The Slug Gene Is Not Essential for Mesoderm or Neural Crest Development in Mice

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The Slug gene encodes a zinc finger protein, homologous to the product of the Drosophila Snail gene, that is implicated in the generation and migration of both mesoderm and neural crest cells in several vertebrate species. We describe here the cloning and genetic analysis of the mouse Slug (Slugh) gene. Slugh encodes a 269-amino-acid protein that shares 92% amino acid identity with the product of the chicken Slug gene. We have characterized Slugh expression during early mouse embryogenesis by whole mount in situ hybridization of Slugh mRNA and through detection of β-galactosidase expression from an in-frame SlughlacZ allele generated through homologous recombination. Slugh expression is first detected in extraembryonic mesoderm and is later detected in many mesodermal subsets, although it is not detected in the primitive streak. In contrast to many other vertebrates, the mouse Slug gene is not expressed in premigratory neural crest cells but is expressed in migratory neural crest cells. Analysis of a targeted null mutation that deleted all Slugh coding sequences revealed that Slugh is not required for mesoderm formation or for neural crest generation, migration, or development in mice. These results indicate that neither the expression pattern nor the biological function of the Slug gene is conserved among all vertebrates. These data also raise interesting questions about the regulation of neural crest generation, which is one of the distinguishing characteristics of the vertebrate subphylum.

INTRODUCTION

Cell migration and epithelial–mesenchymal transitions are intimately involved in the development and differentiation of vertebrate embryos. One of the morphogenetic processes involving both epithelial-mesenchymal transition and cell migration is gastrulation. Gastrulation in a wide variety of organisms results in the transformation of an embryo with one or two embryonic germ layers into an embryo with three embryonic germ layers (ectoderm, mesoderm, and endoderm). Gastrulation also establishes both the anterior-posterior and the dorsal–ventral embryonic axes in vertebrates. In birds and mammals, gastrulation begins with formation of the primitive streak. Individual cells in the primitive streak undergo an epithelial–mesenchymal transition to form free mesenchymal cells that migrate laterally away from the streak and populate the paraxial and lateral mesoderm (Viebahn, 1995).

Neural crest cells undergo a similar epithelial–mesenchymal transition during their formation (Bellairs, 1987; Duband et al., 1995). Neural crest cells delaminate from the dorsal part of the neural folds or neural tube and migrate into the periphery of the embryo, where they subsequently differentiate into a wide variety of different cell types and structures. Derivatives of the neural crest include most neurons and glia of the peripheral nervous system, the facial skeleton, pigment cells, chromaffin cells of the adrenal medulla, and other cell types (for recent reviews, see Bronner-Fraser, 1995; Baker and Bronner-Fraser, 1997a, b). Although neural crest migratory pathways and developmental fates are well documented, less is known about the molecular mechanisms that govern formation and emigration of neural crest cells from the neural tube. However, recent identification in the chick of the Slug gene and its characterization as a potential regulator of the epithelial–mesenchymal transition involved in both mesoderm and neural crest formation has greatly facilitated studies of these processes (Nieto et al., 1994; Liem et al., 1995; Sechrist et al., 1995; Buxton et al., 1997).

The Slug gene encodes a zinc finger protein of the Snail family (Boulay et al., 1987) and has been demonstrated to be a specific and sensitive marker for both premigratory and migrating neural crest cells in Xenopus, zebrafish, and

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chick embryos [Nieto et al., 1994; Liem et al., 1995; Mayor et al., 1995; Sechrist et al., 1995; Thissee et al., 1995; Mancilla and Mayor, 1996; Buxton et al., 1997; Mayor et al., 1997]. Slug is also expressed in early gastrulating mesoderm in chick and zebrafish [Nieto et al., 1994, Thissee et al., 1995], but in Xenopus Slug is not expressed in early gastrulating mesoderm but is expressed later during embryogenesis in lateral plate mesoderm [Mayor et al., 1995].

