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Spatiotemporal, Allelic, and Enforced Expression of *Ximpact,* the *Xenopus* Homolog of Mouse Imprinted Gene *Impact*

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Mouse *Impact* is an imprinted gene encoding an evolutionarily conserved protein of unknown function. We isolated cDNA for the *Xenopus* homolog of *Impact* (*Ximpact*), since the clawed frog not only provides an excellent model for the study of gene function in early development but also allows the generation of interspecific F1 hybrids required for the examination of allelic expression status. The predicted product of *Ximpact* shows an extreme sequence similarity to those of mouse *Impact* and its homologs in nematoda, fission yeast, and budding yeast. The transcript of *Ximpact* is present in oocytes as well as in early embryos, and its spatial distribution is ubiquitous in both embryonic and adult stages. An RT-PCR-RFLP assay using the reciprocal interspecific F1 hybrids and a DNA polymorphism between *X. laevis* and *X. borealis* showed that *Ximpact* is expressed biallelically when analyzed as a whole embryo. Overexpression of *Ximpact* by RNA microinjection resulted in a higher than normal rate of gastrulation defects, suggesting the need for tight control of its dosage in early development.

Genomic imprinting is the phenomenon that confers differential functions on the two genomes according to their parental origins. The best example of genomic imprinting can be seen in the so-called "imprinted genes" which are expressed only from either paternal or maternal allele in contrast to most genes expressed biallelically (1, 2). While the biological roles of genomic imprinting are not fully understood, both paternal and maternal genomes are required for the accomplishment of normal development in mammals (3, 4). This is, in part, due to the imprinted genes, since many genes playing pivotal roles in mammalian development are imprinted (5–8). Genomic imprinting is implicated not only in embryonic development but also in postnatal growth (9), behavior (10, 11), and the pathogenesis of various diseases (12).

While genomic imprinting was originally described in an insect (13), its demonstration at the molecular level has been so far achieved only in mammals. Interestingly, it was recently reported that a transgene in the zebrafish shows methylation imprinting or parent-of-origin-dependent DNA methylation (14–16). Since the imprinting of transgenes is frequently observed in mouse, it is conceivable that nonmammalian vertebrates also have a similar molecular mechanism for genomic imprinting and bear endogenous imprinted genes in their genomes. In fact, parent-of-origin effects have been reported in interspecific crosses of birds, frogs, and fishes (17). The presence of imprinted genes in nonmammalian vertebrates is an issue of particular interest in the field of genomic imprinting research. If the presence of endogenous imprinted genes is demonstrated in non-placental animals or those which can develop through parthenogenesis such as the zebrafish, hypotheses on the roles of genomic imprinting claiming the prevention of parthenogenesis (18) or conflicts between the parents (19) have to be reconsidered.

We decided to analyze homologs of the mammalian imprinted genes in the clawed frog *Xenopus laevis* for the following two reasons. First, many imprinted genes play important roles in early development, and hence invaluable information on their functions would be obtained from the analysis of their homologs in *X. laevis,* which allows one to perform detailed observation of early developmental stages and various fine embryonic manipulations. Second, *X. laevis* allows one to generate interspecific F1 hybrids with *X. borealis,* which would enable one to examine the allelic expression status using sequence polymorphisms easily found between the two species (20). Notably, a parent-of-

