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Identification of Distinct Classes and Functional Domains of Wnts through Expression of Wild-Type and Chimeric Proteins in *Xenopus* Embryos

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Wnts are secreted signaling factors which influence cell fate and cell behavior in developing embryos. Overexpression in *Xenopus laevis* embryos of a *Xenopus* Wnt, Xwnt-8, leads to a duplication of the embryonic axis. In embryos ventralized by UV irradiation, Xwnt-8 restores expression of the putative transcription factor goosecoid, and rescues normal axis formation. In contrast, overexpression of Xwnt-5A in normal embryos generates defects in dorsoanterior structures, without inducing goosecoid or a secondary axis. To determine whether Xwnt-4 and Xwnt-11 fall into one of these two previously described classes of activity, synthetic mRNAs were introduced into animal caps, normal embryos, and UV-treated embryos. The results indicate that Xwnt-4, Xwnt-5A, and Xwnt-11 are members of a single functional class with activities that are indistinguishable in these assays. To investigate whether distinct regions of Xwnt-8 and Xwnt-5A were sufficient for eliciting the observed effects of overexpression, we generated a series of chimeric Xwnts. RNAs encoding the chimeras were injected into normal and UV-irradiated *Xenopus* embryos. Analysis of the embryonic phenotypes and *goosecoid* levels reveals that chimeras composed of carboxy-terminal regions of Xwnt-8 and amino-terminal regions of Xwnt-5A are indistinguishable from the activities of native Xwnt-8 and that the reciprocal chimeras elicit effects indistinguishable from overexpression of native Xwnt-5A. We conclude that the carboxy-terminal halves of these Xwnts are candidate domains for specifying responses to Xwnt signals.

The *Wnt* gene family encodes a group of cysteine-rich secreted glycoproteins implicated in cell signaling (22). Members of this family have been found in all vertebrates examined and in some invertebrates (22). The independent discovery of *Wnt-1*, a proto-oncogene of mice, and the segment polarity gene *wingless* in *Drosophila melanogaster* led to studies implicating the entire *Wnt* family as regulators of cell fate during embryonic development. Analysis of *wingless* mutants reveals that the gene is required for normal segmentation of embryos (1, 20). Mutation of both mouse *Wnt-1* alleles prevents normal development of the midbrain and cerebellum (15, 30).

Overexpression of Wnts through mRNA injection has been used to investigate Wnt activities and potential functions in *Xenopus* embryos (19). Two distinct phenotypes are observed. Injection of mRNAs encoding Xwnt-8 (4), Xwnt-3A (31), mouse Wnt-1 (16), chicken Wnt-8 (7), or the *Drosophila wingless* protein (2) leads to duplication of the embryonic axis in *Xenopus* embryos. In contrast, overexpression of Xwnt-5A affects morphogenetic movements without producing a duplication of the axis (17). UV irradiation of *Xenopus* embryos blocks formation of the embryonic blastula organizer, the Nieuwkoop center, and results in an embryo lacking dorsal axial structures (12, 25). In such embryos, overexpression of Xwnt-8 (26, 27) but not Xwnt-5A (17) mimics the Nieuwkoop center and restores normal development, rescuing expression of the putative transcription factor goosecoid (28). The distinct effects of overexpression of Xwnt-8 and Xwnt-5A in normal and UV-irradiated embryos, their distinct effects on gap junction permeability in early-stage embryos (23), and their distinct effects on activin-treated explants of embryos (19) support the hypothesis that these Xwnts work through separate mechanisms. Xwnt-11, which like Xwnt-5A is present in the egg, has been reported to represent a third class of Xwnt activity in that it partially rescues UV-irradiated embryos (11). Xwnt-4, which is normally expressed in the central nervous system, was reportedly inactive when overexpressed (14). These results suggest the existence of multiple functional classes of Wnts.

Despite the wealth of information about Wnt expression and activities, there is considerable interest in discovering potential Wnt receptors and signaling pathways (22). Apparently Wnts can provide a signal to neighboring cells, as fibroblasts expressing Wnt-1 can transform neighboring mammary epithelial cells in coculture, suggesting that a paracrine mechanism can occur (8). This suggestion has been substantiated by evidence that cells expressing Wnt-1 chimeras tethered to the plasma membrane by a transmembrane domain are still able to transform neighboring mammary epithelial cells (24). It is plausible that the activities of Wnts in cultured mammary epithelial cells (24) and in embryos (22) are attributable to activation of receptor-mediated signaling pathways by Wnts. Notch, a transmembrane protein, may interact at some level with a Wnt signaling pathway (5), but it is still unclear whether this represents a direct biochemical interaction.

In the present study, we initially investigate whether reported *Xenopus* Wnts have overlapping activities, as such information should be useful in dissecting Wnt signaling pathways and cellular responses to Wnt signals. Two functional classes are observed: Xwnt-8-like activity, displayed by Xwnt-1, -3A, and -8, and Xwnt-5A-like activity, displayed by Xwnt-4,

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-5A, and -11. We also exploit the different responses to overexpression of Xwnt-8 and Xwnt-5A of *Xenopus* embryos to identify regions of these Wnts that are sufficient for eliciting these responses. A series of chimeric Xwnts were generated, assayed for their effects on embryonic phenotype after overexpression in normal or UV-irradiated embryos, and assayed for their effects on goosecoid levels in UV-irradiated embryos. The results demonstrate that the carboxy-terminal halves of both Xwnt-8 and Xwnt-5A are sufficient for eliciting the responses of each native Xwnt.