In addition to being a specific marker for neural crest in lower vertebrates, recent studies also suggest that Slug may play a functional role in the formation of premigratory neural crest in the dorsal neural tube, and in the epithelial-mesenchymal transition accompanying the delamination of both mesoderm cells from the primitive streak and neural crest precursors from the neural tube. Two lines of evidence suggest a functional role for Slug during these processes. First, chick embryos treated with Slug antisense oligonucleotides exhibit impaired delamination of neural crest cells from the neural tube and of mesoderm cells from the primitive streak. In the antisense-treated embryos, the presumptive neural crest cells remain integrated into the neuroepithelium dorsally without any sign of the normal epithelial-mesenchymal transition, and mesodermal cells collect in the primitive streak and exhibit impaired migration laterally away from the streak [Nieto et al., 1994]. Second, Slug expression is upregulated in regenerating neural crest precursors subsequent to surgical ablation of the neural folds or dorsal neural tube [Sechrist et al., 1995; Buxton et al., 1997]. The temporal and spatial localization of Slug RNA induction in the ablated embryos is consistent with the model that Slug may play a role in the formation and delamination of neural crest precursors from the neural tube. The data from these two experiments suggest that Slug may play a regulatory role during neural crest formation in chick embryos.

The ability to specifically inactivate genes by targeted disruption in embryonic stem cells has become a powerful tool in the analysis of gene function in mice. We have been studying the mouse Snail (Snai) gene [Smith et al., 1992], which is closely related to the Slug gene, and were interested in determining whether the Slug gene is evolutionarily and functionally conserved in mammals. We therefore cloned the mouse homolog (termed Slugh) of the chick Slug gene and have analyzed its expression and function by making two targeted mutant alleles. Using both whole mount in situ hybridization and an in-frame lacZ fusion allele, we characterized expression of Slugh during early embryogenesis. Slugh expression is first detected in extraembryonic mesoderm and is later detected in many mesodermal subsets of the embryo. However, expression of the Slugh gene is not detected in the primitive streak. Slugh is also expressed in migratory neural crest cells, but is not expressed in premigratory neural crest precursors in the dorsal neural tube. Mice homozygous for a targeted null allele that deletes all coding sequences of the Slugh gene are viable and fertile, although they exhibit postnatal growth deficiency. Our data demonstrate that while the amino acid sequence of the Slugh gene is evolutionarily conserved in mice, important aspects of its expression pattern are not conserved. Our data also indicate that Slugh function is not conserved because the Slugh gene is dispensable for both mesoderm formation and neural crest development in mice.

MATERIALS AND METHODS

Isolation of cDNA and Genomic Clones of the Mouse Slugh Gene

Degenerate PCR primers corresponding to the amino-terminal seven amino acids (MPRSFLV) and the carboxy-terminal seven amino acids (SGCCVAH) of the chick Slug protein [Nieto et al., 1994] were used in a PCR amplification with cDNA template reverse-transcribed from total RNA of E9 mouse embryos. The reverse transcription reaction followed standard protocols [Sambrook et al., 1989]. The PCR reaction was carried out in a Perkin-Elmer Cetus thermocycler for 40 cycles: 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1.5 min extension at 72°C. PCR product was fractionated on a 1% agarose gel and the fragment of desired size was purified, subcloned into pBluescript II [KS+] [Stratagene], and sequenced.

A PCR subclone with extensive sequence identity with the chick Slug cDNA was used as a probe to screen a random-primed mouse brain cDNA phage library [Stratagene]. Hybridizing phage were plaque purified and their inserts subcloned into pBluescript II (SK+) and sequenced. The full-length mouse Slug cDNA sequence (GenBank Accession No. U79550) is a composite of two cDNA clones, clone BR2.5 [nucleotides 1-359] and clone BR1.4 [nucleotides 169-956], which encompass the entire protein coding region, and genomic clone slughG1 [nucleotides 957-1945 in the 3' untranslated region]. The gene symbol Slugh was assigned to the mouse Slugh gene by the Mouse Genome Data Base Mouse Nomenclature Committee.

To obtain genomic clones of the Slugh gene, PCR primers corresponding to sequences specific to the mouse Slugh cDNA were used to screen a P1 genomic library made from genomic DNA of 129/Sv strain mouse embryonic stem cells [Genome Systems, St. Louis, MO]. Two P1 clones were obtained which appeared identical by restriction mapping. The Slugh genomic DNA were subjected to restriction digestion with different enzymes and Southern hybridization. An XbaI fragment of about 8 kb and an NsiI fragment of about 8 kb specifically hybridized to a Slugh cDNA probe and were subcloned. These two genomic clones were analyzed by restriction mapping, hybridization with mouse Slugh cDNA probes and nucleotide sequence analysis, and were shown to contain the entire coding sequence of the Slugh gene.

