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The anterior determinant *bicoid* of *Drosophila* is a derived *Hox* class 3 gene

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ABSTRACT **The *Drosophila* gene *bicoid* functions as the anterior body pattern organizer of *Drosophila*. Embryos lacking maternally expressed *bicoid* fail to develop anterior segments including head and thorax. In wild-type eggs, *bicoid* mRNA is localized in the anterior pole region and the *bicoid* protein forms an anterior-to-posterior concentration gradient. *bicoid* activity is required for transcriptional activation of zygotic segmentation genes and the translational suppression of uniformly distributed maternal *caudal* mRNA in the anterior region of the embryo. *caudal* genes as well as other homeobox genes or members of the *Drosophila* segmentation gene cascade have been found to be conserved in animal evolution. In contrast, *bicoid* homologs have been identified only in close relatives of the schizophoran fly *Drosophila*. This poses the question of how the *bicoid* gene evolved and adopted its unique function in organizing anterior–posterior polarity. We have cloned *bicoid* from a basal cyclorrhaphan fly, *Megaselia abdita* (Phoridae, Aschiza), and show that the gene originated from a recent duplication of the direct homolog of the vertebrate gene *Hox3*, termed *zerknüllt*, which specifies extraembryonic tissues in insects.**

Drosophila body pattern formation is initiated in response to asymmetrically distributed proteins, emanating from prelocalized mRNA in the pole regions of the egg (1–3). The factor required for the establishment of the anterior body part, including head and thorax, is encoded by the homeobox gene *bicoid* (3). *bicoid*, which is located within the homeotic gene complex (Hox-C) next to *zerknüllt* (Fig. 1*A*; reviewed in refs. 4–7), is expressed maternally in response to a general transcription factor, encoded by the gene *serendipity*, in the nurse cell/oocyte syncytium (8). The *bicoid* mRNA is transferred into the oocyte and becomes localized in the anterior pole region of the egg. After egg deposition and translation of the transcript, the *bicoid* protein (Bicoid), and to a lesser degree the mRNA, spread posteriorly, thereby generating a concentration gradient of the protein (3).

The Bicoid gradient controls two regulatory aspects of gene expression in the early embryo. Firstly, it acts as a threshold-dependent transcriptional activator of zygotic segmentation genes, which are required to metamerize the anterior region of the embryo and to specify the segments and pattern elements (3). Secondly, Bicoid prevents the translation of uniformly distributed maternal *caudal* mRNA in the anterior region of the early embryo and thereby causes a second homeodomain transcription factor gradient, that of Caudal, in the opposite direction to Bicoid (2). The combined activities of the two transcription factors are necessary to activate the zygotic segmentation gene cascade in the precellular blastoderm embryo (2). Whereas *caudal* genes have been identified in a large variety of species and consistently show posterior-to-anterior protein concentration gradients (refs. 9 and 10 and references therein), *bicoid* genes have not been found in species other than *Drosophila* or some related schizophoran flies (11, 12). Here we present evidence for the evolutionary origin of *bicoid* by a gene duplication event involving the insect *Hox3* homolog *zerknüllt*.

MATERIALS AND METHODS

Cloning of Homologs. PCR clones of *Ma-bcd* and *Ma-zen* were obtained by using the primer pairs ATGMGTMGTCC-DMGDMGNAC/GCKGCKRTTYTTRAACCA (6) and CARCTBGTDGARCTIGARAAYGARTT/TTYTTRWA-YTTCATICKICKRTTYTG, respectively, on genomic DNA. Genomic sequence was obtained from partially *Mbo*I digested DNA cloned in the phage vector Lambda FixII (Stratagene). For the *Ma-bcd* cDNA 3′ and 5′ rapid amplification of cDNA ends (RACE) experiments were performed. cDNA was prepared from 3 μg of poly(A)$^+$ RNA according to the instructions of the Marathon cDNA amplification kit (CLONTECH). The ORF of *Ma-zen* was deduced from 2.1 kb of genomic sequence by using splice site and promotor prediction software from the Berkley Drosophila Genome Project Sequence Analysis Tools (www.fruitfly.org/reg_tools) package. The sequence includes two promotor consensus sequences and a polyadenylation signal in the 3′ untranslated region. The *Ma-bcd* and *Ma-zen* sequences are deposited in the GenBank database (accession nos. AJ133024, AJ133025).

