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RAPID COMMUNICATION

Functional Conservation of the *Wnt* Signaling Pathway Revealed by Ectopic Expression of *Drosophila dishevelled* in *Xenopus*

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Wnt genes encode secreted growth factors that exhibit potent effects on both embryonic and postembryonic development in vertebrates and invertebrates. Recently, the *dishevelled* (*dsh*), *shaggy/zeste-white 3*, and *armadillo* genes have been shown to participate in *Wnt* (*wingless; wg*) signaling in *Drosophila*. Vertebrate genes that have sequence similarities to all of these *Drosophila* genes have been identified. To determine whether these structurally conserved components of insect *wg* signaling represent a functionally conserved *Wnt* signaling pathway in vertebrates, we investigated the role of *Drosophila dsh* in *Xenopus Wnt* signaling. *Xenopus* embryos ectopically injected with *Drosophila dsh* mRNA developed duplicated axes similar to those seen in embryos injected with *Wnt* mRNAs. The involvement of *dsh* function in the *Wnt* signaling pathway in *Xenopus* was demonstrated using two assays which are specifically sensitive to *Wnt* signaling: synergistic induction of dorsal mesoderm with bFGF and the specific induction of a *Wnt*-responsive reporter gene. These findings support the notion that the intracellular response to the *Wnt* signal has been conserved during evolution to such an extent that its components may be interchanged between distantly related species.

INTRODUCTION

Wnt genes are recognized as mediators of cell-cell signaling events essential during pattern formation and are thought to be involved in tumorigenicity (reviewed by Klingensmith and Nusse, 1994). Ectopic expression of *Wnt-1*-related genes (from flies to mice) induces axis duplications in frog and fly embryos and mammary cancer in mice. Loss of function of the mouse *Wnt-1* gene interferes with brain development, and a similar deficiency in *Drosophila* affects segmentation patterns. In *Drosophila*, the *dishevelled* (*dsh*), *shaggy/zeste-white 3* (*sgg/zw3*), and *armadillo* (*arm*) genes have been shown to participate in *Wnt* (*wingless; wg*) signaling (reviewed by Siegfried and Perrimon, 1994). Intracellular transmission of the *wg* signal is thought to be mediated through *dsh*, resulting in the inactivation of the *sgg/zw3* protein, thereby increasing the level of active *arm* protein. Vertebrate genes that have sequence similarities to all of these *Drosophila* genes have been identified. *arm* resembles vertebrate plakoglobin and β-catenin proteins. *sgg/zw3* is related to the mammalian serine/threonine kinase, glycogen synthase kinase 3. *dsh* encodes a novel protein of unknown biochemical function which is structurally conserved in vertebrates (Sussman *et al.*, 1994).

Several lines of evidence suggest that *dsh* in *Drosophila* is involved in reception or transduction of the *wg* signal in target cells. Genetic epistasis studies have indicated that *dsh* functions downstream of the *wg* signal (reviewed in Siegfried and Perrimon, 1994). Furthermore, the requirement of *dsh* was found to be cell autonomous, suggesting that each cell must have *dsh* to achieve its appropriate fate (Theisen *et al.*, 1994). We decided to investigate the ability of *Drosophila dsh* to function in *Xenopus Wnt* signaling to determine whether the structurally conserved intracellular components of *wg* signaling molecules, such as *dsh*, were part of a functionally conserved *Wnt* signaling pathway in vertebrates. The induction of mesoderm and subsequent axis formation in *Xenopus* was chosen since this is one of the most thoroughly examined inductive events in vertebrates and the action of *Wnt* genes has been studied extensively in this system.

In *Xenopus*, induction of mesoderm is thought to be mediated by a substance(s) released from yolky vegetal endodermal cells that induces equatorial cells to become mesoderm. Dorsal mesoderm (Spemann's organizer), presumably induced by dorsal endoderm (Nieuwkoop center), is a tissue known to cause axis duplication after

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transplantation in amphibian embryos. *Wnt* proteins are thought to be involved in this process. *Wnt* proteins alone are incapable of inducing differentiation of mesodermal tissues in naive ectoderm, but instead they can alter the response of the tissue to mesoderm inducers. Thus, microinjection of mRNA encoding *Drosophila wg* or its orthologs in mice (*Wnt-1*) and *Xenopus* (*Xwnt1* and *Xwnt8*) into early cleavage stage *Xenopus* embryos affects the formation of the dorsal mesoderm and results in induction of secondary axes, similar to that seen in the organizer transplantation experiments (reviewed in Klingensmith and Nusse, 1994). This observation is consistent with the idea that *Xenopus Wnt* proteins mimic the activity of the Nieuwkoop center, the part of the embryo that regulates the induction of Spemann's organizer. However, a note of caution is that neither *Xwnt1* nor *Xwnt8* are naturally expressed during the 16- to 32-cell stage when Nieuwkoop center activity and mesoderm induction commence, suggesting that as yet unidentified members of the *Xenopus Wnt* family participate in the organizer induction process.