Generation of Mice Carrying a SlughlacZ Fusion Allele or a Deletion of the Slugh Gene

The SlughlacZ fusion construct was generated by replacing a 2 kb PstI-NsiI genomic fragment, corresponding to the zinc finger coding region, with the β-galactosidase gene and the PGKneoPA cassette [Soriano et al., 1991]. The resulting construct carries a 2.5-kb homologous region 5' to and a 5.5-kb homologous region 3' to the replaced region. An HSV-1k cassette [Mansour et al., 1988] was further introduced to allow negative selection against random integration into the genome. This replacement vector, when integrated into the Slugh locus, would result in the expression of a...
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FIG. 1. Comparison of the mouse and chicken Slug proteins. The deduced amino acid sequences of the mouse Slugh protein and the chicken Slug protein (Nieto et al., 1994) are compared. Amino acids identical in the two proteins are boxed. The GenBank Accession No. for the mouse Slug cDNA sequence is U79550.

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RESULTS

Cloning and Sequence Analysis of the Mouse Slug (Slugh) cDNA

A PCR reaction using degenerate oligonucleotide primers corresponding to chick Slug amino acid sequences yielded an 810-bp product with an overall nucleotide sequence identity of 85% to the chick Slug cDNA. This PCR fragment was used to screen a mouse brain cDNA library. Two cDNA clones were isolated. Sequence analysis revealed that these two cDNA clones overlapped and contained the complete protein coding sequence of the mouse Slugh cDNA (assigned the gene symbol Slugh; GenBank Accession No. U79550). The open reading frame encodes a protein of 269 amino acids, with 92% amino acid sequence identity to the chick Slug protein (Fig. 1). The mouse Slugh protein contains five zinc fingers and is 56% identical to the mouse Sna protein, which has only four zinc fingers (Nieto et al., 1992; Smith et al., 1992).
Expression of the Slugh Gene

We first attempted to characterize the spatial and temporal localization of Slugh transcripts during embryogenesis in mice by whole mount in situ hybridization. Slugh transcripts proved difficult to visualize by whole mount in situ hybridization, but after optimization of the prehybridization embryo treatment protocol (see Materials and Methods), we detected weak staining of presumptive migratory neural crest cells in embryonic day E8.5 and E9.5 embryos (Figs. 3B, 3D, and 3F). Surprisingly, we could not detect Slugh expression in premigratory neural crest cells (Fig. 3D). It appears, therefore, that the Slugh gene in mice is not expressed in a developmental pattern or at levels comparable to those of the Slugh genes in chick, Xenopus, and zebrafish (Nieto et al., 1992; Mayor et al., 1995; Sechrist et al., 1995; Thise et al., 1995).

To better characterize the cell types that express the Slugh gene in early mouse embryos, we generated a targeted knock-in of a lacZ reporter. We cloned the bacterial β-galactosidase (lacZ) gene in frame into the second exon of the Slugh gene, just upstream of the zinc finger coding region. This Slugh–lacZ fusion construct was targeted into the Slugh locus through homologous recombination. We designate this targeted allele SlughlacZ (Fig. 2). In numerous cases, expression of targeted in-frame lacZ fusions has been shown to faithfully reflect expression of the endogenous gene (e.g., Fong et al., 1995; Bernex et al., 1996; Offield et al., 1996; Hrabé de Angelis et al., 1997). Staining for β-galactosidase is easy to conduct and gives low backgrounds in early mouse embryos. β-galactosidase detection of lacZ fusion alleles also provides better resolution of gene expression in individual cells, particularly migrating neural crest cells (e.g., Mansour et al., 1996).