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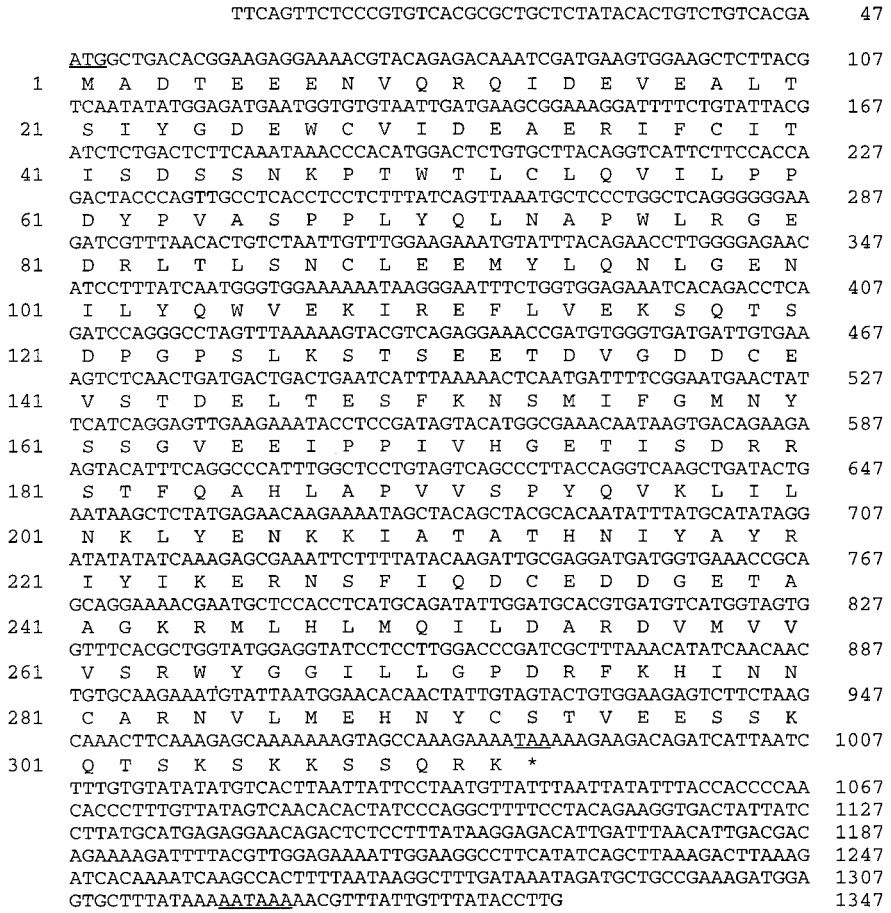


FIG. 1. The primary structure of the *Ximpact* cDNA. Nucleotide sequence and deduced amino acid sequence are shown. Numbers shown in the left and right indicate those for amino acid residues and nucleotides, respectively. Initiation codon (ATG), termination codon (TAA), and polyadenylation signal (AATAAA) are underlined. The nucleotide sequence of *Ximpact* cDNA is deposited in DDBJ, EMBL, and GenBank (Accession No. AB020319).

origin effect on the body and head size was reported in crosses between the bullfrog and the minkfrog (17), and amphibians may also have imprinted genes.

As the first target to be analyzed, we isolated the *Xenopus* homolog of the mouse imprinted gene *Impact* because of its extreme evolutionary conservation (21). The *Impact* is a paternally expressed gene isolated by our unique screening method termed the allelic message display (21). Recent advance of genome projects has revealed that hypothetical proteins homologous to *Impact* throughout the reading frame are found in the genomes of the nematoda *C. elegans*, the fission yeast *S. pombe*, and the budding yeast *S. cerevisiae*. In addition, genes encoding hypothetical proteins that share a highly conserved domain with the predicted product of *Impact* are found in the genomes of most eubacteria. These proteins comprise the "YCR059c/yigZ hypothetical protein family" or the "Uncharacterized Protein Family 29 (UPF0029)" with completely unknown functions (22, 23).