Whole-Mount *in Situ* Hybridization. Hybridization to *Megaselia* embryos was done with DNA probes at 48°C as described for *Drosophila* (13) with the following modifications. To burst the vitelline layer, −80°C cold methanol was used in the devitellinization step, and the embryos were heated in methanol for 1 min to +70°C. The temperature shock was repeated two to three times during the following methanol washes.

Phylogenetic Analysis. Sequence similarities in percentage were calculated with the PAM250 residue weight table by using MEGALIGN/DNASTAR software. Molecular phylogenetic trees were constructed from protein sequences to avoid possible distortion by codon usage differences. The Parsimony method and the Neighbor Joining method on a Dayhoff distance matrix, implemented by using PHYLIP 3.572c, were applied (14). Topology robustness was assessed by 500 bootstrap resamplings. Statistical likelihood of alternative user-defined trees was assessed by a resampling of estimated likelihood method implemented with PROTML in MOLPHY 2.2 (15).

RESULTS AND DISCUSSION

Cloning and Expression Patterns of *Megaselia bicoid*. In a search for *bicoid* homologs in lower dipteran species, we used

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Abbreviation: Hox-C, homeotic gene complex.
The sequences reported in this paper have been deposited in the GenBank database (accession nos. AJ133024, AJ133025).
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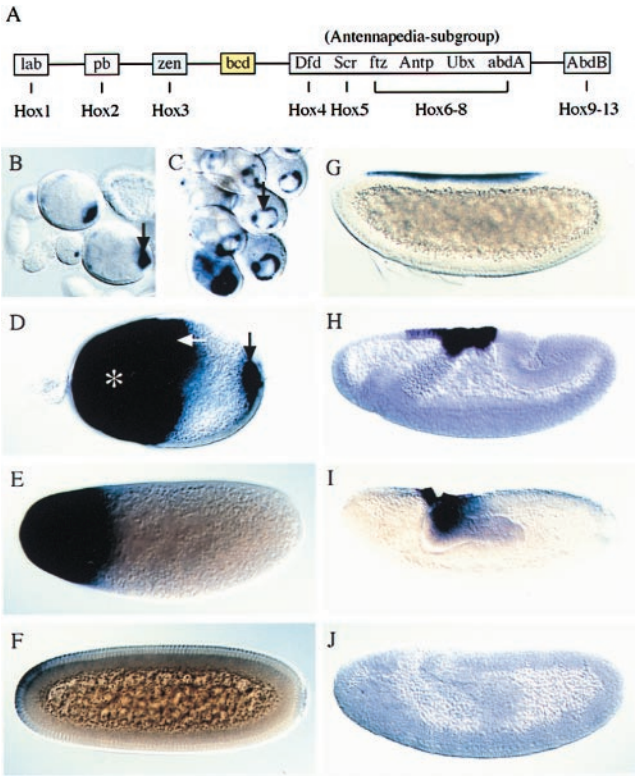

FIG. 1. *Hox* gene cluster and expression patterns of *Ma-bcd* and *Ma-zen* in *Megaselia*. (*A*) The Hox-C of *Drosophila* and homologous genes in vertebrates (*Hox1–13*) (4–7). *Dfd* (*Deformed*), *Scr* (*Sex combs reduced*), *ftz* (*fushi tarazu*), *Antp* (*Antennapedia*), *Ubx* (*Ultrabithorax*), and *abdA* (*abdominal A*) form a subgroup of *Antennapedia* related genes. lab, *labial*; pb, *proboscipedia*; zen, *zerknüllt*; bcd, *bicoid*; AbdB, *Abdominal B*. (*B* to *F*) *In situ* hybridization showing the transcript distribution of *Ma-bcd* in the oocyte (*B–D*; arrows), nurse cells (*D*; asterisk), and embryos before (*E*) and during (*F*) cellularization. (*G–J*) Patterns of *Ma-zen* transcripts before (*G*), during (*H*), and after (*I*, *J*) gastrulation. In *Drosophila*, *zerknüllt* is expressed also in the pole cells (22).

