). During metamorphosis, the larval epithelial cells undergo apoptosis and are replaced by a multiple-folded adult epithelium with elaborate connective tissue and muscles (Shi et al., 2001; Shi and Ishizuya-Oka, 2001). We previously used a PCR-based subtractive hybridization screen to isolate TH-regulated genes from pre-metamorphic intestine treated with or without TH (Shi and Brown, 1993). Previously published genes from this screen fell into three gene expression patterns (Shi, 2000). Most genes are up-regulated transiently during metamorphic climax, e.g. TH/bZIP, stromelysin 3, sonic hedgehog, and others. Some genes have the opposite pattern, where they are expressed before and after but are down-regulated during metamorphosis, e.g. intestinal fatty acid binding protein (Ishizuya-Oka et al., 1994). Still other genes are up-regulated during pro-metamorphosis and this high expression is maintained into the juvenile froglet, e.g. TH/aromatic amino acid transporter (Liang et al., 1997; Ritchie et al., 2003). Here, we report a novel gene, ID14, with a unique expression pattern.

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1.1. Sequence comparisons

A subtractive hybridization screen designed to isolate TH-regulated genes in the intestine (Shi and Brown, 1993) led to the isolation of a PCR fragment, ID14, from a gene expressed in the post-metamorphic intestine (see below). This cDNA was used to screen an intestinal cDNA library, yielding a cDNA clone with a length of 2371 base pairs (accession no. AY363162). The longest open reading frame predicts a 315-amino acid protein (Fig. 1A), implying a 25 base 5′ UTR and a 1401 base 3′ UTR, containing a polyadenylation signal and poly(A) tail. Searching the *Xenopus* expressed sequence tag databases using

BLAST obtained no hits with significant sequence similarity.

To search for potentially homologous genes to ID14, we performed a BLAST search with the coding sequence or protein sequence and obtained human and mouse α-tectorins. Using the web-based Simple Modular Architecture Research Tool (SMART) (Letunic et al., 2002; Schultz et al., 1998), both ID14 and the α-tectorins were found to contain an N-terminal conserved domain, the NIDO domain or 'extracellular domain of unknown function in nidogen (entactin) and hypothetical proteins'. Analysis by SMART also predicted a signal sequence of 22 amino acids at the amino-terminus of ID14. The sequence

A

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MKTVLPLLLV ATVCFGDLAT DPILYPYGPS EGDKVTPKKD DGATSAIPIS   50
TVFIFFGKKH NSLYVNNNGV ISFGVAVSSY TPNAFPLADG SPFVAPYWGD  100
VNNEIAGTVY YRESKDPNLL ERISNDMRKY YPNFNYRATW AFVATWDKVA  150
YYGSRSKKTN TFQAVLTTDG KLAFIFLNYG IITWTTGKAS GGDPLTGLGG  200
IPAQAGFNSG DKTNYFNIPG SRTPDIVNIQ KTSNVNTPGR WLFEVDKFRV  250
AGGCVYEANF VKYNETFWKD PTCETKCRCN MDGDVECEEE WCSGNLLCQP  300
STWHYSCRIG IGQCF*                                       315
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B

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Hs   MNYSSFLRIWVSFIFALVQHQAQPRELMYPFWQNDTKTPKVDDGSSSEIK   50
Mm   ~~~~~L~~~~~~~~~~~~~~R~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~   50
Gg   ~~TR~L~SA~AALLVVT~R~R~~AMASL~~~~P~~~~~~~~~~~~~~~~~~   50
Fr                      LYP~GPSHR~QE~~~M~~~~~P~~T   25
ID14    MKTV~PLLLVATVCFGDLATD~ILYP~GPSEG~KV~~~K~~~AT~A~P   48

Hs   LAIPVFFFGVPYRTVYVNNNGVVSFNVLVSQFTPESFPLTDGRAFVAPFW  100
Mm   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~I~~~~  100
Gg   ~SV~FI~~RS~~~~~~~~~~~~I~~~S~~~~~~~~A~~~A~~~~~~~~~~C  100
Fr   ~S~QFI~~NS~~~SI~~~~~~~I~~~~Q~~~~~~~~A~~~S~S~S~I~~L~   75
ID14 ISTVFI~~~KKHNSL~~~~~~~I~~G~A~~SY~~NA~~~A~~SP~~~~Y~   98