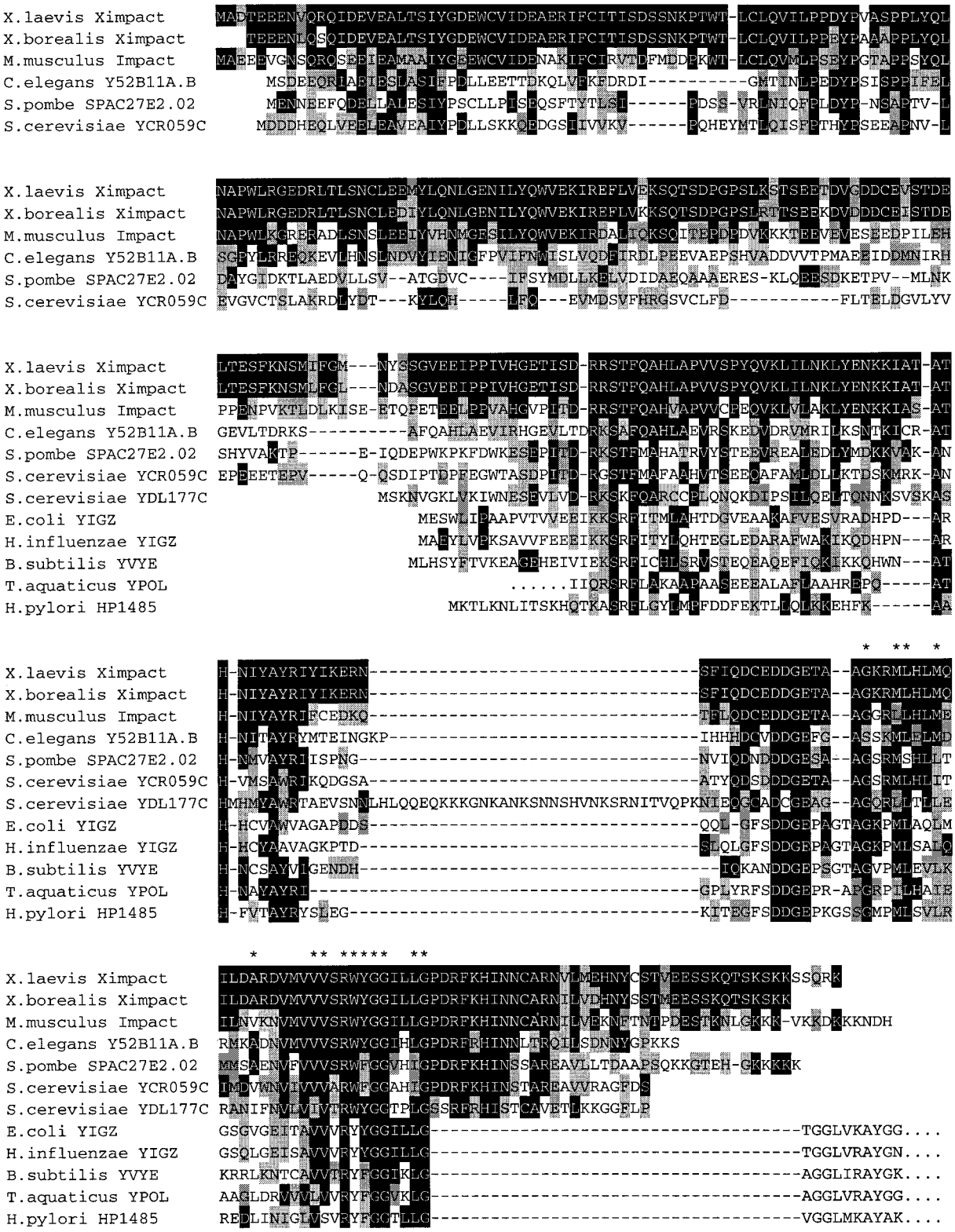
In this report, we describe cDNA cloning of the *Xenopus* homolog of *Impact*, its spatial and temporal expression patterns as well as the effect of its overexpression by RNA microinjection. We also examined its allelic expression status using the reciprocal F1 hybrids and a DNA polymorphism between *X. laevis* and *X. borealis*, thereby providing, to the best of our knowledge, the first report on the molecular analysis of the possibility of allele-specific expression of imprinted gene homologs in nonmammalian vertebrates.

MATERIALS AND METHODS

Preparation of embryos and adult tissues. Unfertilized eggs were laid from *X. laevis* females that had been injected with 150 units of gonadotropic hormone, Gonadropin (Teikokuzoki, Tokyo). Eggs were artificially fertilized, dejellied in 2% L-cysteine-HCl (pH 7.9), cultured in 0.1× Steinberg's solution [6 mM NaCl, 0.067 mM KCl, 0.034 mM $Ca(NO_3)_2$, 0.083 mM $MgSO_4$, 1 mM Hepes (pH 7.4)] at 20~21°C until the desired stages, and kept frozen until analyzed as described previously (24).

Embryos at 8-cell stage (stage 4) were dissected with a glass needle and a hair loop into the four portions, namely dorsal animal, dorsal vegetal, ventral animal and ventral vegetal portions, in Steinberg's solution containing collagenase at 1 mg/ml as described (24). Simi-

A


X.laevis Ximpact
X.borealis Ximpact
M.musculus Impact
C.elegans Y52B11A.B
S.pombe SPAC27E2.02
S.cerevisiae YCR059C
S.cerevisiae YDL177C
E.coli YIGZ
H.influenzae YIGZ
B.subtilis YVYE
T.aquaticus YPOL
H.pylori HP1485

