Expression of MHC Class Ia and Class Ib During Ontogeny: High Expression in Epithelia and Coregulation of Class Ia and \textit{Imp7} Genes

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Expression of MHC Class Ia and Class Ib During Ontogeny: High Expression in Epithelia and Coregulation of Class Ia and Imp7 Genes

Luisa Salter-Cid,* Masaru Nonaka,† and Martin F. Flajnik2*

The amphibian *Xenopus* permits the examination of immune responses in a species that progresses through two distinct lives, tadpole and adult, in which animals are free-living and immunocompetent. MHC gene expression as well as general features of the immune system change profoundly at metamorphosis. In this study gene expression of class Ia, class Ib, and the immune proteasome component Imp7 was investigated by Northern blotting at all stages of development. Class Ia genes are expressed in most adult tissues, with highest levels in intestine. Class Ib genes are expressed at lower levels, and their tissue distribution is somewhat more restricted than that of class Ia. Consistent with the idea that particular class Ib isotypes can perform distinct functions, preferential expression of class Ib genes is found in some tissues, with one family being expressed exclusively in epithelia. The onset of MHC expression is not simultaneous in all tissues: class Ia transcripts are first present in tadpole lung, gill, and intestine, organs with epithelial surfaces in contact with the environment. In every tissue except colon and muscle, class Ia expression increases markedly after metamorphosis. Interestingly, expression of the MHC-linked proteasome component Imp7 mirrored class Ia expression, while the constitutive Imp7 homologue X was expressed ubiquitously at all stages. Class Ib transcripts were never detected before metamorphosis, suggesting that the *Xenopus* class Ib proteins identified to date do not subserve class Ia functions in tadpole life. The Journal of Immunology, 1998, 160: 2853–2861.
APCs, but also on several epithelial surfaces, such as pharynx, gut, skin, and gills (20, 22). The expression of adult levels of class II Ags on cells of the premetamorphic lymphoid system is not surprising, considering that tadpoles are capable of generating specific Ab responses, albeit with a more restricted repertoire than that of adults (21, 23, 24), but their presence in regions that contact with the external environment suggests that class II molecules may also be used by the tadpole for direct protection, possibly by enabling several different cell types to present Ags to T cells (22).

One particularly interesting feature of MHC expression in *Xenopus* is that, in contrast to the early larval appearance of class II molecules, class I proteins are found at low levels on the surface of most cells before metamorphosis (20, 25). Thus, high class I expression is essential neither for early larval development nor for the tadpole immune system to function. It should be noted, however, that class I MHC α-chains are expressed in thymic epithelial cells of young tadpoles (20), perhaps leading to the expression of a putative CD8 molecule that is expressed on larval T cells (26). In contrast to class I expression (27), the changes in class I levels seem to be more dependent on time than on metamorphosis; in “blocked” tadpoles there is a steady increase in the expression of cell surface class I molecules, suggesting that class I expression is independent of thyroid-regulated developmental changes (25, 28).

*Xenopus*, an amphibian last sharing a common ancestor with mammals 300×10⁹ yr ago, with its well-characterized MHC, is a phylogenetically important model to examine the developmental regulation and evolution of MHC-linked genes (29). Furthermore, because the *Xenopus* immune system displays different characteristics during tadpole and adult lives, one may analyze MHC function both during ontogeny and in concert with the extensive tissue reorganization that occurs at metamorphosis. Herein we include studies on the tissue distribution and ontogenetic expression of the *Xenopus* classical and nonclassical class I molecules and the pro- teasome subunit Imp7. Since the function of most nonclassical class I genes is poorly understood, examining the tissue distribution, expression during ontogeny, and phylogenetic distribution of these molecules may give an indication of their functions, and begin to elucidate whether they represent a primitive system of recognition.

Materials and Methods

Animals

Partially inbred MHC homozygous *X. laevis* frogs with the haplotypes f,g (30), and r (31), were bred and maintained at the University of Miami School of Medicine, Miami, FL. All animals were anesthetized in water containing 0.02% (tadpoles) or 0.3% (adults) MS222 (tricaine methane-sulfonate) before being killed.