Hs   ADVHNGIRGEIYYRETMEPAILKRATKDIRKYFKDMATFSATWVFIVTWE  150
Mm   ~~~~~~~~~~~~~~~~~D~~~~R~~~~~~~~~~~~~T~~~~~~~~~~~~~  150
Gg   G~~A~~~~~~~~~~~STN~EL~GESS~~~~~~~~~~~S~~~S~~~~~~~~  150
Fr   ~~~~~~~~~~DV~~~~S~~~E~~QK~~Q~~~~H~~N~~A~M~~~~~~A~~H  125
ID14 G~~N~E~A~TV~~~~SKD~NL~E~ISN~M~~~YPNFN YR~~~A~VA~~D  147

Hs   EVTFYGGSSTTPVNTFQAVLVSDGSYTFTLFNYYEINWTTGTASGGDPLT  200
Mm   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~  200
Gg   ~~Q~~~~~~~~~~~~~~~~~IT~~VSS~AI~~~Q~~S~~~~~~~~~~~~~  200
Fr   Q~~~~~~~Q~~~~~~~~~~I~~~TKS~CM~~~G~~S~S~~~~~~~~~~~~  175
ID14 K~AY~~  ~RSKKT~~~~~~~TT~~KLA~IFL~~GI~T~~~~K~~~~~~~~  196

Hs   GLGGVMAQAGFNGGNLTNFFSLPGSRTPEIVNIQETTNVNVPGRWAFKVD  250
Mm   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~  250
Gg   ~~~~~~~~~~~~~~IS~~~~I~~~~~~D~~~~EQ~~~~~I~~~~~~~I~  250
Fr   ~~~~TT~~S~~~~~~EIGL~~N~~~~~SNDM~~~EQ~~~~~T~~~~V~RI~  225
ID14 ~~~~IP~~~~~~S~DK~~Y~NI~~~~~~D~~~~~K~S~~~T~~~~L~E~~  246

Hs   GKEIDPANGCTSRG    QFLRRGEVFWDDLNCTVKCRCLDFNNEIYCQEA  297
Mm   ~~~~~~~~~~~~~~    ~~~~~~~~~~~~~I~~~~~~~~~~~~~~~~~~~  297
Gg   ~~~~~~~~ LSL~~    ~~~HQ~~I~~ENS~~ST~~~~~~~~~~~F~~~M  296
Fr   TEL~~~~~~~SYN~KCRR~Y~~~~I~~LSDQ~SQR~~~~~IE~~VH~~~~  275
ID14 KFRV  ~G~~VYEA    N~VKYN~T~~K~PT~ET~~~~N MDGDVE~E~E  290

Hs   SCSPYEVCEPKGKFFYCSAVETSTCVVFGEPHYHTFDGFLFHFQGSCAYL  347
Mm   ~~~~~~~~~~~R~~~~~P~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~  347
Gg   LA ~F~TV~~~I~~~Q~VP~~~ A~~~~~D~~~~~~~~~~~~~~~~~~S~~  344
Fr   P~GQL~T~~QQEGA~~~QPTR~~~~~~~~D~~~~~~~~~~Y~~~~T~S~~  325
ID14 W~~GNLL~Q~STWHYS~RIGIGQCF*                         315
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Fig. 1. ID14 encodes a novel protein sharing sequence similarity with α-tectorin proteins. (A) Deduced amino acid sequence of *Xenopus laevis* ID14. The inferred signal peptide is underlined, and the NIDO domain (extracellular domain of unknown function in nidogen and hypothetical proteins) is in bold. The asterisk indicates the termination site. (B) The NIDO domain of *Xenopus* ID14 is homologous to that in α-tectorin proteins from *Homo sapiens* (*Hs*), *Mus musculus* (*Mm*), *Gallus gallus* (*Gg*), and *Fugu rubripes* (*Fr*). The tilde indicates the same amino acid as in *H. sapiens*, spaces indicate gaps, and the bold indicates the NIDO domain. The full-length ID14 protein is shown, whereas only the comparable portion is shown for the α-tectorin proteins. The N-terminal sequence for *F. rubripes* α-tectorin was not available.