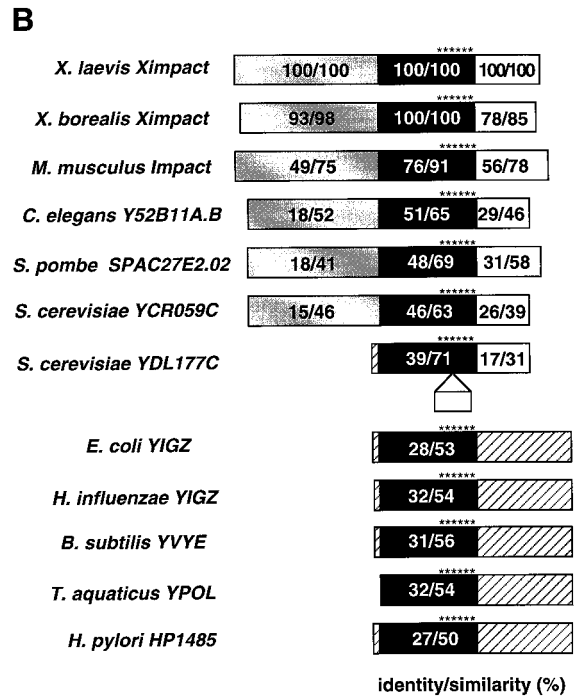


FIG. 2—*Continued*

larly, embryos at neurula stage (stage 18) were divided into notochord, somite, dorsal ectoderm, and belly (24).

All the adult tissues except testis were excised from mature females chilled in ice water using forceps and scissors. Tissues were rinsed with 0.1 × Steinberg's solution and kept frozen until analyzed (25).

RNA isolation. Total RNAs were isolated from embryos and adult tissues by a modified single-step acid guanidine thiocyanate phenol–chloroform method using TRIZOL reagent (Gibco BRL, MA) followed by further purification with salt and ethanol precipitation (24).

Molecular cloning and sequencing of full-length cDNA for Xenopus Impact. Using an oligo-$(dT)_{12-18}$ primer and total RNAs extracted from tailbuds, the first strand cDNA was synthesized and subsequently used as the template for the PCR with various combinations of primers synthesized for the amplification of mouse *Impact* under the following thermal cycling conditions: 94°C for 3 min + (95°C for 15 s + 50°C for 1 min + 72°C for 1 min) × 2 cycles + (95°C for 15 s + 55°C for 1 min + 72°C for 1 min) × 28 cycles + 72°C for 5 min. The PCR products were subcloned into pT7 Blue vector (Novagen, WI) and subjected to DNA sequencing. Both 5′- and 3′-RACE were performed for the isolation of full-length cDNA using Marathon-RACE kit (CLONTECH, CA). The gene-specific primers used were shown below: first sense primer, 5′-GCAGATATTGGATGCACGTG-3′; nested sense primer, 5′-ATATTGGATGCACGTGATGTCATGG-3′; first anti-sense primer, 5′-TGATATGTTTAAAGCGATCG-3′; and nested anti-sense primer, 5′-CGATCGGGTCCAAGGAGGATAC-3′. Amplified products were subcloned and sequenced as described. The full-length cDNA was amplified by RT-PCR using the following two primers: sense primer, 5′-CAGTTCTCCCGTGTCAC-3′; and nested anti-sense primer, 5′-CCATCTTTCGGCAGCATC-3′. The amplified product was subjected to direct cycle sequencing to eliminate the effect of misincorporated nucleotides at the step of PCR.

Semiquantitative RT-PCR assay. The RT-PCR mixture (20 μl) containing cDNA corresponding to 50 ng of total RNA and primers (10 pmol each) was subjected to the following thermal cycling: 94°C for 3 min + (95°C for 15 s + 50°C for 30 s + 72°C for 30 s) × ~30 cycles + 72°C for 5 min. Primers used for *Ximpact*, cytoskeletal actin, and EF1α were as follows: *Ximpact* sense, 5′-ATATTGGATGCACGTGATGTCATGG-3′; *Ximpact* anti-sense, 5′-CGATCGGGTCCAAGGAGGATAC-3′; actin sense, 5′-CTGAGTTCATGAAGGATCAC-3′; actin anti-sense, 5′-AAATTTACAGGTGTACCTGC-3′; EF1α sense, 5′-TACTCAATCACCCAGGACAG-3′; and EF1α anti-sense, 5′-GGAATCATTTCCACTATAGC-3′. Products were electrophoresed on a polyacrylamide gel, stained with SYBR Green I (Molecular Probe, OR), and visualized by FluorImager (Molecular Dynamics, CA).

PCR-RFLP assay. The primers used for the amplification of the fragment containing the polymorphic *Eco*RI site were shown below: 5′-GAGAACATCCTTTATCAATG-3′, 5′-CCATGACATCACGTGCATC-3′. The PCR with these primers was performed under the following thermal cycling condition (94°C for 3 min + [95°C for 15 s + 54°C for 30 s + 72°C for 30 s] × 30 cycles + 72°C for 5 min). The amplified products were completely digested with *Eco*RI (Gibco BRL), subjected to 6% PAGE, and stained with SYBR Green I (Molecular Probes).

RNA microinjection. The microinjection experiments were performed as described previously using the RNAs synthesized by *in vitro* transcription reactions with T7 RNA polymerase as described previously (25).