Oligonucleotides

The following oligonucleotides were synthesized based on the sequence of the *Xenopus* class Iα (32) and class Iβ XNC1(33) clones: class Iα RF, 5'-CGAGCCTTTTGGGCTCCAGAGTT-3' (nucleotides 165–189); class Iα 3R, 5'-TGATCGAGGGGCGTGAA-3' (complementary to nucleotides 611–631); class Iα Sp-R, 5'-CCAGATGAGAGGAGGTGT-3' (complementary to nucleotides 1162–1179); class Iβ Fa3, 5'-CTCTCCAAAAGT GGAAGATTT-3' (nucleotides 595–615); and class Iβ Ra3, 5'-TGC CAATGAGGTCTACACGAGATTTG-3' (complementary to nucleotides 816–839).

The following oligonucleotides were synthesized based on the sequences of XNC1 (GSP2), XNC6/XNC8 (GSP3), and XNC consensus (GSP1): GSP1, 5'-CCTCTGGTTGTACCTCCACACC-3' (complementary to nucleotides 779–799); XNC1; GSP2, 5'-TATGGAGGTAGCAAAGT GAC-3' (nucleotides 148–168, XNC1); and GSP3, 5'-CAACCY(C/T)TC TTCGGTAGACAT-3' (complementary to nucleotides 542–574 (XNC6); nucleotides 569–600 (XNC8)).

DNA probes

Two XenoProbes class Ia probes were used in these studies: a 750-bp insert generated by PCR using 10 μM of the RF and α3R primers encoding the three external domains (probe A) and a 1015-bp insert using the RF and Stp-R primers encoding the entire coding region (probe B). Both probes were amplified from the class Iα plasmid clone (32) under buffer conditions recommended for the Taq DNA polymerase (Stratagene, La Jolla, CA; Life Technologies, Gaithersburg, MD). The *Xenopus* class Iβ α3 domain-specific probe was generated by PCR using as a template the XNC1 clone (13), and the Fa3 and Ra3 primers. XNC6 and XNC8 isotype-specific probes were generated from 5' RACE--amplified clones using the 5' RACE adaptor primer and the GSP3 oligonucleotide, both at a concentration of 10 μM. Two nanograms of plasmid DNA containing either the XNC6 or XNC8 inserts were used as a template in a PCR under the conditions used in the 5' RACE procedure (see below). The *Xenopus* cytoskeleton actin cDNA (full-length) is cloned into the plasmid SV40/pBR322 (p30) before being killed. All probes were radiolabeled with 32P by random priming with the Random Prime labeling kit (Boehringer Mannheim, Indianapolis, IN). The sp. act. ranged from 1 to 4×10⁹ cpm/μg.

Total RNA isolation

Total RNA was extracted using the Trizol reagent (Life Technologies) following the manufacturer’s protocol for purification from tissues or cells grown in suspension. The total RNA concentration was determined by optical density, and the integrity of the RNA samples confirmed by electrophoresis (see below).

Northern blot analysis

Twenty micrograms of total RNA from various tissues or cells was lyophilized and resuspended in 10 μl of RNA loading buffer (1% running buffer, 50% deionized formamide, 2.2 M formaldehyde, 3% Ficoll, 0.1% bromo- phenol blue in DEPC water), and 1 μl of ethidium bromide (EtBr; 400 μg/ml stock), was added. The samples were heated at 65°C for 10 min and kept on ice until loading onto a formaldehyde gel (1% agarose, 2.2 M formaldehyde, and 1% running buffer in DEPC water). Electrophoresis was performed at 23 V overnight in 1× running buffer (20× RNA running buffer is 0.4 M MOPS, 0.02 M EDTA, 0.1 M Na acetate, and 0.019 N NaOH in DEPC water). Transfer was carried out overnight by capillary action in 10× SSC onto a Zeta-Probe membrane (Bio-Rad, Richmond, CA). RNA was immobilized onto the nylon membrane by baking at 80°C under vacuum. The membrane was then washed in 2× SSC for 10 min, followed by prehybridization in a solution containing 50% deionized formaldehyde, 5× SSC (1× SSC is 150 mM NaCl and 15 mM trisodium citrate), 5× Denhardt’s solution (× Denhardt’s is 0.02% BSA, 0.02% Ficoll, and 0.2% polyvinylpyrrolidone), 1% SDS, and 200 μg/ml denatured salmon sperm DNA for at least 2 h at 42°C. Twenty-five nanograms of radiolabeled probe was subsequently added to the solution, and hybridization took place for at least 16 h at 42°C. After that period the membrane was washed with 2× SSC/0.1 SDS twice for 5 min each time, followed by one wash with 0.1× SSC/0.1 SDS at 65°C for 20 min. Autoradiography was performed using Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