Table 1
Protein sequence comparisons among NIDO domain-containing proteins

	α-Tectorin	Nidogen-1	Nidogen-2	Apomucin	Tumor endothelial marker 7	FLJ00133
(a) *Percent identity of the NIDO domains of ID14 and all proteins in the human genome containing the domain*						
ID14	56	24	26	25	21	45
α-Tectorin		27	29	23	17	48
Nidogen-1			46	25	16	21
Nidogen-2				25	15	23
Apomucin					17	18
Tumor endothelial marker 7						16
(b) *Percent identity of the NIDO domains of ID14 and all known vertebrate α-tectorins*						
	ID14	*Homo sapiens*	*Mus musculus*	*Gallus gallus*		
Homo sapiens	56					
Mus musculus	57	98				
Gallus gallus	57	78	78			
Fugu rubripes	54	70	69	69		

similarity between the α-tectorins and ID14 was restricted to the NIDO domain, and nidogens were also retrieved in BLAST searches but with a much lower similarity score. A BLAST search using only the long 3′ UTR retrieved no matches in any database. In order to confirm α-tectorin was the most similar, we performed a pair-wise sequence similarity comparison between the NIDO domains of ID14 and all six proteins in the human genome containing the NIDO domain (Table 1a). Only α-tectorin had a similarity score over 50%.

This result was puzzling because mammalian α-tectorins are expressed in the middle ear (Legan et al., 1997; Mustapha et al., 1999), an organ lacking in frogs. However, a search of the genome of the fish *Fugu rubripes*, which also lacks a middle ear, identified an α-tectorin homologous to the human α-tectorin. In addition to sequence similarity, this homology assessment was based on the conserved domain structure of the α-tectorins from SMART analysis and the conserved intron–exon structure in the fish and human genes. Furthermore, these α-tectorins are approximately 2500 amino acids long, compared to 315 amino acids for ID14. Percent sequence similarity comparisons between ID14 and the known vertebrate α-tectorins, which includes the mouse (Legan et al., 1997) and chicken (Coutinho et al., 1999) homologs, indicated that the α-tectorins of fish and other vertebrates are more similar amongst each other than any is to ID14 (Table 1b). The sequence alignments of the vertebrate α-tectorins with ID14 show sequence conservation from 35 amino acids N-terminal to, and throughout, the NIDO domain (Fig. 1B). There is little sequence similarity outside this region, except for a curious stretch of seven amino acids at the N-terminus of the *Fugu* sequence. These comparisons suggest that ID14 is a novel gene, derived within the amphibian lineage, and we predict the existence of a true α-tectorin homolog in frogs.

1.2. Larval expression patterns

Northern blot analysis of ID14, using a developmental series of intestinal RNA, showed this gene is expressed at the very end of the metamorphic transformations induced by TH (Fig. 2A). At the end of metamorphosis, all of the larval intestinal epithelial cells have been replaced by adult cells, and the connective tissue and muscles have increased in cell number (Ishizuya-Oka and Ueda, 1996). The Northern blot for the whole body across the same developmental stages revealed a very different pattern compared to the intestine (Fig. 2B). In the whole body, ID14 is detectable in pre-metamorphosis, stage 50, and is highly expressed throughout metamorphosis. This constitutive expression in the whole animal is also consistent with the inability of 1-day TH treatment to alter the mRNA level in whole animals during pro-metamorphosis, stages 54–58 (Fig. 2B).