RESULTS AND DISCUSSION

Molecular Cloning of Xenopus Impact cDNA

To isolate cDNA encoding the homolog of *Impact* from *Xenopus laevis*, we tested primers for the mouse *Impact* cDNA (21) in various combinations in RT-PCR using tailbud cDNA as a template. Using a primer pair derived from the most highly conserved region of *Impact*, we obtained a PCR product, termed Xim14, showing the expected size (21). Subsequent cloning and sequencing of Xim14 revealed that it can encode a polypeptide highly homologous to the corresponding region of *Impact* product. We thus synthesized primers based on the Xim14 sequence and used them for 5′- and 3′-RACE (rapid amplification of cDNA ends) for the isolation of cDNAs flanking Xim14. Combining both RACE fragments with Xim14, we reconstructed the entire nucleotide sequence of a candidate cDNA for the *Xenopus* homolog of *Impact*. To eliminate the possibility of artifacts in RACE, we designed primers derived from both ends of the reconstructed sequence and used them for RT-PCR. The expected product of ~1.3 kb long was readily amplified, and the authenticity of the

FIG. 2. Alignment of the Ximpact homologs. (A) Comparison of deduced amino acid sequences between Ximpact and other homologs. Identical and similar positions are shaded in black and gray, respectively. Asterisks indicate the motif [G-X(2)-{LIMV}(2)-X(2)-{LIMV}-X(4)-{LIMV}-X(5)-{LIMV}(2)-X-R-{FYW}(2)-G-G-X(2)-{LIMV}-G] commonly shared by the members of YCR059c/yigZ family. (B) Schematic presentation of *Ximpact* homologs. The gray, black, and white boxes indicate the N terminal, central, and C terminal domains, respectively. Hatched boxes are nonhomologous regions. Numbers in boxes represent identity and similarity expressed in percentile.

reconstructed sequence was verified. Northern blot hybridization with this cDNA probe detected an RNA of ~1.3 kb, suggesting that the cDNA is of full or nearly-full length (data not shown). We thus subjected the RT-PCR product to direct cycle sequencing for the elimination of the effects of misincorporation during PCR to define the final sequence data shown in Fig. 1.

The cDNA contains an open reading frame (ORF) composed of 312 amino acid residues. Although the sequence around the first methionine of this ORF does not match to the typical Kozak's consensus sequence (26) and no in-framed stop codons are found in its upstream, we assume it to be the initiation codon, judging from the sequence homology to its homologs from other species. The predicted product of this ORF shows a high sequence homology (56% identity, 90% similarity) to that of mouse *Impact* (21). We thus designated the gene from which this cDNA was derived as *Ximpact* for *Xenopus Impact*.

Structural Features and Molecular Evolution of Ximpact

We compared the predicted structure of *Ximpact* product with those of its homologs from various other species (Fig. 2). These proteins apparently consist of three domains, namely N-terminal, central, and C-terminal domains, corresponding to amino acid residues 1~175, 176~272, and 273~312 of the predicted product of *Ximpact*, respectively. While the N- and C-terminal domains are found only in eukaryotes, the central domain is highly conserved even in various eubacteria. It is found in all the eubacteria whose genomes are fully sequenced except mycoplasma and cyanobacterium. The protein family sharing this highly conserved domain was designated initially as YCR059c/yigZ hypothetical protein family (22), and, more recently, as Uncharacterized Protein Family 29 (UPF0029) (23). Despite its striking evolutionary conservation, no clues are currently available for their functions.

For the allelic expression study described below, we cloned the cDNA encoding the ORF of *Ximpact* from *X. borealis* using PCR. We found that 58 nt of the 889 nt, which comprises the ORF sequence except those corresponding to the primers, are different between the two species (6.5% divergence). At the amino acid level, 22 out of 304 residues (7.2%) are diverged between the two frogs. As mentioned above, *Ximpact* protein consists of three domains, and the DNA regions encoding N-terminal, central, and C-terminal domains showed 6.8, 3.7, and 10.3% of divergence between the two species, respectively. To our interest, all of the amino acid substitutions are found in the eukaryote-specific N- and C-terminal domains, which show 9.9 and 13.8% divergence, respectively. It should be, however, noted that most of these substitutions are of conservative ones. On the other hand, all the nucleotide sequence polymorphisms found in the region encoding the central domain are neutral; the amino acid sequence of this domain is completely identical between the two species. These observations also indicate the extreme conservation or slow molecular evolution of the central domain, suggesting its evolutionarily conserved and, presumably, important function.