In this paper, we show that the *Drosophila wg* intracellular signaling molecule *dsh* is capable of changing the character of mesoderm in *Xenopus*, resulting in the induction of secondary axes as seen with the *Wnt* gene products. We further provide evidence suggesting that *dsh* acts in the *Wnt* signaling pathway in *Xenopus*. These results indicate that, despite the large phylogenetic distance between *Drosophila* and *Xenopus*, the signaling cascades of *Drosophila wg* and vertebrate *Wnt* gene products share a common mechanism of action.

MATERIALS AND METHODS

Synthetic mRNAs and Microinjection

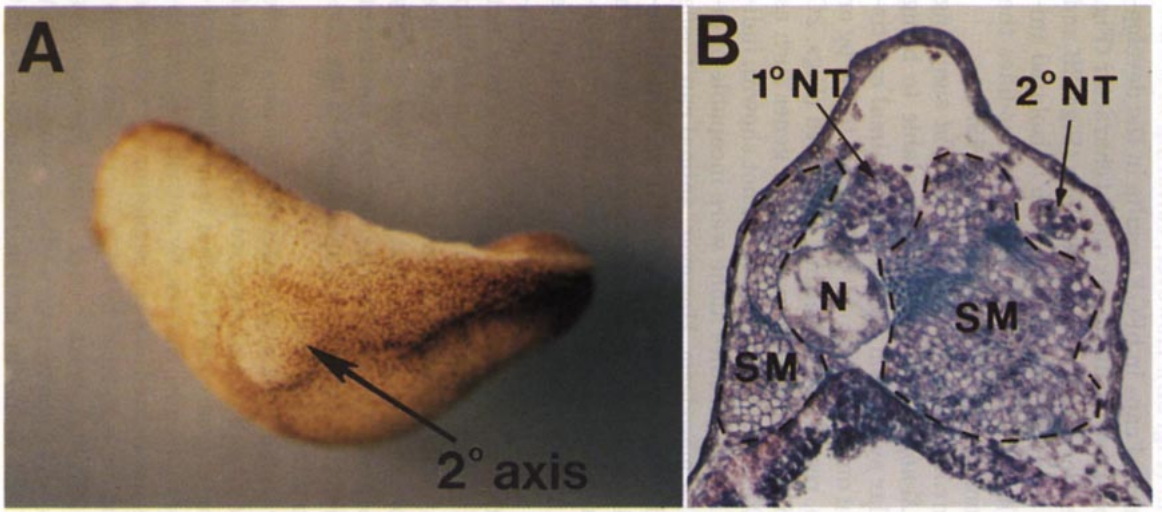
Culture of embryos, preparation of synthetic mRNA, microinjection, and dissection of explants were as described previously (Cho *et al.*, 1991). *dsh* mRNA was synthesized using the T7 MEGAscript Kit (Ambion) from linearized plasmid (*Bam*HI). One nanogram of capped synthetic *dsh* mRNA in a volume of 4 nl was injected per ventral blastomere as indicated in Fig. 1C and explants were cultured in 1× modified Barth's saline.

RT-PCR

RT-PCR was performed essentially as described previously (Blitz and Cho, 1995). All primer sequences used are described (Blitz and Cho, 1995) with the exception of