degenerated PCR primers to amplify DNA fragments containing the homeodomain-encoding region from a primitive cyclorrhaphan fly, *Megaselia abdita.* The cyclorrhaphan flies are divided into two subordinate groups: the Aschiza and the Schizophora. The phorid *Megaselia* is an aschizan fly (16). Therefore, it is different from the monophyletic group of schizophoran flies that includes *Drosophila* and the few other species where *bicoid* could be identified so far (11, 12). We isolated genomic DNA encompassing the transcription unit and performed 5′ and 3′ rapid amplification of cDNA ends (RACE) experiments to clone the cDNA. The cDNA and corresponding genomic DNA were sequenced to establish the identity and structure of the *bicoid* homologous gene, termed *Ma-bcd*, and the transcript distribution during oogenesis and embryogenesis was visualized by *in situ* hybridization.

Ma-bcd transcripts accumulate first in the oocyte, where a transient ring-shaped pattern is observed (Fig. 1 *B* and *C*). Later, during oogenesis, transcripts are expressed in the nurse cells; they accumulate in the anterior region of the oocyte and transiently at its posterior pole (Fig. 1*D*). In the early embryo, *Ma-bcd* transcripts spread from the anterior pole forming an enlarging anterior cap until the onset of cellularization (Fig. 1*E*). Subsequently, transcripts disappear rapidly (Fig. 1*F*). No zygotic expression was observed during embryogenesis (not shown). These findings establish identical expression patterns of *bicoid* and *Ma-bcd* in *Drosophila* (3, 17) and *Megaselia*.

The Homeodomains of *Ma-bcd* and *zerknüllt* Are Closely Related. A comparison of the *Ma-bcd* homeodomain and homeodomain proteins of *Drosophila* indicates that aside from *bicoid*, *Ma-bcd* is most similar to *zerknüllt* (48.3%), whereas the similarity to homeodomains encoded by the other members of the *Drosophila* Hox-C is less pronounced (45.0%–36.7%) (Fig. 2 and data not shown). The homeodomain of *Ma-bcd* is related only distantly to the homeodomains encoded by *orthodenticle* and the *Drosophila* homologs of *goosecoid* and *Ptx1* (38.3%–33.3%), which have been classified in the past as *bicoid*-related genes (Fig. 2). These proteins share common DNA-binding properties that depend on the diagnostic lysine in position 50 of the homeodomain (18–21). Notably, also the *zerknüllt* homologs of other insects and their orthologs in various animal

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                1        10        20        30        40        50        60
                |--------|---------|---------|---------|---------|---------|
Ma-bcd          RRRTRTTFTSSQIAELEEYFRQGKYLNNIRLSELTGRLNLGQAQVKIWFKNRRRRFKIEQ
Dm-bcd          PRRTRTTFTSSQIAELEQHFLQGRYLTAPRLADLSAKLALGTAQVKIWFKNRRRRHKIQS 70.0%

Ma-zen          TKRSRTAFTSIQLLELENEFKKNKYLNRPRRIEISLRLSLSERQVKIWFQNRRMKSKKDR 50.0% (46.7%)
Dm-zen          LKRSRTAFTSVQLVELENEFKSNMYLYRTRRIEIAQRLSLCERQVKIWFQNRRMKFKKDI 48.3% (43.3%)
Tc-zen          GKRARTAYTSAQLVELEREFHHGKYLSRPRRIQIAENLNLSERQIKIWFQNRRMKHKKEQ 48.3% (45.0%)
Dm-zen2         SKRSRTAFSSLQLIELEREFHFNKYLARTRRIEISQRLALTERQVKIWFQNRRMKLKKST 46.7% (45.0%)
Cs-hox3         AKRARTAYTSAQLVELEKEFHFNRYLCRPRRIEMANLLNLSERQIKIWFQNRRMKYKKEQ 46.7% (43.3%)
Al-hox3         GKRARTAYTSAQLVELEKEFHFNRYLCRPRRVEMAAMLNLTERQIKIWFQNRRMKYKKEQ 46.7% (45.0%)
Al-Xlox         NKRTRTAYTRGQLLELEKEFHFNKYISRPRRIELAAMLNLTERHIKIWFQNRRMKWKKEQ 46.7% (41.7%)
Sg-zen          SKRARTAYTSQQLIELEKDFSINRYLCRPRRIELAAQLGLTERQIKIWFQNRRMKYKKEK 45.0% (46.7%)
Ls-hox3         PKRSRTAYTSAQLVELEKEFHFNRYLCRRRRIEMAALLNLSERQIKIWFQNRRMKYKKDQ 45.0% (45.0%)
Hox3            SKRARTAYTSAQLVELEKEFHFNRYLCRPRRVEMANLLNLTERQIKIWFQNRRMKYKKDQ 45.0% (43.3%)