Temporal and Spatial Distribution of Ximpact Transcript

For the examination of temporal expression pattern of *Ximpact*, we performed semi-quantitative RT-PCR assays using RNAs isolated at various developmental stages (Fig. 3A). The *Ximpact* transcript is already detected at stage 3 as a maternal RNA. While the amount of *Ximpact* transcript is kept constant until stage 9, it is suddenly decreased at stage 10. This is presumably due to the midblastula transition. Then, the *Ximpact* transcript is gradually increased after stage 10, when the zygotic gene expression is activated. Since mouse *Impact* is also expressed in early embryonic stages (21), it is conceivable that it plays some role in early development as other imprinted genes.

We also examined the spatial distribution of *Ximpact* transcript in embryos and adult frog. The embryos at the 8-cell stage were dissected into the 4 parts as shown in Fig. 3B. The *Ximpact* transcript was detected evenly in the dissected portions (Fig. 3B, upper panel). The neurula embryos were divided into notochord, somite, dorsal ectoderm, and belly (Fig. 3B, lower panel). The results of RT-PCR indicated that these four tissues contain roughly similar amount of *Ximpact* transcript (Fig. 3B). The *Ximpact* transcript was detected in all the adult tissues examined, showing no evidence for tissue preference (Fig. 3C). This is in good contrast with the observation that mouse *Impact* is expressed much more abundantly in adult brain than other tissues examined (21). The difference in tissue distribution pattern of *Impact* transcript between mouse and frog may indicate some functional differentiation of this gene between the two species. Alternatively, there are two or more homologs of *Impact* and that the one we obtained is a paralog rather than an ortholog of mouse *Impact*. Efforts to examine the presence of other *Impact* homologs are currently underway.

Allelic Expression Status of Ximpact

To examine whether *Ximpact* is imprinted or not, one has to distinguish each parental allele by nucleotide polymorphisms. For this, we took advantage of the fact that interspecific F1 hybrids can be generated between *X. laevis* and *X. borealis*, which display a considerable level of nucleotide sequence divergence. Among the polymorphic sites revealed between the ORFs of *Ximpact* cDNA from *X. laevis* and *X. borealis*

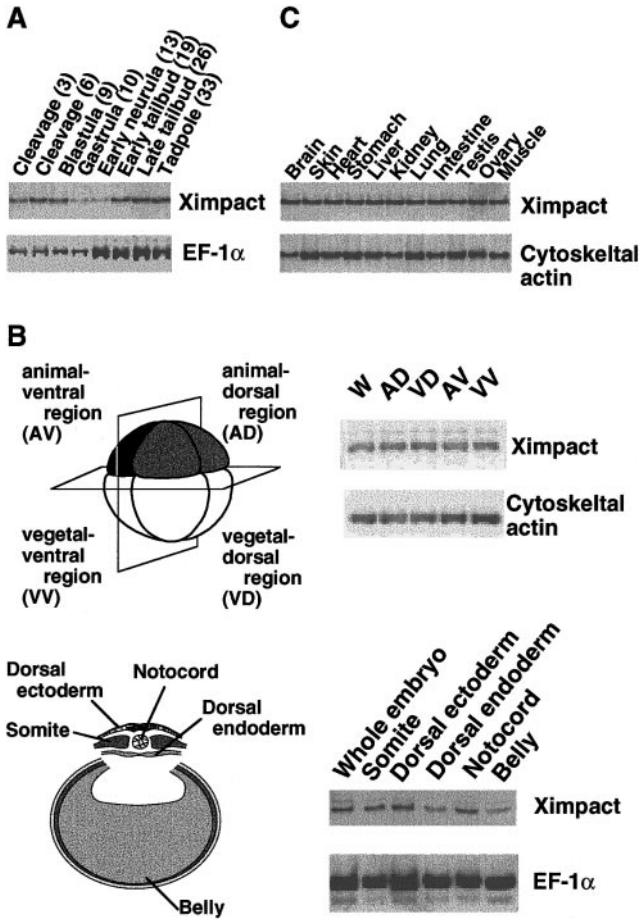


FIG. 3. Temporal and spatial expression of *Ximpact*. (A) Expression of *Ximpact* during embryogenesis. Total RNAs (25 ng) isolated from embryos at different stages (stages 3, 6, 9, 10, 13, 19, 26, and 33) were used for RT-PCR assay. (B) Spatial distribution of *Ximpact* transcript in embryonic tissues. Embryos at 8-cell stage were dissected into four portions, namely, animal-dorsal (AD), vegetal-dorsal (VD), animal-ventral (AV), and vegital-ventral (VV) regions as shown in the upper left panel. Total RNAs isolated from these portions were subjected to the RT-PCR assay (upper right panel). Embryos at the neurula stage were dissected into notochord, dorsal ectoderm, dorsal endoderm, somite, and belly (lower left), and RNA from each portion was examined by the RT-PCR assay (lower right). (C) Distribution of *Ximpact* transcript in adult tissues. The RNAs from adult brain, skin, heart; stomach, liver; kidney, lung, intestine, testis, ovary, and muscle were examined by RT-PCR.