FIG. 1. Ectopic expression of *Drosophila dsh* induces secondary axes by changing the fate of ventral marginal zones (VMZs) to that of dorsal marginal zones (DMZs). (A) Embryo injected with *Drosophila dsh* mRNA into ventral blastomeres of the 4-cell stage exhibits induction of a secondary axis. Anterior is left and dorsal is up. The secondary axis with dark pigment is indicated with an arrow. Note, however, the absence of anterior-most structures in the secondary axis. The partial phenocopying effect of *Drosophila dsh* may be attributed to the heterospecific nature of the *dsh* molecule used in this experiment or to the possibility that *dsh*-mediated signaling constitutes only part of the *Wnt* signaling cascade in *Xenopus* dorsal-ventral patterning. (B) Transverse section through trunk of a *dsh*-injected embryo. Note the enlarged somite (SM) to the right (closer to the secondary neural tissue) compared to the somite of the left. Dashed lines mark the somite boundary. Abbreviations: N, notochord; 1°NT, primary neural tissue; 2°NT, secondary neural tissue; SM, somite. (C) Schematic diagram showing the injection assay and isolation of explants. *Drosophila dsh* mRNA was injected in the equatorial region of the two ventral blastomeres at the 4-cell stage. VMZs and DMZs were dissected at early gastrula of stage 10.25 and cultured until uninjected sibling embryos reached the indicated stages and RNA was isolated for analysis by RT-PCR assays. Some injected embryos were also grown to stage 30 for phenotypic examination. (D) RT-PCR of RNA from explants injected with *dsh*. DMZ (D) and VMZ (V) explants from embryos injected ventrally with *dsh* (+) were compared to uninjected controls (−) at stage 11 (gastrula, lanes 1–4) and stage 25 (tadpole, lanes 5–8) equivalents. PCR was performed to detect the expression of *goosecoid, orthodenticle 2*, cardiac actin, and N-CAM. Histone H4 primers were included as controls showing that the amount of amplification does not vary significantly between samples. The entire experiment was performed twice with the same results.