MATERIALS AND METHODS

Wild-type plasmid construction. Subcloning of the coding sequences of p*Xwnt-4* (14) (GenBank accession number U13183) into the expression vector pSP64T was accomplished in two steps. First, the 5′ untranslated region was removed by linearizing p*Xwnt-4* at the *Sca*I site prior to ligation of a modified *Bgl*II linker (5′-AGATCTCCATGGCCCCAGAGTACT-3′) containing the ATG start codon and then isolating the entire coding region by cleavage with *Pst*I in the polylinker of pGEM2. The resulting fragment was cloned into pSP64T cut with *Bgl*II and *Pst*I to create pSP64-*Xwnt-4*. The 3′ untranslated region was removed from pSP64-*Xwnt-4* by PCR amplification of a 665-bp fragment corresponding to the 3′ portion of the *Xwnt-4* coding sequence by using the upstream primer, A.11 (5′-GAGAAATGTGGCTGTGA-3′), and the downstream primer, X4.S (5′-GGCAGATCTGGATCACCGGCATGTGTGCAT-3′; containing a stop codon followed by a *Bgl*II site). The template for amplification was the plasmid, p*Xwnt-4*. The PCR product was cleaved at the internal *Eco*RI site, gel purified, ligated to a *Bgl*II-*Eco*RI fragment from pSP64-*Xwnt-4*, and cloned into pSP64T at the *Bgl*II site. The resulting construct, pX4.2, contains the entire coding region of *Xwnt-4* cDNA flanked by β-globin 5′ and 3′ untranslated regions from pSP64T.

Xwnt-11 was initially obtained by using degenerate *Wnt* primers and PCR using oocyte cDNA (4, 31). This 450-bp fragment was used to screen an oocyte λgt10 library (generously provided by Doug Melton, Harvard University), and 12 clones were obtained. PCR primers flanking the coding region were made by using the published sequence of *Xwnt-11* (11) and were used to amplify the complete coding region of *Xwnt-11* from two different bacteriophage isolates (the primer sequences were 5′-GGGGGAGATCTCCAGTACCAATGGCTC CG and 5′-AAAAAGATCTATTATGGCTTTACTTGCA). The coding regions were subcloned into expression vector pT7ts (generously provided by Andrew Johnson and Paul Krieg, University of Texas at Austin), and the products were partially sequenced and tested independently for activity.

Construction of chimeric Xwnts. Alignment of the amino acid and nucleotide sequences of *Xwnt-8* and *Xwnt-5A* revealed the presence of two in-frame restriction sites (*Nco*I and *Pvu*II) at corresponding conserved regions in each *Xwnt* (see Fig. 4). Simple restriction enzyme digests and religation were used to generate some constructs, all of which were prepared in pSP64T to provide the 5′ untranslated sequence of β-globin, as previously employed for *Xwnt-8* (4) and *Xwnt-5A* (17). Chimeras 3 and 4 were generated by PCR-based gene splicing by the overlap extension protocol of Horton et al. (6). For each chimera, a minimal restriction fragment of the amplification products, which included the chimeric splice junction, was ligated together with appropriate portions of wild-type *Xwnt-8* or *Xwnt-5A* cDNAs to minimize sequence errors introduced by the polymerase. These cDNAs were partially sequenced around the splice junction. As shown in Fig. 4, chimera 1 contains the N-terminal half of Xwnt-8 (amino acids 1 to 183) and the C-terminal half of Xwnt-5A (amino acids 240 to 380); in contrast, chimera 2 contains the N-terminal half of Xwnt-5A (amino acids 1 to 239) and the C-terminal half of Xwnt-8 (amino acids 184 to 358). Chimera 3 has a longer N-terminal region (amino acids 1 to 260) from Xwnt-8 and a shorter C-terminal portion (amino acids 310 to 380) of Xwnt-5A than chimera 1, while chimera 4 has a longer N-terminal region (amino acids 1 to 309) from Xwnt-5A and a shorter C-terminal region (amino acids 261 to 358) from Xwnt-8 than chimera 2. In chimera 5, both the N-terminal (amino acids 1 to 239) and the C-terminal regions (amino acids 310 to 380) are derived from Xwnt-5A; however, an internal portion (amino acids 184 to 260) is from Xwnt-8. Chimera 6 contains both the N-terminal sequences (amino acids 1 to 183) and C-terminal sequences (amino acids 261 to 358) from Xwnt-8, but the internal region corresponding to the first portion of the C-terminal half (amino acids 240 to 309) is derived from Xwnt-5A. Construct 7, which contains the signal peptide and the complete C-terminal half of Xwnt-8 (amino acids 184 to 358), was generated by deleting the N-terminal half of the *Xwnt-8* sequence from the wild-type *Xwnt-8* cDNA with *Sty*I, and the rest of the plasmid was blunted with Klenow DNA polymerase and was then religated. The DNA sequence at the junction site was confirmed by DNA sequencing.