(see above), we decided to use an *Eco*RI site, which is found on the *X. laevis* allele but lost from the *X. borealis* one (Fig. 4A). Using an RT-PCR-RFLP assay developed for this site, we examined the allelic expression status of *Ximpact* in both reciprocal F1 hybrids. As shown in Fig. 4B, both alleles were amplified from the whole embryo cDNAs prepared at stages 26 and 33. These results indicated that *Ximpact* is expressed biallelically, or from both alleles, when analyzed as whole embryos at these stages. Since stage-specific and tissue-specific imprinting is often observed in mammalian imprinted genes (27), the possibility of monoallelic expression of *Ximpact* in other stages or in particular tissues cannot be excluded.

To the best of our knowledge, this is the first report in which the allelic expression status is analyzed at the DNA level to examine the possibility of genomic imprinting in nonmammalian vertebrates. While no evidence was obtained for the imprinting of *Ximpact*, our results proved that the interspecific F1 hybrid frogs used here work as a useful resource for the examination of allelic expression status. Since genomic imprinting is not always conserved evolutionarily and species-specific imprinting has been reported on several imprinted genes (27), homologs of other imprinted genes would be worth testing using the F1 hybrid frogs. Besides these candidate gene approaches, it is also possible to use these reciprocal F1 hybrid frogs in the search of imprinted genes by the Allelic Message Display, a screening method which is based on the polymorphisms in the transcripts (21).

Effect of Overexpression of Ximpact

As an initial step to the elucidation of *Ximpact* function, we microinjected its mRNA into the fertilized eggs and examined its effect on development. The microinjection of *Ximpact* RNA reproducibly results in a higher than normal rate of gastrulation defect: the gastrulation failure was observed in 27 of 83 embryos injected with *Ximpact* mRNA (Figs. 5A and 5B), whereas none of the 79 embryos injected with the same amount of *lacZ* mRNA showed the defect (Figs. 5C and 5D). Such a high rate of gastrulation failure was not observed in the control embryos injected with much

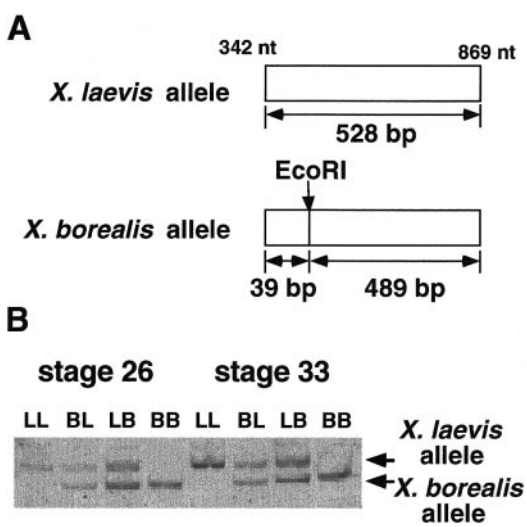


FIG. 4. Allelic expression of *Ximpact*. (A) The schematic map of the region containing a polymorphic *Eco*RI site. (B) RT-PCR-RFLP assay. RT-PCR products from tailbud and tadpole cDNAs prepared from *X. laevis* (LL), reciprocal F1 hybrids between *X. laevis* and *X. borealis* (LB and BL), and *X. borealis* (BB) were digested with *Eco*RI and subjected to 4% PAGE.