Germline transmission of the SlughlacZ allele was obtained from three correctly targeted ES cell clones. Embryos heterozygous for the SlughlacZ allele were isolated and were stained with XGAL to reveal β-galactosidase activity. Embryos from the three independently targeted lines of mice gave identical staining patterns. At E8.5, β-galactosidase staining was detected in migratory cephalic neural crest cells at the level of the midbrain and hindbrain and was transiently detected in newly formed somites (Figs. 3A and 3E). This expression pattern was also detected by whole mount in situ hybridization of Slugh RNA (Figs. 3B and 3F). At E9.5, in addition to expression in cephalic neural crest, β-galactosidase-positive cells in the trunk region were observed over the rostral half of each somite (Fig. 3C). Previous work has shown that neural crest cells are inhibited from migrating through the caudal portion of the somite (reviewed in Keynes and Stern, 1988). The location of these SlughlacZ-expressing cells over the rostral halves of the somites indicates that they are migrating neural crest cells. Expression of Slugh RNA in trunk neural crest cells was also detected by whole mount in situ hybridization (Fig. 3D). However, unlike the pattern of Slug gene expression reported in other vertebrates, Slugh expression was not detected in premigratory neural crest cells along the dorsal midline of mouse embryos by either β-galactosidase detection of the lacZ allele or whole mount in situ hybridization of Slugh RNA (Figs. 3E and 3F, and data not shown).

β-Galactosidase staining was also detected in the extraembryonic membranes of E8.5 and E9.5 embryos (Figs. 3A and 3C). Staining in the extraembryonic membrane was also detected by whole mount in situ hybridization of Slugh RNA (data not shown). We then examined SlughlacZ expression in E7.5 embryos. At this stage, β-galactosidase staining was observed only in the extraembryonic region (Fig. 4A). Sectioning of stained E7.5 embryos showed that the β-galactosidase staining was exclusively in the extraembryonic mesoderm of the amnion and visceral yolk sac (Fig. 4B). No β-galactosidase staining was observed in the primitive streak or other portions of the embryo proper at this stage. Sectioning of XGAL-stained E9.5 embryos revealed SlughlacZ expression in lateral mesoderm (Fig. 4C). Sagittal sections of stained E9.5 embryos show streams of individual neural crest cells migrating through the rostral halves of the somites (Fig. 4D). At E10.5, SlughlacZ expression was observed in neural crest cells in the branchial arches and around the optic vesicle (Fig. 4E). A sagittal section.
through the developing eye revealed $\text{Slugh}^{\text{lacZ}}$ expression in the lens epithelium (Fig. 4F).

Another major site of $\text{Slugh}$ expression is in the limb bud. $\text{Slugh}^{\text{lacZ}}$ expression in the limb bud was first detected at E10 in the ventral ectoderm (Figs. 5A and 5B) and in the anterior region of the limb mesenchyme (Fig. 5C). Slightly weaker expression was also observed in the posterior mesenchyme of the limb bud at this stage (Fig. 5D). This expression pattern was confirmed by whole mount in situ hybridization of $\text{Slugh}$ RNA (Figs. 5E, and 5F, and data not shown).

![Fig. 3. Comparison of expression patterns of the $\text{Slugh}^{\text{lacZ}}$ allele and $\text{Slugh}$ RNA expression is observed in neural crest-derived craniofacial mesenchyme, and in mesoderm of the somites, yolk sac, and allantois. (C, D) At E9.5, both $\text{Slugh}^{\text{lacZ}}$ and $\text{Slugh}$ RNA expression is observed in presumptive neural crest cells migrating through the rostral portion of the somite. (E, F) No $\text{Slugh}^{\text{lacZ}}$ or $\text{Slugh}$ RNA expression is observed in premigratory neural crest along the dorsal midline of the neural tube (arrows) at E8.5. al, allantois; c, caudal; r, rostral.

![Fig. 4. $\beta$-Galactosidase expression in embryos heterozygous for the $\text{Slugh}^{\text{lacZ}}$ allele. (A, B) $\beta$-Galactosidase expression in headfold-stage embryos. (A) Whole mount preparation. (B) Sagittal section. $\text{Slugh}^{\text{lacZ}}$ expression is observed in extraembryonic mesoderm of the visceral yolk sac and the mesoderm layer of the amnion. No expression is observed in the embryo at this stage. (C, D) $\text{Slugh}^{\text{lacZ}}$ expression is observed in the lateral mesoderm (C) and in presumptive neural crest cells (D) migrating through the rostral portion of the somite. (E) At E10, expression is observed in periocular mesenchyme, and in neural crest-derived mesenchyme populating the maxillary and mandibular portions of the first branchial arch. (F) In and around the eye at E10, $\text{Slugh}^{\text{lacZ}}$ expression is observed in periocular mesenchyme, the lens vesicle, and the overlying epithelium. No expression is observed in the developing retina. am, amnion; c, caudal; em, extraembryonic mesoderm; lm, lateral mesoderm; nt, neural tube; r, rostral; rt, retina.