In addition to the different temporal expression profiles, the intestine and whole body also express different forms of the ID14 mRNA. The most abundant species in the whole body is 2.4 kb, which corresponds in size with the length of the ID14 cDNA. The dominant band in the intestine is 1.1 kb and is just larger than the coding sequence. This band is likely produced through the use of an alternative polyadenylation signal or alternate splicing of a putative intron in the 3′ UTR. The largest and least abundant band ($>$5 kb) may be a pre-processed form. To investigate the possibility of alternate mRNA processing in the intestine, we assayed for the presence of a coding region fragment and a 3′ UTR fragment in the mRNA in the intestine, skin, and tail of tadpoles at different stages throughout metamorphosis by reverse transcriptase (RT)-PCR (Fig. 3). Based on the transcript sizes from the Northern analysis, we expected that intestinal transcripts would lack most of the long 3′ UTR but that other tissues of the body would have both the coding sequence as well as the long 3′ UTR. Our RT-PCR
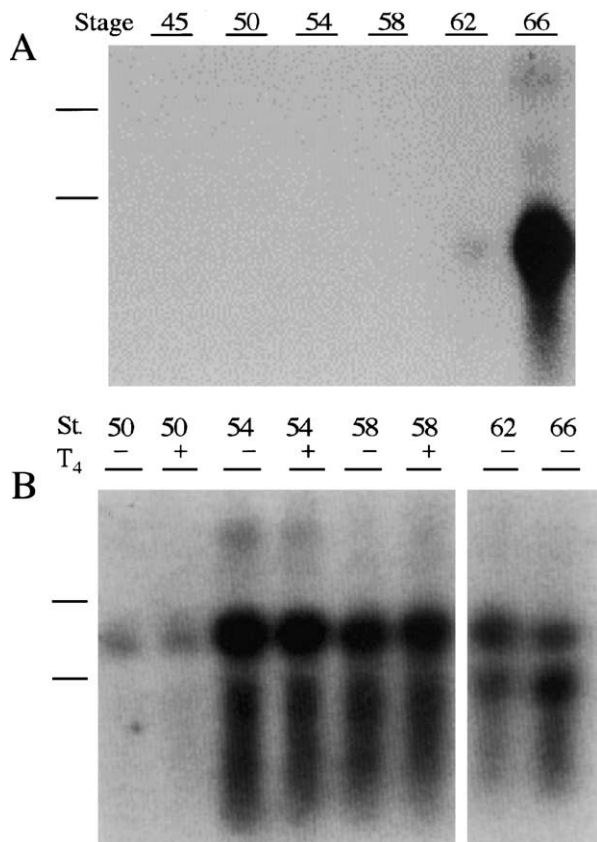
Fig. 2. Larval expression pattern of ID14 in spontaneous metamorphosis. (A) Northern blot of total intestinal RNA at the indicated stages shows that ID14 is up-regulated towards the end of metamorphosis (stage 66). (B) Northern blot of whole body RNA at the indicated stages reveals constitutive expression of ID14 during development. For stages 50–58, both control animals and animals treated with 26 nM T4 for 1 day were used, and no change in ID14 expression was induced by TH. Note that the predominant band in A is the 1.1 kb band while that in whole body is the 2.4 kb band, although at stage 66 the 1.1 kb band is more abundant than the 2.4 kb even in the whole body. The short lines on the left in both A and B indicate the positions of the 18S and 28S rRNA.
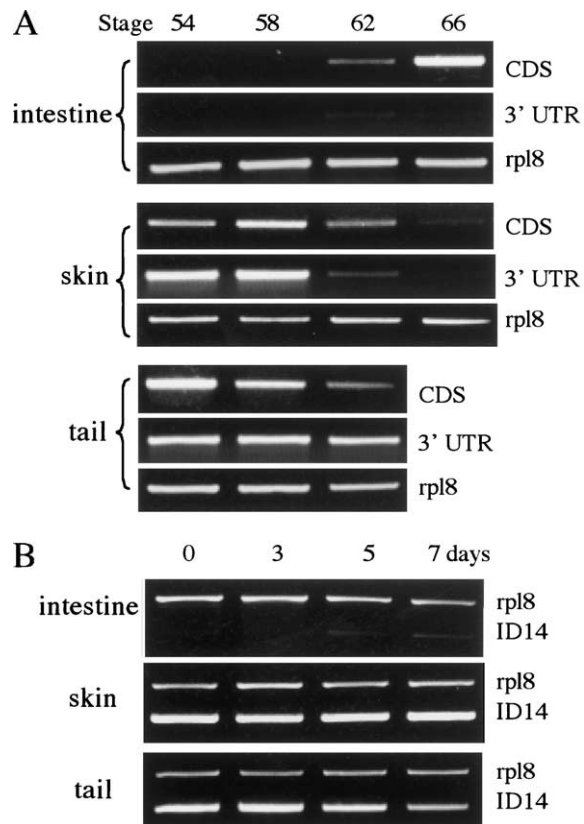

Fig. 3. Tissue-dependent TH-regulation and processing of ID14 mRNA. (A) The temporal regulation of ID14 differs across tadpole organs, and processing of ID14 mRNA is also tissue-specific. RT-PCR was performed using total RNA from the intestine, skin, and tail from the indicated developmental stages with primers in the ID14 coding sequence or $3'$ UTR, and the control gene rpl8. ID14 is up-regulated during metamorphosis only in the intestine, and the $3'$ UTR is highly expressed in the skin and tail but not the intestine. (B) ID14 is a late TH-inducible gene in the intestine but not tail or body skin. Total RNA from intestine, tail, and skin from stage 54 tadpoles treated with 5 nM T3 for 0, 3, 5, or 7 days was subjected to RT-PCR analysis. Two primer pairs were used in each reaction tube, rpl8 control primers (0.04 μM) and primers for the ID14 coding sequence (0.4 μM for intestine, and 0.2 μM for tail and skin). Note that ID14 is up-regulated by TH after 5–7 days only in the intestine.