Dm-Ubx          RRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHALCLTERQIKIWFQNRRMKLKKEI 45.0% (41.7%)
Dm-pb           PRRLRTAYTNTQLLELEKEFHFNKYLCRPRRIEIAASLDLTERQVKVWFQNRRMKHKRQT 43.3% (48.3%)
Dm-Antp         RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN 43.3% (41.7%)
Dm-lab          NNSGRTNFTNKQLTELEKEFHFNRYLTRARRIEIANTLQLNETQVKIWFQNRRMKQKKRV 41.7% (43.3%)

Dm-Ptx1         QRRQRTHFTSQQLQELEHTSSRNRYPDMSTREEIAMWTNLTEARVRVWFKNRRAKWRKRE 38.3% (36.7%)
Dm-otd          QRRERTTFTRAQLDVLEALFGKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQL 36.7% (38.3%)
Dm-gsc          KRRHRTIFTEEQLEQLEATFDKTHYPDVVLREQLALKVDLKEERVEVWFKNRRAKWRKQK 33.3% (36.7%)
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FIG. 2. Homeodomain alignment and percent sequence similarity relative to *Ma-bcd* and *Dm-bcd* (in brackets). Numbers refer to amino acid position. Abbreviations: *Ma-bcd*, *bicoid* of *Megaselia* (this work); *Dm-bcd*, *bicoid* of *Drosophila* (27); *Ma-zen*, *zerknüllt* of *Megaselia* (this work); *Dm-zen*, *zerknüllt* of *Drosophila* (22);*Tc-zen*, *zerknüllt* of beetle (7); *Cs-hox3*, *Hox3* of spider (24); *Al-hox3* (28) and *Al-Xlox* (29), *Hox3* and *Xlox* of cephalochordate, respectively; *Dm-zen2*, *zerknüllt-2* of *Drosophila* (22); *Sg-zen*, *zerknüllt* of grasshopper (7); *Ls-hox3*, *Hox3* of ribbonworm (30); *Hox3*, consensus reported for the *Hox3* paralogy group (7). Other abbreviations refer to *Hox* genes (cf. legend to Fig. 1; 31–34), *Ptx1*, *orthodenticle* and *goosecoid* of *Drosophila* (18–21). Identical amino acids (reference to *Ma-bcd*) are underlaid.

classes, the *Hox3* genes of chordates, ribbonworm, and spider, show a higher degree of similarity to the *Ma-bcd* homeodomain than do the *bicoid*-related genes (Fig. 2). These observations suggest that, in spite of the considerable sequence divergence exhibited by the *Drosophila* genes, *bicoid* and *zerknüllt* are closely related.

***Ma-bcd* and *Ma-zen* Are Sister Genes.** To address the hypothesis that *bicoid* and *zerknüllt* are the closest relatives among *Hox* genes, we cloned the *Megaselia zerknüllt* gene (*Ma-zen*) and asked whether *Ma-zen* provides a link between *bicoid* and the *Hox3* genes of the vertebrate Hox-Cs.

We isolated *Ma-zen* by a PCR approach and isolated the genomic DNA encompassing the transcription unit. Whole-mount *in situ* hybridization of *Megaselia* embryos reveals that *Ma-zen* transcripts are expressed only zygotically. They form a restricted pattern at the dorsal side of the blastoderm embryo, covering the area of the amnioserosa precursor cells, and disappear in the extended germ-band stage (Fig. 1 *G–J*). Thus, *Ma-zen* is expressed like *zerknüllt* in *Drosophila*. This suggests that *Ma-bcd* and *Ma-zen* have separate functions in *Megaselia,* similar to the functions of their homologs in *Drosophila* (3, 22).