FIG. 2. Specificity of *Drosophila dsh* in *Xenopus Wnt* signaling. (A–F) *Drosophila dsh* synergizes with bFGF. While uninjected control animal cap tissues remain ectoderm (A), they differentiate into ventral type mesoderm and do not elongate after bFGF treatment (100 μg/ml) (B). Animal caps receiving either *Xwnt8* or *dsh* mRNA alone (200 pg/embryo) failed to elongate (C and E), whereas animal caps that received *dsh* or *Wnt* mRNA together with bFGF treatment became elongated (D and F). The synergism of *dsh* with bFGF was observed using *dsh* concentrations as low as 40 pg per embryo. (G–I) *dsh* activates a *Wnt*-responsive reporter gene. (G) Schematic map showing the *gsc* WT/Luc and M4/Luc constructs. Note that the activin-responsive element (ARE) and *Wnt*-responsive element (WRE) are physically distinct and separable. M4/Luc contains a 6-bp substitution in the ARE. (H) The mutations in M4/Luc inactivated the ARE. After microinjecting either the *gsc* WT/Luc or M4/Luc construct into 4-cell stage embryos (20 μg/ml), animal caps were isolated at blastula stage and incubated with or without activin. After 3 hr, animal caps were homogenized in 50 m*M* Tris (pH 7.5) and reporter gene activities (luciferase) were measured to quantitate the relative induction of the *gsc* promoter by activin. While the WT/Luc was induced over 20-fold by activin treatment, the M4/Luc was not induced. (I) The M4/Luc gene is induced by both *Xwnt8* and *dsh*. M4/Luc was either injected alone or co-injected with the indicated mRNAs into either the dorsal or the ventral side of 4-cell stage embryos. Dorsal and ventral marginal zones were removed at early gastrula (stage 10.25), incubated for 1 hr, homogenized as above, and assayed for luciferase activity. Comparison of dorsal and ventral explants injected with M4 alone revealed that the dorsal explants induce the M4 reporter gene 5- to 10-fold higher than the ventral explants. Co-injection of *Xwnt8* mRNA caused a 10-fold induction of M4 in ventral tissues, which is indicative of dorsalization of these tissue. Activation of M4/Luc was also seen in ventral explants injected with *dsh*. Activation of M4/Luc in the dorsal explants were similar whether the tissue received *Xwnt-8*, *dsh*, or M4 alone. The experiments using ventral explants were repeated four times and essentially identical relative activation by *Xwnt8* and *dsh* was observed in all cases.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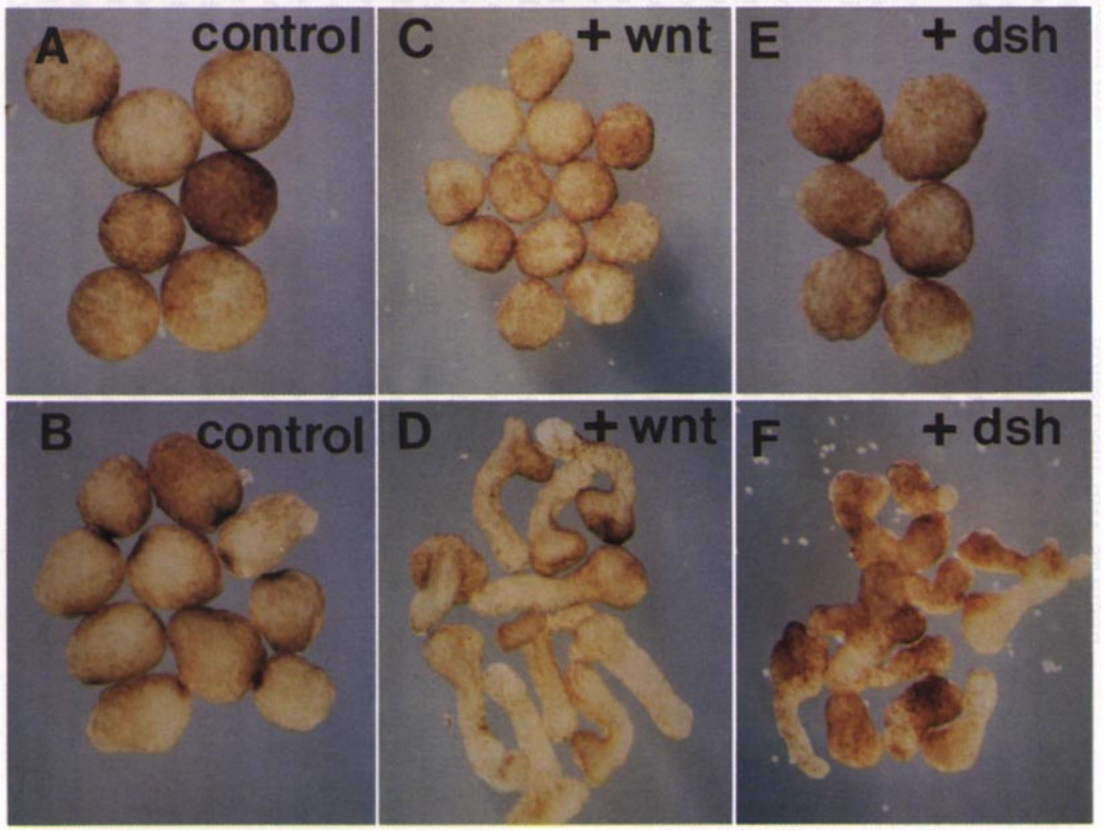
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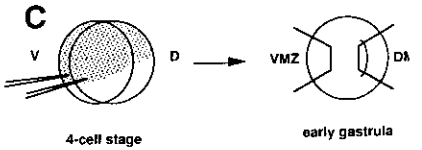
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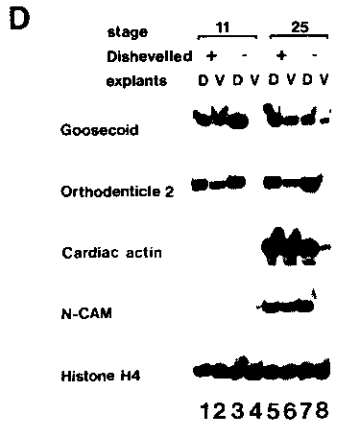
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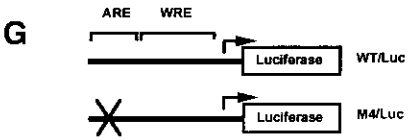
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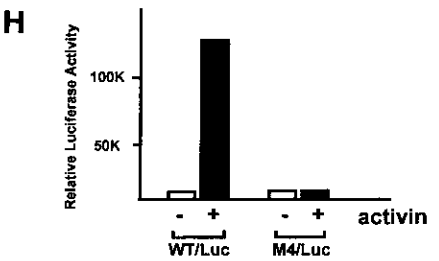
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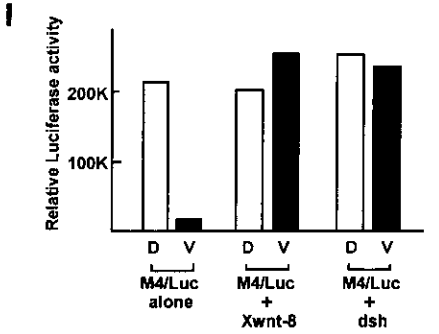
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M4/Luc + dsh

goosecoid primers (F:5′-GGACGCAGCAATGCTCG-3′; R:5′-GTGCCCACATCTGGGTAC-3′).