$\text{Slugh}$ Mutant Mice Are Viable but Show Growth Retardation

Since the $\text{Slugh}^{\text{lacZ}}$ allele deletes the zinc finger coding region, it is expected to create a loss-of-function mutation.
Mice heterozygous for Slugh\textsuperscript{lacZ} were intercrossed and the genotypes of F2 progeny were determined 2 weeks after birth. Mice homozygous for the Slugh\textsuperscript{lacZ} allele were viable, although they appeared significantly smaller than their littermates. To ensure that we had created a null mutation of the Slugh gene, we constructed a second Slugh targeting vector. Integration of this targeting vector into the Slugh locus results in a 9.7-kb deletion that removes genomic sequences of the entire Slugh protein-coding region (we designate this mutant allele Slugh\textsuperscript{A1}; Fig. 6A). Germine transmission was obtained from four correctly targeted ES clones. Southern blot analysis of progeny of the Slugh\textsuperscript{A1} mutant mice confirmed that the protein-coding sequences of the Slugh gene were deleted (Fig. 6B).

F1 mice heterozygous for the Slugh\textsuperscript{A1} mutation were intercrossed and the genotypes of the F2 progeny were analyzed. Slugh\textsuperscript{A1} homozygous mutant mice were born alive and survived the early postnatal period. Almost all Slugh\textsuperscript{A1} homozygotes develop swollen eyelids (Fig. 7A), which often led to infection of the eyes in adults. This phenotype was also observed in the Slugh\textsuperscript{lacZ} homozygotes. Histological analyses of the eyes and eyelids revealed a variety of conjunctival and periorbital abnormalities in the Slugh\textsuperscript{A1} homozygotes. While the structure of the eyes was essentially normal (Figs. 7B and 7C), mutant mice exhibited various degrees of suppurative conjunctivitis (data not shown). This consisted of various degrees of neutrophilic infiltration that exuded into the lumen between the eyelid and cornea. The eyelids themselves ranged from being normal to having various degrees of inflammation. We also carried out extensive histopathological analyses of Slugh\textsuperscript{A1} mutant mice at newborn and adult stages but did not detect defects in any differentiated derivatives of the neural crest in the Slugh\textsuperscript{A1} homozygotes.

Another phenotype observed in both Slugh\textsuperscript{lacZ} and Slugh\textsuperscript{A1} homozygous mutants was moderate postnatal growth retardation. At birth, no clear differences in the
weight of the pups could be detected. At weaning age (around 3 weeks), however, the average weight of the \textit{Slug} homogous and wild-type littermates. The growth retardation of \textit{Slug} mutant mice occurred in the first 3 weeks of life (Fig. 8). After weaning, the growth rates of the homogous mutant mice parallel those of their wild-type and heterozygous littermates. However, the mutants never catch up with their control littermates. Histopathological analyses did not reveal the cause of the growth retardation. Both male and female \textit{Slug} homögotes were fertile, and the mutant allele could be maintained as a \textit{Slug} homozygous mutant line.

\section*{DISCUSSION}

We describe here the cloning and genetic analysis of a mouse homolog (\textit{Slug}) of the chick Slug gene. The cDNA clones we have isolated encode a protein with 92\% amino acid identity to the chick Slug protein. After the initiation of this work, the sequence of a mouse cDNA clone identical to our \textit{Slug} sequence was published by Savagner et al. (1997). In addition, we have sequenced many subclones from the degenerate PCR cloning screen, and all have been identical to the \textit{Slug} sequence. Thus, it seems probable that we have isolated the mouse ortholog of the chick Slug gene, and that no other gene in the mouse more closely resembles chick Slug.

In contrast to the evolutionary conservation of the amino acid sequence of the chick and mouse Slug proteins (92\% amino acid identity), our results indicate that the expression pattern of the mouse \textit{Slug} gene is quite different from that of chick Slug. In chick embryos, Slug gene expression is first detected in delaminating mesodermal cells in the primitive streak (Nieto et al., 1994). While \textit{Slug} expression in mice is observed in the extraembryonic mesoderm at E7.5 and later in lateral mesoderm, it is not detected in the primitive streak of mouse embryos. Slug is also expressed in both premigratory neural crest precursor cells and in migratory neural crest cells of chick embryos (Nieto et al., 1994). In contrast, in the neural crest of mice \textit{Slug} expression is observed only in migratory neural crest cells.