Sequence comparison of the predicted *Ma-bcd* and *Ma-zen* proteins clearly establishes a sister relationship of the two proteins (Figs. 2–4). Evidence for this is based on the following findings. The homeodomain of *Ma-bcd* shows a higher sequence similarity to *Ma-zen* (50.0%) than to any other non-orthologous homeodomain (Fig. 2). In addition, molecular phylogenetic trees involving the homeodomains of the Hox-C genes of *Drospohila* resolve with high confidence when the *Ma-bcd* and *Ma-zen* instead of the *bicoid* and *zerknüllt* sequences were used for the analysis (Fig. 3). It is important to note that the *Drosophila* homeodomains included in the tree in Fig. 3 evolve very slowly (except *fushi tarazu*) and can be assumed to be identical or almost identical in amino acid sequence in *Megaselia* and *Drosophila* (ref. 23; U.S.-O., unpublished results). To further test the statistical significance of this finding, we estimated likelihood values for the tree shown in Fig. 3 and for 11 modified trees where the position of *Ma-bcd* was changed. The tree shown in Fig. 3 is supported over the modified trees with a bootstrap value of 94.8%. Finally, the alignment of the *Ma-bcd* and *Ma-zen* proteins shows conservation of sequences not only in the homeodomain but also N-terminal to it (Fig. 4). The conserved sequences in front of the homeodomain are not evident from the comparison of *bicoid* and *zerknüllt* of *Drosophila.* Thus, the sister-gene relationship of *bicoid* and *zerknüllt* revealed by the *Megaselia* genes remains hidden when the obviously more diverged sequences of the *Drosophila* genes are compared.

***bicoid* Is a Derived *Hox* Class 3 Gene.** The newly established sister-gene relationship implies that *bicoid* genes, like the *zerknüllt* genes (7, 24, 25), are direct homologs of the *Hox3* genes in the Hox-C of noninsect animal classes. Thus, *bicoid* is a *Hox* gene in the phylogenetic sense, and the location of *bicoid* in the Hox-C of *Drosophila* (Fig. 1*A*) is an ancestral trait. The consistent failure to isolate *bicoid* from insects other than flies, which has been attempted in various laboratories (4, 26) including our own, suggests a recent origin of the *bicoid* gene. The fact that *Ma-bcd* is more similar to *zerknüllt* genes of higher insects than to other *Hox3* homologs (Fig. 2) is consistent with

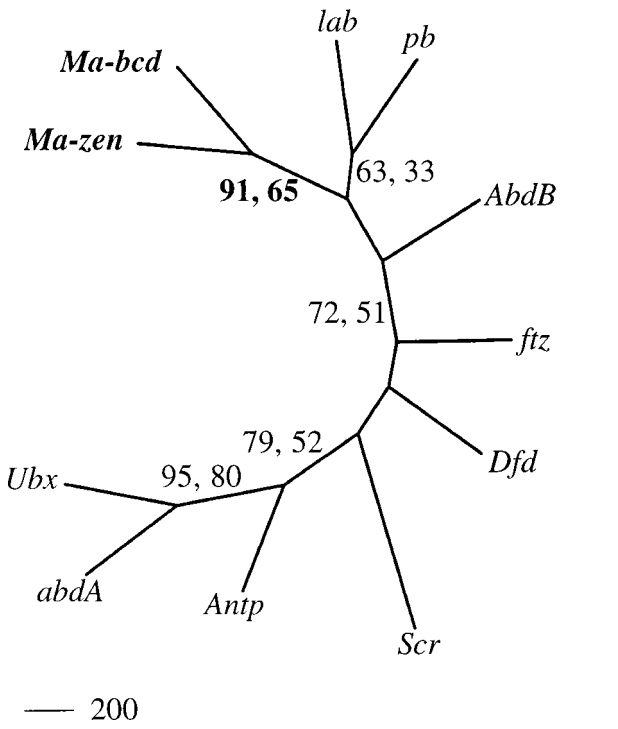

FIG. 3. Evolutionary relationship of *Ma-bcd*, *Ma-zen,* and Hox-C genes of *Drosophila*, as deduced by neighbor-joining analysis. Numbers refer to bootstrap percentages obtained from neighbor-joining (first value) and maximum parsimony analysis. Trees including the more diverged *bicoid* and *zerknüllt* genes of *Drosophila* remain unresolved with respect to the monophyletic cluster of *bicoid* and *zerknüllt* orthologs (bootstrap value below 50%; data not shown). For abbreviations, see legend to Figs. 1 and 2.