analysis across developmental stages was consistent with this view (Fig. 3A) and was in agreement with the Northern blot analysis (Fig. 2). The intestine expresses predominantly the coding region of ID14, whereas the skin and tail RNA contain both the coding region and the $3'$ UTR with a much higher ratio of $3'$ UTR to coding region than that in the intestine. These results suggest that the different mRNA species identified by Northern analysis (Fig. 2) are due to different lengths of the $3'$ UTR. Furthermore, the RT-PCR results confirmed the Northern data that the temporal regulation of ID14 differs between the intestine and other tissues.

The up-regulation of the ID14 expression by the end of metamorphosis in the intestine (Figs. 2A,3A) suggests that ID14 is a late TH-response gene in the intestine. We tested this using a TH-induction assay where gene expression was analyzed from pre-metamorphic tadpoles treated with TH for 0–7 days. RT-PCR analysis of total intestinal RNA

confirmed the absence of ID14 expression during pre-metamorphosis (Fig. 3B, upper panel). Expression of ID14 appears after 5–7 days of TH treatment, which induces the development of adult intestine (Shi and Ishizuya-Oka, 2001), corroborating the Northern blot data that ID14 is expressed in the post-metamorphic intestine. RT-PCR analysis of total RNA from tail and skin show that ID14 is not regulated by TH in these tissues (Fig. 3B, lower panels), consistent with the whole body Northern blots (Fig. 2B).

1.3. Adult expression patterns

The developmental changes in the relative levels of the 1.1 and 2.4 kb mRNA species in the whole body and the predominance of the 1.1 kb mRNA in the intestine suggest
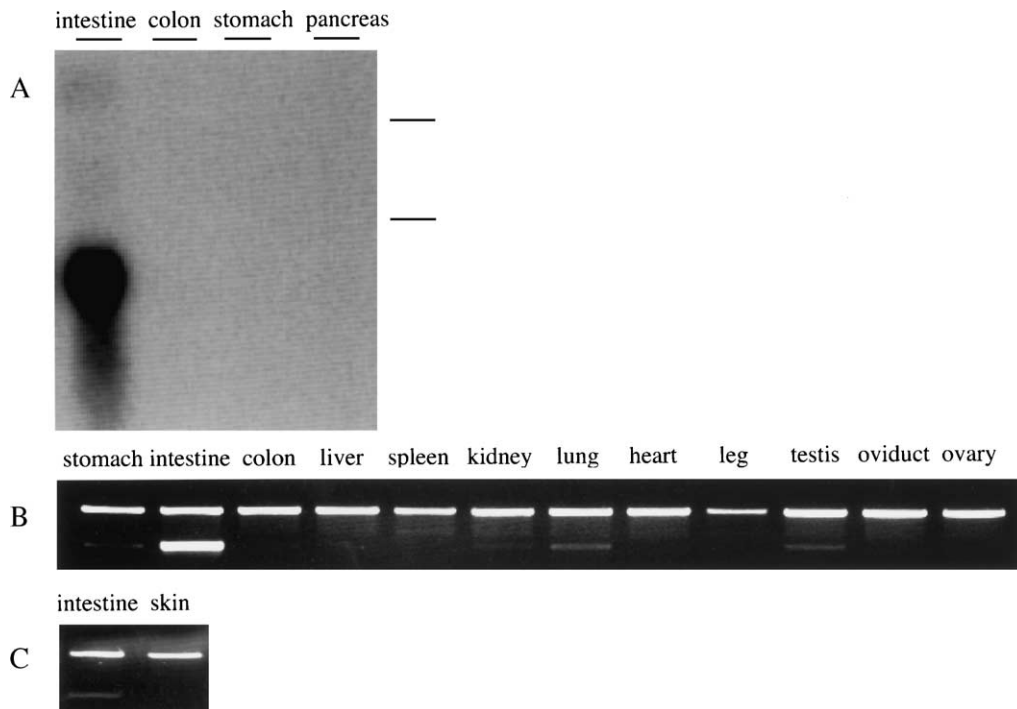
Fig. 4. Tissue-specific expression of ID14 in post-metamorphic frogs. (A) Northern blot of total RNA from froglet gastrointestinal tract tissues revealed intestine-specific expression. (B) RT-PCR analysis on total RNA from adult tissues showed strongest expression in the intestine, less expression in the lung and testis, weak expression in the stomach, colon, liver, and kidney, and no detectable expression in the other tissues/organs. (C) The adult skin has little or no ID14 expression compared to the intestine. To obtain these data for the skin, different RT-PCR conditions were required as detailed in Section 2 and the instestine was used as an internal control for comparison with the data in B.