cDNA amplification by 5' RACE

Amplification of XNC isotypes from several tissues was conducted using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). First-strand cDNA synthesis was performed using 1 μg of total RNA and the cdna synthesis primer provided by the kit. Second-strand cdna synthesis and ligation of the Marathon cdNA Adaptor were conducted according to the manufacturer’s instructions. 5' RACE was carried out as follows. For each PCR reaction (50 μl total volume), 5 μl of the diluted adaptor-ligated cdNA mix was added with 1 μl of 10 mM dNTPs, 5 μl of 10 × PCR buffer (Life Technologies), 3 μl of 25 mM MgCl2, and 1 μl of each primer (GSP1 and adaptor primer 1) at a concentration of 10 μM. The reactions were heated at 94°C for 5 min, after which they were maintained at 80°C (hot start). Taq DNA polymerase (Life Technologies) was diluted in 1× PCR reaction buffer, and 5 μl (2 U) was added to reactions equilibrated at 80°C. The mix was submitted to 30 amplification cycles of denaturation for 30 s at 94°C, annealing for 2 min at 55°C, and extension for 2 min at 72°C.

3 Abbreviations used in this paper: 5' RACE, rapid amplification of 5' complementary DNA ends; DEPC, diethylpyrocarbonate; EtBr, ethidium bromide; UT, untranscribed region.
A full-length Xenopus β-actin probe was used as a positive control. The actin gene is not expressed in the same amounts (33) in all tissues, so in some cases we have included EtBr-stained gels in the figures to display the amount and the quality of RNA. Class Ia transcripts were detected in all tissues studied, except colon (Fig. 1) and muscle (not shown), and expression varies among organs, with the highest levels in intestine (Figs. 1A and 4). The very high expression of class Ia in intestine in relation to that in spleen suggests that such expression is due not only to infiltrating lymphoid cells but also to the epithelium itself.

As described, Xenopus class Ib genes make up a large family of loci falling into at least nine subfamilies (XNCI–XNC9) (13), six of which have open reading frames and potentially could be expressed as proteins. Thus, with the hypothesis that each class Ib subfamily is expressed in a tissue-specific fashion and encodes a protein with a unique function (11, 13), the XNC isotype distribution was examined in different organs from adult Xenopus.

Northern blotting analysis was conducted using a probe encoding the conserved class Ib α3 domain. Hybridization was first conducted with the class Ib and class Ia probes (note that under the hybridization conditions employed here, the class Ia and class Ib genes do not cross-hybridize) on RNA prepared from two Xenopus thymic tumor cell lines (36), B3B7 (Fig. 2, lanes A-1 and B-1) and f/f-2 (Fig. 2, lanes A-2 and B-2). Many bands were shown to hybridize to the class Ib probe from both cell lines (Fig. 2B), but only the f/f-2 line expressed any class Ia mRNA (high m.w. band in lane A-2). In tissue distribution analyses, XNC genes were shown to be expressed in adult lung, intestine, spleen, and thymus (AD lanes in all panels of Fig. 3), while liver, colon, and muscle were clearly negative (data not shown). Overall, the tissue distribution of Xenopus class Ib expression is similar to that of class Ia, except that individual class Ib isotypes are expressed in lower amounts than the classical molecules. Consistent with the wide range of mRNA sizes predicted from the previously isolated cDNA clones, several class Ib bands were detected in all tissues except lung. While in some tissues such as intestine the relative expressions of the different transcripts are similar, in others some isotypes seem to be preferentially expressed (e.g., thymus; 2.0- and
1.3-kb bands). In lung, only one band of 3.9 kb was detected, suggesting that only one set of class Ib genes is predominantly expressed there.