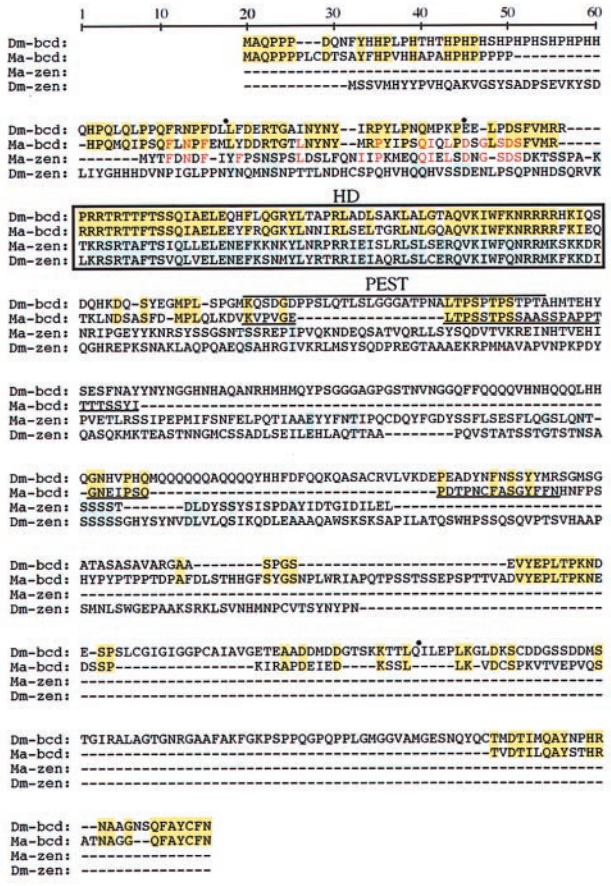

FIG. 4. Proteins encoded by *bicoid* and *zerknüllt* of *Megaselia* (*Ma-bcd*, *Ma-zen*) and *Drosophila* (*Dm-bcd*, *Dm-zen*) (27, 22). Dashes indicate sequence gaps for optimal alignment. Amino acid identities of the *bicoid* proteins (yellow boxes) or the *zerknüllt* proteins (blue boxes) and between *Ma-bcd* and *Ma-zen* in front of the homeodomain (red letters) are highlighted. Dots above *Dm-bcd* mark intron positions; only the first two are conserved in *Ma-bcd* (64 bases and 11.5 kb). Homeodomains (HD) are boxed, PEST domains (35) are overlined (*Dm-bcd*) and underlined (*Ma-bcd*).

the assumption that *bicoid* originated recently during insect radiation.

bicoid is expressed in the anterior egg region, where it exerts its role in patterning the anterior body of the larval fly. In contrast, *zerknüllt* and its orthologs function in extraembryonic anlagen. Although the extraembryonic anlage in flies, the amnioserosa, is located at the dorsal side of the blastoderm fate map (22), extraembryonic anlagen in other insects, such as the beetle *Tribolium*, are formed in an anterior egg position (7). This suggests that initially the sister genes *bicoid* and *zerknüllt* may have been coexpressed in the anterior egg region. The subsequent recruitment of *bicoid* in patterning the embryo, instead of determining the dorsally shifted extraembryonic anlagen, changed the selection conditions for the gene. Ensuing adaptations must have resulted in a new set of target genes (1, 2), as reflected by the characteristic lysine in Bicoid′s homeodomain position 50 that specifies DNA recognition (Fig. 2). The newly acquired functions of Bicoid entrained a significant change in the developmental mechanism of axis specification and furnish an outstanding model of molecular evolution in a patterning process.

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