Reporter Gene Assays

*gsc*WT/Luc was constructed by ligating a PCR-amplified 238-bp *gsc* promoter fragment into the *Bam*HI site of the pOLuc vector. M4/Luc was constructed by PCR amplifying the same *gsc* promoter with the following oligonucleotides (F:GGATCCCAGTCAGCAGCTGACCG, R:GGATCCCAGACTGCAGTCCTCTT) and cloning into pOLuc. The animal cap and explant assays were carried out as described in the legend to Fig. 2.

RESULTS AND DISCUSSION

In order to investigate the possible involvement of *dsh* protein in *Wnt* signaling in *Xenopus*, synthetic *Drosophila dsh* mRNA was microinjected into the ventral side of 4-cell stage embryos (see Fig. 1C). Axis duplication was observed at a high frequency (49%, $n = 112$) in *dsh*-injected embryos. Although similar to the secondary axes induced by *Wnt-1* or *Xwnt8* injection, the secondary axes induced by *dsh* were not complete, as they lack the most anterior structures (see Fig. 1A). Histological examination of the *dsh* mRNA-injected embryos confirmed the presence of a secondary axis containing excess somitic mesoderm as well as secondary neural tissue (Fig. 1B). However, the induced neural tissue was often structurally disorganized and a secondary notochord was missing.

The induction of a secondary axis indicates that ectopic expression of *dsh* appears to change the cell fate of the ventral mesoderm to that of a more dorsal character. To further explore this possibility, we examined the character of *dsh*-expressing tissues using molecular assays in explant culture. Following injection of *dsh* mRNA into the ventral side of embryos, both ventral and dorsal marginal zone tissues were cultured in isolation (Fig. 1C) until the indicated stage and subjected to RT-PCR analysis (Fig. 1D). The results demonstrate that expression of *dsh* in cells of the ventral mesoderm led to the induction of marker genes (*goosecoid; gsc*, and *orthodenticle 2; Xotx2*) (Cho *et al.*, 1991; Blitz and Cho, 1995) normally expressed in the organizer (dorsal mesoderm), but not in the ventral mesoderm (Fig. 1D; compare lanes 2 and 4, and lanes 6 and 8). Cardiac actin (a marker for muscle differentiation) and N-CAM (a neural marker), whose expression were also absent in ventral control explants, were also induced in the *dsh* mRNA-injected ventral explants (Fig. 1D; compare lanes 6 and 8). These results are consistent with the idea that ectopic expression of the *dsh* protein mimics the *Wnt* signal in *Xenopus*, thereby causing the ventral mesoderm cells to adopt a dorsal mesodermal (organizer) fate (Christian *et al.*, 1992).

In addition to *Wnt*, injection of either activin or *BVg-1* mRNA (both TGF-β related molecules) can induce secondary axes (reviewed by Vize and Thomsen, 1994), presumably via their cognate serine/threonine kinase receptors. In order to demonstrate that *dsh* acts specifically in the *Wnt* signaling pathway, rather than by cross-activating TGF-β-related signaling cascades, the specificity of the *dsh* effect was investigated. Previously, it was shown that *Wnt* (*Xwnt8*) can collaborate with FGF in the induction of *Xenopus* mesoderm in explants (Christian *et al.*, 1992). Animal caps isolated from *Xenopus* embryos when cultured with bFGF alone form ventral mesoderm (Fig. 2B). However, culturing of *Xwnt8*-expressing animal caps in bFGF results in the development of mesoderm with distinctly dorsal character (Fig. 2D). Since neither bFGF nor *Xwnt8* alone (Figs. 2B and 2C) was able to induce dorsal mesoderm in isolated animal caps, it has been suggested that *Wnt* modifies the cellular response to bFGF (Christian *et al.*, 1992).

If *dsh* is involved downstream of the *Wnt* signaling pathway in *Xenopus*, *dsh* too should be able to bring about a similar synergism with bFGF. Animal cap tissues receiving only *Xwnt8* or *dsh* mRNA (up to 800 pg or 2 ng/embryo, respectively) neither elongated (Figs. 2C and 2E) nor induced mesodermal marker genes such as *gsc* or *Xbra* (*Xenopus brachyury*) (data not shown), indicating that these molecules alone were incapable of directly inducing mesoderm. However, animal cap tissue that received both *dsh* mRNA and bFGF treatment became elongated and induced dorsal mesoderm (as shown in Figs. 2E and 2F). Histological examination of FGF-treated caps microinjected with *dsh* mRNA revealed the presence of abundant segmented muscle tissue compared with that of FGF-treated caps alone (data not shown). This synergistic interaction of *Wnt* and *dsh* with bFGF is distinctly different from that of TGF-β-like growth factors such as activin and *BVg-1* that directly induce mesoderm. These observations are consistent with the notion that *dsh* is acting specifically in the *Xenopus Wnt* signaling pathway.