**Ontogenic expression of class I genes**

These studies were initiated to determine whether transcription of the class Ia genes is induced at metamorphosis and therefore coincides with the previously obtained protein data (20, 26, 28), or whether there is high class Ia gene expression but some type of post-transcriptional regulation in immunocompetent tadpoles. Additionally, it was our purpose to determine whether in the absence of class Ia expression, the identified class Ib family might substitute for class Ia during tadpole life.

The developmental studies were performed using Northern hybridization procedures employing total RNA isolated from tissues of animals at various stages of development. The organs were from young adults (from 5 mo to 1 yr of age), animals at 2 mo and 2 wk after metamorphosis, tadpoles at the metamorphic climax (stages 62–65, stages are from the normal table of *Xenopus* development by Nieuwkoop and Faber (37)), tadpoles just entering metamorphosis (stages 58–60), and tadpoles at stages when the tadpole-like immune system is most active (stages 54–56).

The results of these studies indicate that although in most tissues class Ia transcripts are hardly detectable before metamorphosis, expression of the classical genes during ontogeny is not initiated simultaneously in all organs (Fig. 4). Whereas no class Ia mRNA can be detected in thymus, spleen, and skin before metamorphosis, class Ia message is present in intestine and lung during the mature tadpole’s life. Moreover, adult levels of the class Ia mRNA are observed in tadpole gill, an organ that obviously disappears at...
metamorphosis. In all other tissues expression dramatically increases after the metamorphic climax. These results indicate that a major control of class Ia expression during development is at the mRNA level. Further studies are needed to determine whether such regulation is strictly at the transcriptional level or whether there are other factors (e.g., mRNA stability) responsible for the differential expression. It is of interest that as in previous studies of class II expression (22), class Ia transcripts first are detected in tissues whose epithelial surfaces are in direct contact with the environment.

Class Ib developmental studies were performed with RNA isolated from thymus, spleen, intestine, and lung (Fig. 3). During ontogeny the pattern of XNC expression is similar to that of class Ia, with exception of mucosal tissues, in which no class Ib transcripts were detected (data not shown). Such results suggest that these two sets of class I genes, although not genetically linked and, as far as has been analyzed, having very different putative promoters (M. F. Flajnik, unpublished observations), are subject to a similar type of regulation in lymphocytes. Furthermore, these studies show that proteins encoded by this class Ib family seem not to
TABLE I. Identification of XNC isotypes of 5’ RACE

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of Clones Analyzed</th>
<th>XNC1</th>
<th>XNC2</th>
<th>XNC3</th>
<th>XNC4</th>
<th>XNC5</th>
<th>XNC6</th>
<th>XNC7</th>
<th>XNC8</th>
<th>XNC9</th>
<th>U1a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>31</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Intestine</td>
<td>23</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Spleen</td>
<td>23</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lung</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>24</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

*a* UI, unidentified (novel?) isotypes.
findings are consistent with studies in mammals suggesting that epithelial surfaces are in direct contact with the environment. Such seems to be favored in skin, lung, and intestine, tissues whose isotypes is found in some organs. Namely, a form of controls in different tissues, and preferential expression of cer-

2.0-kb bands in thymus detected with the conserved is 1.7 kb, and thus cannot account for the highly expressed 1.3- and genes.

alternative splicing or are encoded by recently diverged class Ib mine whether the 1.3- and 3.9-kb transcripts are generated from class Ib transcript in the lung. Future studies are needed to deter-
tissues. Thus, these data confirm that 5 is also expressed in the skin and intestine, but not in lymphoid tissues. Therefore, main in thymus, of several as yet unidentified clones needs to be studied further.