that in post-metamorphic frogs, the intestine may be the major organ that expresses ID14, thus increasing the relative abundance of the 1.1 kb mRNA in the whole animals (Fig. 2B). Indeed, Northern blot analysis of four different organs of the gastrointestinal tract, the stomach, small intestine, colon, and pancreas showed that only the small intestine expresses ID14 in post-metamorphic frogs (Fig. 4A). Furthermore, using the more sensitive RT-PCR assay, we showed that in adult frogs, the intestine has by far the highest level of ID14 expression. Much lower levels of ID14 expression are present in the lung and testis, while little or no expression was found in other organs analyzed. Under the PCR conditions, we failed to obtain a signal from the internal control gene rpl8, from the adult skin RNA, likely due to some contaminant inhibiting the PCR. Thus, we used less RNA and a higher PCR cycle number to analyze the expression in the skin and intestine. As shown in Fig. 4C, again the intestine has much higher levels of ID14 expression than in the skin, consistent with the reduced levels of ID14 in the skin of animals at tail resorption, stage 66. These results suggest that ID14 has very restricted organ-specific functions in adult frogs.

1.4. Tissue-specific expression

The late up-regulation of ID14 expression in the intestine correlates with the development of the adult intestinal

epithelium (Shi and Ishizuya-Oka, 2001). This suggests that ID14 is expressed in the differentiating and/or differentiated adult intestinal epithelial cells. Adult epithelial cells first appear as nests of proliferating cells at climax of metamorphosis (stage 60) and replace the apoptotic larval epithelial cells (Shi and Ishizuya-Oka, 2001). Consistent with the Northern blot and RT-PCR analyses above, no larval cells expressing ID14 mRNA were detected by in situ hybridization in the intestine at stage 54 (Fig. 5A). Also, ID14 is not detectable at stages 61 and 62, when larval cells undergo apoptosis and adult stem cells begin proliferating (Fig. 5B). At stage 63, when the adult epithelium completely replaces the larval epithelium and begins to differentiate, ID14 mRNA became detectable in the epithelium but not the connective tissue or muscles (Fig. 5C), and its expression was up-regulated towards the end of metamorphosis (stage 66) (Fig. 5D). In contrast, all control hybridizations with the sense RNA probe gave only background levels of signals (Fig. 5E). Similar to its epithelial-specific expression in the intestine, ID14 mRNA was also restricted to the epithelium of the skin (Fig. 6A) and epithelium of the tail (Fig. 6B) in tadpoles. Again, control hybridizations using the sense RNA probe showed no specific signals (Fig. 6C,D).