To further support the hypothesis that *dsh* acts in the *Xenopus Wnt* signaling pathway, we tested the effect of *dsh* on a reporter gene construct that is specifically activated by *Wnt* molecules, but not by activin. The *gsc* homeobox gene, implicated as a key regulator of vertebrate axis specification, is induced by both *Xwnt-8* and activin (Cho *et al.*, 1991; Steinbeisser *et al.*, 1993). Recently, we have succeeded in separating a *Wnt*-responsive DNA element (*WRE*) from an activin-responsive element (*ARE*) within the promoter of *gsc* (T.W., U.R., and K.W.Y.C., manuscript in preparation). Specific mutations in the ARE of the wild-type *gsc* promoter construct

(WT/Luc) provided a reporter gene construct (M4/Luc) that could no longer respond to activin (Figs. 2G and 2H).

We further examined the specific response of the M4/Luc construct toward the *Wnt* signal using a blastomere injection assay. Previously, it has been shown that *Xwnt8* expression dorsalizes the ventral blastomere and activates *gsc* (Steinbeisser *et al.*, 1993). Co-injection of *Xwnt8* mRNA with the M4/Luc construct into ventral blastomeres reveals that *Xwnt8* expression activates the M4/Luc reporter gene 5- to 10-fold higher than control blastomeres injected with reporter alone (Fig. 2I). The activation of the M4/Luc construct by *Xwnt8* in ventral tissue demonstrates the presence of a functional WRE in the M4/Luc construct that is distinct from the ARE (Fig. 2G). We then examined whether injection of *dsh* could mimic the *Wnt* signal and induce the expression of the M4/Luc reporter gene. Ventral tissues co-injected with M4/Luc and *dsh* mRNA showed 5- to 10-fold increases in reporter gene activity compared with that of reporter gene injection in the absence of *dsh* (Fig. 2I). These findings are consistent with the idea that *dsh* induces expression of the *goosecoid* promoter (M4/Luc) construct via the WRE and not via the ARE in a manner similar to that seen with *Xwnt8*.

Intracellular signaling molecules suggested to be involved in *Drosophila wg* signaling are all present in *Xenopus*. *Xgsk-3* (a *zeste-white 3/shaggy* homolog) and *β-catenin* (an *arm* homolog) genes thought to act in the cascade downstream of *wg* have been identified in *Xenopus* (Pierce and Kimelman, 1995; McCrea *et al.*, 1993). In *Drosophila*, *shaggy* encodes a cytoplasmic SER/THR kinase whose activity is antagonized by *wg* in order to propagate the signal. Consistent with this notion, a dominant-negative "kinase dead" mutant of *Xgsk-3*, equivalent to antagonized *shaggy* activity, induces secondary axes (Pierce and Kimelman, 1995). Interference with β-catenin activity also affects dorsal mesoderm. Injection of antibodies to β-catenin into *Xenopus* embryos causes the formation of double axes in *Xenopus* embryos, similar to the phenotype due to ectopic expression of *Wnts* (McCrea *et al.*, 1993). Furthermore, depletion of maternal β-catenin inhibits dorsal mesoderm formation (Heasman *et al.*, 1994). In addition to these genes, we have recently isolated a *Xenopus* homolog (*xdsh*) of *Drosophila dsh* (data not shown). The fact that the *Xdsh* gene was isolated from oocyte and gastrula cDNA libraries suggests that *Xdsh* plays an important role in early embryonic patterning.

Identification of these *Wnt* signaling components in *Xenopus*, taken together with our demonstration of the specificity of *Drosophila dsh* in *Xenopus Wnt* signaling, indicates that the entire intracellular framework of the *Wnt* signaling pathway has been conserved during evolution. The observations also reinforce the view that the discovery of *Wnt* signaling components in one species is likely to be directly relevant to understanding of the signaling pathway in distantly related species. In this regard, availability of a *Wnt*-responsive reporter gene in *Xenopus* will provide us with an excellent opportunity to examine the molecular basis of intracellular *Wnt* signal transduction events in vertebrates.

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