Discussion

In mammals, class I MHC expression is regulated at the transcriptional, post-transcriptional, and translational levels (41). At the time these studies with Xenopus were initiated it was known that class Ia molecules were expressed in low amounts, but it was not clear at which level this regulation of class I expression occurred. Thus, the first step was to determine whether class Ia transcripts were detectable in premetamorphic tissues. In addition to the possible total absence of larval class Ia transcription, another attractive hypothesis was the possible expression of developmentally controlled, tadpole-specific, class Ib genes, perhaps derived from XNC family.

During ontogeny, expression of class Ia genes is not simultaneous in all tissues; transcripts are present in intestine, lung, and especially gills in tadpoles, while no class Ia mRNA is detected in thymus, spleen, and skin until after metamorphosis. In all cases expression dramatically increases after the metamorphic climax. The results of these experiments reveal that the major control of Xenopus class Ia and class Ib developmental expression, at least in hemopoietic tissue, takes place at the mRNA level rather than in the biosynthesis or assembly of class Ia proteins. Possible regulatory mechanisms include changes in the DNA methylation status, differences in mRNA half-life (i.e., stability), transcriptional regulation including availability of specific transcription factors, and/or changes in chromatin structure (developmentally regulated changes in chromosomal composition during Xenopus embryogen-

esis have a determining influence on gene activity (42)).

Studies of ontogenic expression of Xenopus class Ib genes were undertaken to further examine how the tadpole might protect itself from pathogens. We hypothesized that class Ib genes might be expressed before metamorphosis and compensate for the lack of class Ia molecules, i.e., class Ib Ags could be used by the tadpole as restriction elements to present Ags to αβ and/or γδ T cells. The expression data do not support this hypothesis; instead, the failure to detect class Ib mRNA during tadpole life suggests a possible coregulation of expression of class Ia and class Ib genes during ontogeny. While there appears to be no expression of XNC family members, it is still possible that another family of unidentified class I genes could be expressed during tadpole life. Moreover, class Ia and class Ib mRNAs are both up-regulated at metamorphosis, but this co-regulation is not absolute; while class Ia transcripts were detected early in larval intestine and lung, no XNC isotypes were identified in any tadpole tissue. Thus, these results suggest that at least at the level of ontogeny expression in mucosa there is differential regulation of class Ia and class Ib.

One conclusion seems clear from these studies; the low abundance of premetamorphic class I proteins at the cell surface is primarily determined by factors that influence the quantity and/or the type of transcripts produced. Thus, other variables, such as lack of peptide transporter function, that have been determined to influence the biosynthesis and expression of mammalian embryonic class I molecules (43) do not seem to be a primary factor(s) influencing larval class I levels. Our data indicate that expression of the presenting molecules, class Ia and class Ib, and that of the processing molecule, Imp7, are regulated in concert.

mRNA encoded by any other XNC subfamily member. The two isotypes chosen were XNC6, because of its apparent predominant expression in the lung, and XNC8, since it was preferentially amplified from thymus RNA and was previously isolated only from a thymus (not spleen or liver) cDNA library (16).

Northern hybridizations with these two probes revealed that while XNC8 is detected in tissues such as skin, intestine and spleen, it is preferentially expressed in thymus (Fig. 6A). As expected from the size of the cDNA clone, the XNC8 transcript size is 1.7 kb, and thus cannot account for the highly expressed 1.3- and 2.0-kb bands in thymus detected with the conserved a3 probe. It is quite possible that the XNC8 transcripts detected in the skin, intestine, and spleen are derived from infiltrating hemopoietic cells. As predicted, the XNC6-specific probe hybridized to two different-sized transcripts: one of 1.3 kb expressed in almost all tissues that matches the size of the isolated cDNA clone, and another at 3.9 kb expressed in high amounts in the lung (Figs. 3 and 6B). The latter is also expressed in the skin and intestine, but not in lymphoid tissues. Thus, these data confirm that 5’ RACE identified the major class Ib transcript in the lung. Future studies are needed to determine whether the 1.3- and 3.9-kb transcripts are generated from alternative splicing or are encoded by recently diverged class Ib genes.