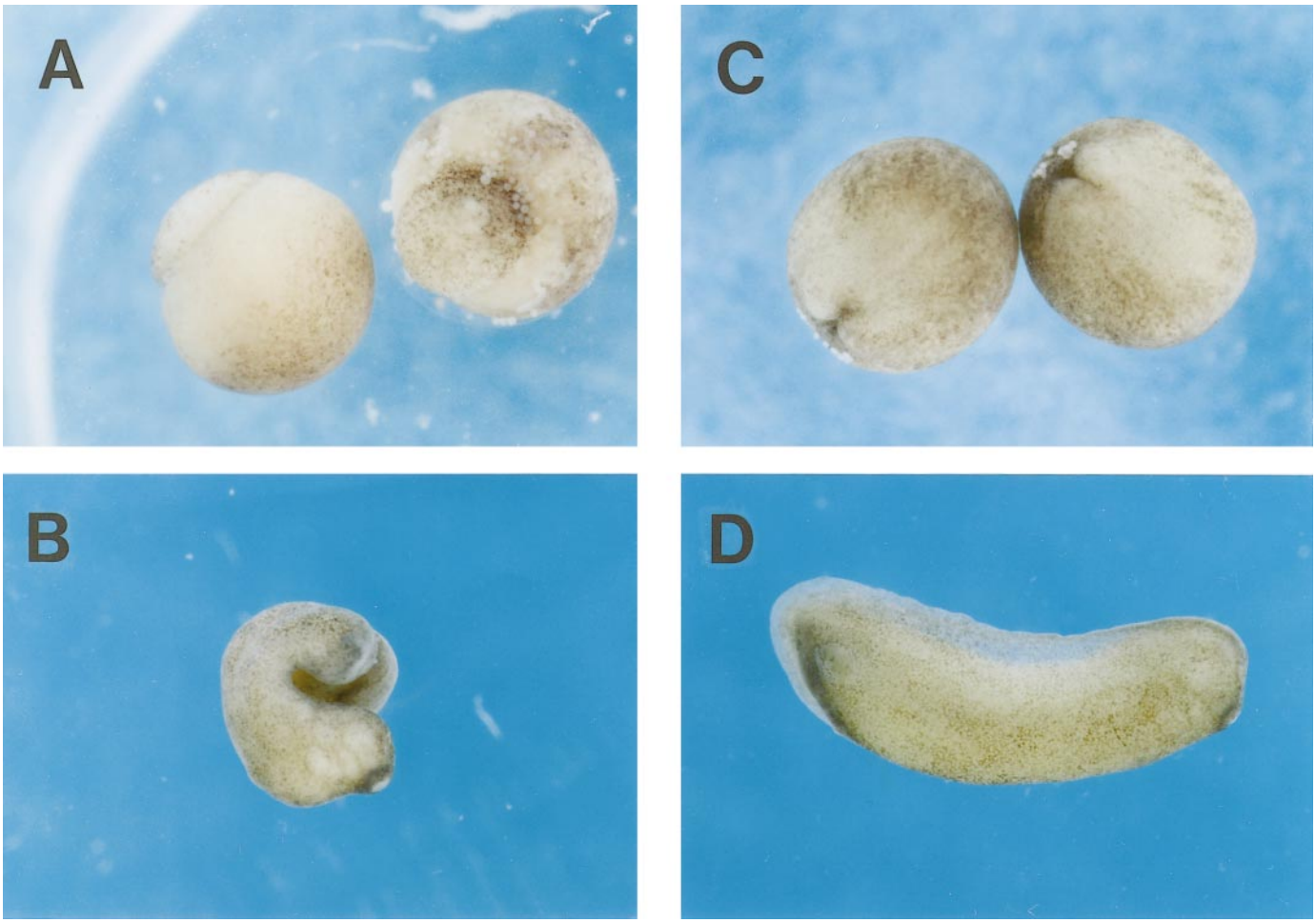


FIG. 5. Effects of microinjection of *Ximpact* mRNA. The embryos injected with *Ximpact* mRNA at the 2-cell stage were observed at early neurula (stage 14) (A) and the tailbud stage (B). The control embryos were injected with the same amount of *lacZ* (β-galactosidase) mRNA and observed at early neurula (C) and the tailbud stage (D).

higher dose of *lacZ* mRNA (not shown). We think that this is an intriguing observation and that detailed analysis of these defects in the future would shed light on the biological roles of *Ximpact* as well as its homologs.

Since the overexpression of *Ximpact* mRNA, and presumably its protein product, is harmful to early embryo, its endogenous expression should be strictly controlled during normal development. If we assume the evolutionary conservation of *Impact* functions, its overexpression may be also hazardous in other species. In this context, it is interesting to note that the overexpression of the budding yeast homolog of *Impact* confers yeast cells on growth defective phenotypes under several stressed conditions (unpublished data). Also, to our interest, a deficiency of paternal chromosome 18 uniparental disomic embryos, in which the expression level of *Impact* would be doubled, was reported along with a higher than normal rate of developmental retardation (28). It may be that mouse (or mammals) has evolved to avoid deleterious overexpression of *Impact*, in part, by limiting its expression only from a single allele whereas the clawed frog (or amphibians) controls the dosage of *Ximpact* by other means than genomic imprinting.

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