In conclusion, ID14 is a novel, NIDO domain-containing gene, expressed late in metamorphosis in the intestine but throughout the larval period in the skin and tail.
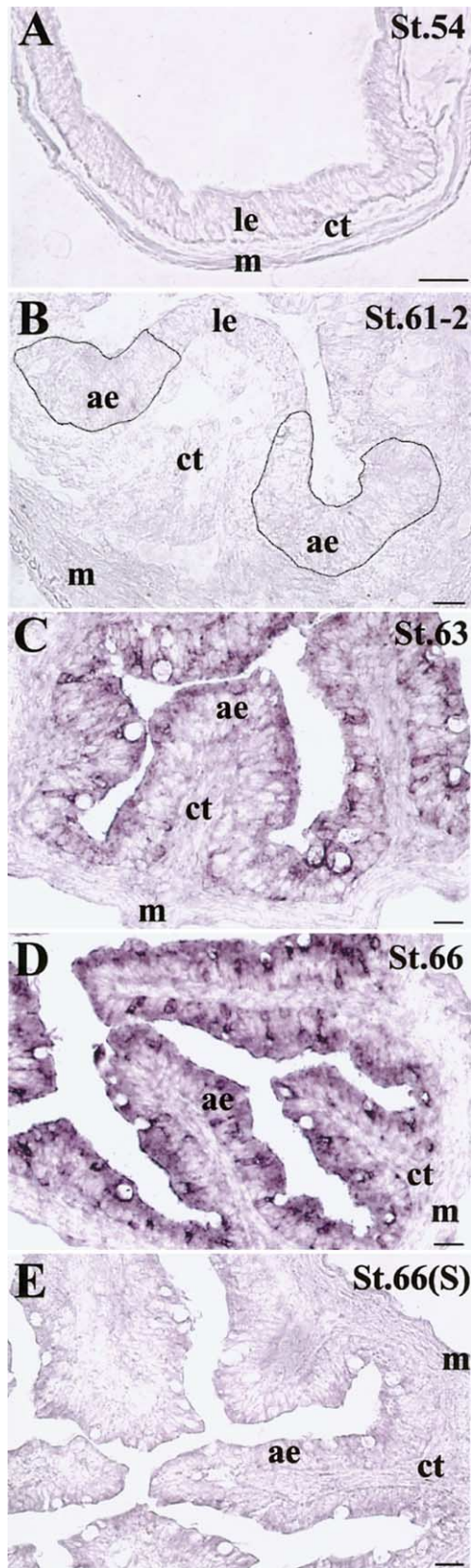
Fig. 5. In situ localization of ID14 mRNA to the intestinal epithelium. Cross-sections of the intestine were hybridized with the antisense (A–D) or sense (E) RNA probes. (A) No specific signals were detected in the larval epithelium (le) at stage 54. (B) No signals were seen in dying larval cells

The expression profile of ID14 across metamorphosis and its epithelial localization make ID14 the first molecularly characterized gene marker for adult differentiated intestinal epithelium. ID14 should be a valuable aid in the study of the cellular/developmental mechanisms of intestinal tissue remodeling and the origin of the adult intestinal epithelial stem cells during development.

2. Materials and methods

2.1. Animal treatments

Larval and adult *Xenopus laevis* were purchased (NASCO, Fort Atkinson, WI) Tadpoles were reared in dechlorinated tap water and staged according to Nieuwkoop and Faber (1994). Tadpoles of indicated stages were treated in the presence or absence of 5 nM T_3 or 26 nM T_4 as described (Shi and Brown, 1993).

2.2. Cloning

A PCR-based subtractive hybridization (Shi and Brown, 1993) led to the isolation of a small PCR fragment of ID14. The cDNA fragment was used to screen a *lambda* Uni-Zap (Stratagene) cDNA library made using intestinal RNA from stages 52–54 tadpoles treated with 5 nM T_3 for 18 h (Patterton and Shi, 1994). A 2.4 kb full-length cDNA clone was isolated and sequenced (accession no. AY363162).

2.3. Expression analyses

For Northern blot hybridization, RNA was isolated from indicated tissues or whole body of tadpoles with or without indicated treatment. The total RNA was analyzed by Northern blot hybridization (10 μg/lane) as described (Patterton and Shi, 1994; Shi and Brown, 1993). To control for RNA loading and quality, the Northern blots were hybridized with the cDNA for the ribosomal protein L8 (rpL8), whose expression is constant during development and independent of T_3 (Liang et al., 1997) (data not shown).

For RT-PCR analysis of gene expression, total RNA was isolated using Trizol (Invitrogen) from organs of adults and staged tadpoles treated with or without 5 nM T3. Two primer pairs were used per reaction tube (Buchholz et al., 2003): the control gene rpl8 and ID14 coding sequence