In conclusion, in adult Xenopus class Ib genes are under different controls in different tissues, and preferential expression of certain isotypes is found in some organs. Namely, a form of XNC6 seems to be favored in skin, lung, and intestine, tissues whose epithelial surfaces are in direct contact with the environment. Such findings are consistent with studies in mammals suggesting that some class Ib molecules (e.g. MIC genes in humans (40)) play an important role in mucosal immunity. By contrast no lymphoid-specific isotype could be unambiguously identified. However, the presence, mainly in thymus, of several as yet unidentified clones needs to be studied further.

FIGURE 6. Identification of particular XNC isotypes that are expressed differentially. Isotype-specific probes (XNC8 in A; XNC6 in B) demonstrate preferential expression of class Ib genes in adult tissues. EtBr staining of total RNA is displayed in the lower panel.
Finally, it is important to point out that although class Ia mRNA was not detected in premetamorphic lymphoid tissues, low amounts of class Ia protein have been detected by FACS in tadpole splenocytes (22, 26, 28) (data not shown). It is not clear why in the present study there is not a perfect correlation between the Northern blotting and protein expression data, i.e., absolutely no detectable mRNA in hemopoietic tissue but low levels of expressed protein. One possibility might be that the tadpole class Ia mRNA half-life is very short, making it difficult to detect, and/or larval class Ia proteins might be very stable, thus requiring only extremely low levels of transcription.

Tadpoles are distinct from mammalian and avian embryos in that they are free-living and must contend with pathogens. Thus, larval Xenopus are immunocompetent, are capable of specific Ab production and skin graft rejection, and are able to mount a MLR (21). All these immune functions may, of course, be explained by the presence of class II molecules, but the question remains: why is there poor expression of class I processing and presenting genes in tadpole cells? A simple hypothesis is that larval Xenopus may not express class I molecules to protect themselves against autoimmunity (21). If tadpoles expressed class I proteins and thus could generate MHC-restricted CTL against Ags derived from pathogens, some CTL could cross-react with the large number of adult Ags that emerge at metamorphosis, eliciting devastating autoimmunity. Thus, to protect themselves from autoaggression, tadpoles may rely more on the class II-mediated humoral immune response. Exceptions to this rule may occur only in tissues such as intestine and gill, which in addition to having epithelial surfaces that are in direct contact with the environment also undergo profound reorganization at metamorphosis. Thus, it may be that class I is expressed only in those organs where it can play an additional role of protection against pathogens.

Xenopus class Ib isoforms display great structural diversity (13) and heterogeneity in their patterns of expression, which may reflect diverse functions that vary from tissue to tissue. Results obtained by 5’ RACE confirm that there is preferential expression of certain class Ib isoforms in some tissues. Namely, one single XNC6-like isotype is preferentially expressed in the lung. Current studies are aimed at identifying the XNC6 protein in epithelial tissues.

Results obtained in recent years suggest that several mammalian class Ib proteins are expressed on epithelial surfaces, such as skin, intestine, and lung. Moreover, consistent with an involvement of these molecules in the first line of defense against pathogens (11), it has been determined that some class Ib proteins apparently bind peptides derived from conserved proteins, such as heat shock molecules (44). In this context, the Xenopus system provides an opportunity to study class Ib function, since one of the XNC subfamilies is expressed exclusively in epithelial tissues, including lung, intestine, and skin. Its sequence is not similar to that of any described mammalian class Ib molecule, but its expression pattern is like that of the human MIC genes (40) which are also preferentially transcribed in epithelial cells. Taken together, these observations suggest that epithelium-specific MHC class Ib molecules have been maintained in different species during evolution, indicating that the nonclassical Ags play an important role in mucosal defense.

Finally, it is striking that not only are some XNC isoatypes preferentially expressed in epithelial surfaces, but Xenopus class Ia molecules, class II β-chains (45), and Imp7 (39) genes are transcribed most abundantly in intestine. These observations also suggest that the mucosal immune system is especially important in Xenopus during both tadpole and adult stages.

Acknowledgments

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References