In the developing limb bud, \textit{Slug} expression is also different from that of chick Slug. During limb development in the chick, Slug expression is initially observed in ectoderm covering the limb bud and is subsequently restricted to the posterior mesoderm (Ros et al., 1997). In the developing mouse limb bud, \textit{Slug} expression is first detected in the ventral ectoderm, and subsequently strong expression is observed in the anterior limb mesenchyme. These data indicate that while the sequence of the Slug gene is evolutionarily conserved, important aspects of the expression pattern are not conserved.

Our analyses of two independent mutations of the \textit{Slug}
gene indicates that the Slugh gene is not essential for prenatal mouse development but is required for early postnatal growth. In chick embryos, expression of the Slugh gene has been implicated in the regulation of the epithelial–mesenchymal transition involved in formation of both the mesoderm and the neural crest (Nieto et al., 1994). Slugh gene expression in the chick embryo precedes the onset of the epithelial–mesenchymal transition of primitive streak mesoderm and the neural crest, and Slugh antisense oligonucleotides specifically inhibited delamination and migration of both mesoderm and neural crest cells. Lack of detectable expression of the Slugh gene in such premigratory cell populations in mouse embryos is consistent with our finding that Slugh is not involved in the regulation of emigration of the mesoderm and the neural crest. However, Savagner et al. (1997) recently reported that transfection of the mouse Slugh cDNA into a rat bladder carcinoma cell line caused cell shape changes characteristic of the initial phase of epithelial–mesenchymal transformation. Given this finding and the widespread expression pattern of Slugh in mouse embryos, it is somewhat surprising that the Slugh mutant mice do not exhibit a more severe phenotypic effect.

One possible explanation for the mild phenotype observed in the Slugh mutant mice is functional compensation by other members of the Snail gene family. Only one other member of this gene family, Sna, has been identified in the mouse. The Sna gene is more widely expressed than the Slugh gene in the early mouse embryo, being expressed in the parietal endoderm, primitive streak mesoderm and its derivatives, and migratory neural crest (Nieto et al., 1992; Smith et al., 1992). However, Sna is not expressed in premigratory neural crest precursors in the dorsal neural tube. It is possible that Sna expression in the primitive streak of mouse embryos might provide the same function that Slugh expression provides in the primitive streak of chick embryos. However, since Sna is not expressed in premigratory neural crest in mice, it could not provide the function that Slugh expression performs in neural crest formation in the chick and other vertebrates.

Formation of the neural crest is one of the distinguishing features of the vertebrate subphylum (Gans and Northcutt, 1983; Northcutt and Gans, 1983). The unique properties of this cell population, and its presence only in vertebrates (although ancestral cell populations may exist in nonvertebrate chordates; see Baker and Bronner-Fraser, 1997b), suggest that the formation of the neural crest evolved only once on the line leading to present day vertebrates. The fact that Slugh gene expression has been demonstrated to be a specific and sensitive marker for both premigratory and migrating neural crest cells in Xenopus, zebrafish and chick embryos (Nieto et al., 1994; Liem et al., 1995; Mayor et al., 1995; Sechrist et al., 1995; Thissee et al., 1995; Mancilla and Mayor, 1996; Buxton et al., 1997; Mayor et al., 1997), and the evidence for a functional role of Slugh during neural crest formation and regeneration in chick embryos (Nieto et al., 1994; Sechrist et al., 1995; Buxton et al., 1997), makes our finding that Slugh is not required for generation of neural crest in mouse embryos quite surprising. Apparently, in mice other genes must have taken over the role performed by Slugh in the generation and delamination of neural crest in chick, zebrafish, and Xenopus embryogenesis.

In recent years, examination of evolutionarily conserved gene families has been a valuable tool in understanding and dissecting complex developmental processes in higher vertebrates, particularly mice. However, despite the well-documented evolutionary conservation of some developmental mechanisms, recent morphological studies have indicated that more variability exists during embryonic development of different vertebrate species than was previously believed (Richardson et al., 1997). Our results demonstrate that gene functions evolutionarily conserved in some vertebrates may not be conserved in all. Therefore, the question of whether a gene conserved at the level of amino acid sequence is also conserved functionally in different vertebrate species needs to be examined critically for each case, and for each species.

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