(le) or proliferating, undifferentiated adult epithelial cells (ae) at stages 61 and 62. Identification of the area of adult cell proliferation demarcated by dark line is based on previous studies (Ishizuya-Oka and Ueda, 1996). (C) Signals became detectable in the adult epithelium (ae) at stage 63, and intensified by stage 66 (D). (E) Background signal was weakly seen for the intestine at stage 66 hybridized with the sense probe. ct, connective tissue; m, muscles. Bars, 20 μm.
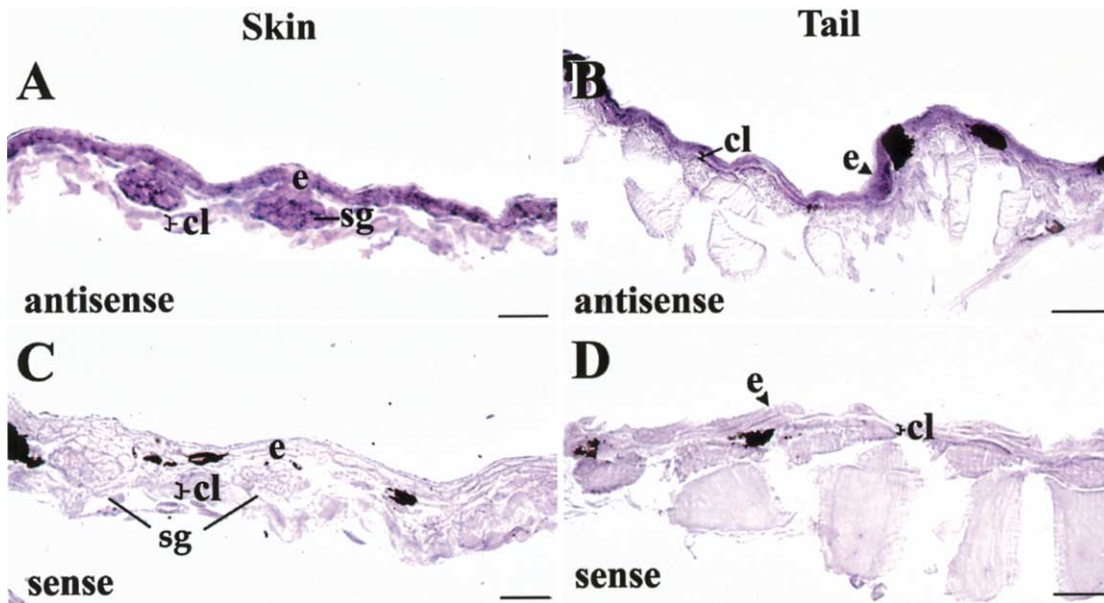
Fig. 6. In situ localization of ID14 mRNA to the epithelia of tadpole skin and tail. Cross-sections of back skin (stage 66) and tail (stage 55) were hybridized with the antisense (A and B) or sense (C and D) RNA probe. Specific signal in the epithelium is seen in sections hybridized with the antisense probe in the skin (compare A and C) and tail (compare B and D). The specific signal in the secretory glands in A is consistent with the epithelial origin of the glands. The dark spots in B–D are melanin pigment granules. e, epithelia; sg, epithelial secretory glands; cl, collagen lamella. Bars, 20 mm.

(5′-TGTTTCGGAGACTTGGCCACC and 5′-GGATAG-TACTTCCTCATGTCG) or ID14 3′ UTR (5′-GGA-TACTTGCCCCCCTACTGG and 5′-GTCACAGCTTT TCTGCTGAGG). Reactions were carried out using Super-script One-Step RT-PCR (Invitrogen) with 0.5 µg RNA, 0.04 µM rpl8 primers, and 0.2–0.4 µM ID14 primers in a 25 µl reaction volume. To avoid unknown inhibition of RT-PCR by adult skin RNA samples, the adult skin RNA (and adult small intestine as a control) was diluted 1/10 and 35 cycles were used. Thermocycler settings were one cycle of 50 °C for 30′ and 94 °C for 2′ followed by 28 cycles of 94 °C for 30″, 55 °C for 30″, and 72 °C for 30″. Products of PCR were run on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Procedures for in situ hybridization were the same as those described previously (Ishizuya-Oka et al., 1994). In brief, digoxigenin (DIG)-11-UTP-labeled single-stranded antisense and sense RNA probes were prepared according to the instructions of the DIG RNA labeling kit (Roche Diagnostics) by using a 2.4-kb ID14 cDNA clone in pBluescript. Tissue fragments isolated from the anterior part of the small intestine, the back skin, and the tail were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) at 4 °C for 4 h. They were frozen on dry ice and cut at 7 µm. Hybridization buffer containing DIG-labeled antisense or sense RNA probe (125 ng/ml) was applied to the sections. After hybridization at 42 °C for 18 h, the sections were treated with 20 µg/ml RNase A (Wako) at 37 °C for 30 min to remove excess unhybridized probes. They were then washed, processed for immunological

detection of the hybridized DIG-probes according to the manufacturer's instructions (DIG-probe Detection Kit, Roche Diagnostics), and examined microscopically.

Acknowledgements

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