In vitro transcriptional and translational analyses. Capped mRNAs were transcribed from linearized DNA template with an SP6 or T7 RNA polymerase in vitro transcription kit (mMESSAGE mMACHINE SP6 or T7; Ambion, Austin, Tex.) according to the manufacturer's instructions. DNA template removal and the isolation of the mRNA product were carried out as described by McMahon and Moon (16). The integrity of the transcribed RNAs was investigated by separating RNAs on formaldehyde–1.5% agarose gels. To investigate whether each chimera produced a translatable product of the expected size, synthetic RNAs were translated in vitro in a rabbit reticulocyte lysate and the products were separated on a sodium dodecyl sulfate-polyacrylamide gel.

Microinjection of RNAs. RNA was microinjected into the marginal zone of the dorsal or ventral two cells of four-cell-stage embryos. When noted, embryos were irradiated at the vegetal pole during the first cell cycle (4, 25) and RNA was injected into the marginal zone before the first cleavage. While approximately a 10-nl volume per embryo was injected, the mRNA amounts used for injections were varied for each construct in order to reach a threshold that produced a phenotype. Thereafter, increased concentrations were tested in multiple experiments to determine whether chimeras that yielded only a partial axis effect would, at increased mRNA doses, yield a different effect. All the results presented for multiple experiments over a range of RNA doses below 1 ng per embryo were consistent, and injection of increased amounts of control RNAs such as bovine prolactin or β-galactosidase had no effects on development. Injected embryos were incubated in either 5% Ficoll in 0.1× MBS (18) overnight or 5% Ficoll in 1× MBS for 2 h before being transferred into 0.1× MBS for continuous culture, as described previously (18). UV-treated embryos were scored according to the dorsoanterior index used by Kao and Elinson (9) (0, completely ventralized; 5, normal embryo; and 10, maximal dorsalization).

RNA isolation and Northern blot analysis. RNA was extracted from a pool of 10 embryos (stage 10.5) by the guanidine method described by Chomczynski and Sacchi (3). The RNA equivalent to three embryos was analyzed by Northern (RNA) blot with a ^{32}P-labeled antisense RNA probe for *goosecoid*. The same blots were stripped and reprobed with a ^{32}P-labeled DNA probe for EF-1α to compare the amounts of RNA loaded.

Animal cap assays. Embryos were maintained in 0.1× MBS solution until stage 8 to 8.5. The animal cap region was dissected away with an eyebrow hair knife and baby hair loop in 1× MBS before being transferred into a dish with a base of 1% agar and 0.5× MBS, with or without activin at a concentration necessary to induce notochord formation (approximately 40 ng/ml; doses varied from batch to batch). Caps were grown until the controls reached stage 25 before they were scored for extension and fixed for histology. Embryos were staged according to the system of Nieuwkoop and Faber (21).

Antibody labeling. Embryos were fixed and labeled according to the protocol of Moon and Christian (18). Horseradish peroxidase 12/101 staining was enhanced by using 0.01% $NiSO_4$ and 0.01% $CoCl_2$ during the peroxidase reaction. Mouse monoclonal 12/101 antibody was generously provided by Chris Kintner (Salk Institute, San Diego, Calif.). Mouse monoclonal neural cell adhesion molecule (NCAM) antibody (6F11) was generously provided by William Harris (University of California at San Diego). Horseradish peroxidase goat anti-mouse secondary antibody (Zymed) was used.

Histology. For histological analysis, embryos were fixed in 3.7% formaldehyde overnight at room temperature and then kept in phosphate-buffered saline at 4°C. The fixed embryos were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin eosin by the method of Kelly et al. (10) or with Janus Green and Neutral Red.

RESULTS

Animal caps expressing wild-type Xwnts. Xwnt-5A has been shown previously to inhibit the extension of animal caps treated with activin, without interfering with the induction of dorsal mesodermal cell types in the explants (17, 19). In contrast, Xwnt-8 enhances the extension of animal caps treated with basic fibroblast growth factor or low concentrations of activin (19). Xwnt-4 and Xwnt-11 were tested for their effects on this type of morphogenetic movement to determine whether they resembled a known Wnt activity. At the two-cell stage, both embryonic blastomeres were injected with either Xwnt-4 or Xwnt-11. Animal caps were isolated at stage 8, and half were placed in medium containing activin A. When control embryos reached stage 25, the caps were scored for extension and then fixed for histology. The presence of a notochord was determined by the morphology in the section. Xwnt-4 and Xwnt-11 affected the extension of caps in a manner nearly identical to that seen for Xwnt-5A (Fig. 1). Extension was inhibited, but differentiation of tissue into notochord was still seen in the unextended caps (Table 1). Animal caps not treated with activin appear the same as uninjected controls and form atypical epidermis. We conclude that Xwnt-4, -5A, and -11 inhibit the morphogenetic movements induced in animal caps by activin without blocking the ability of activin to induce

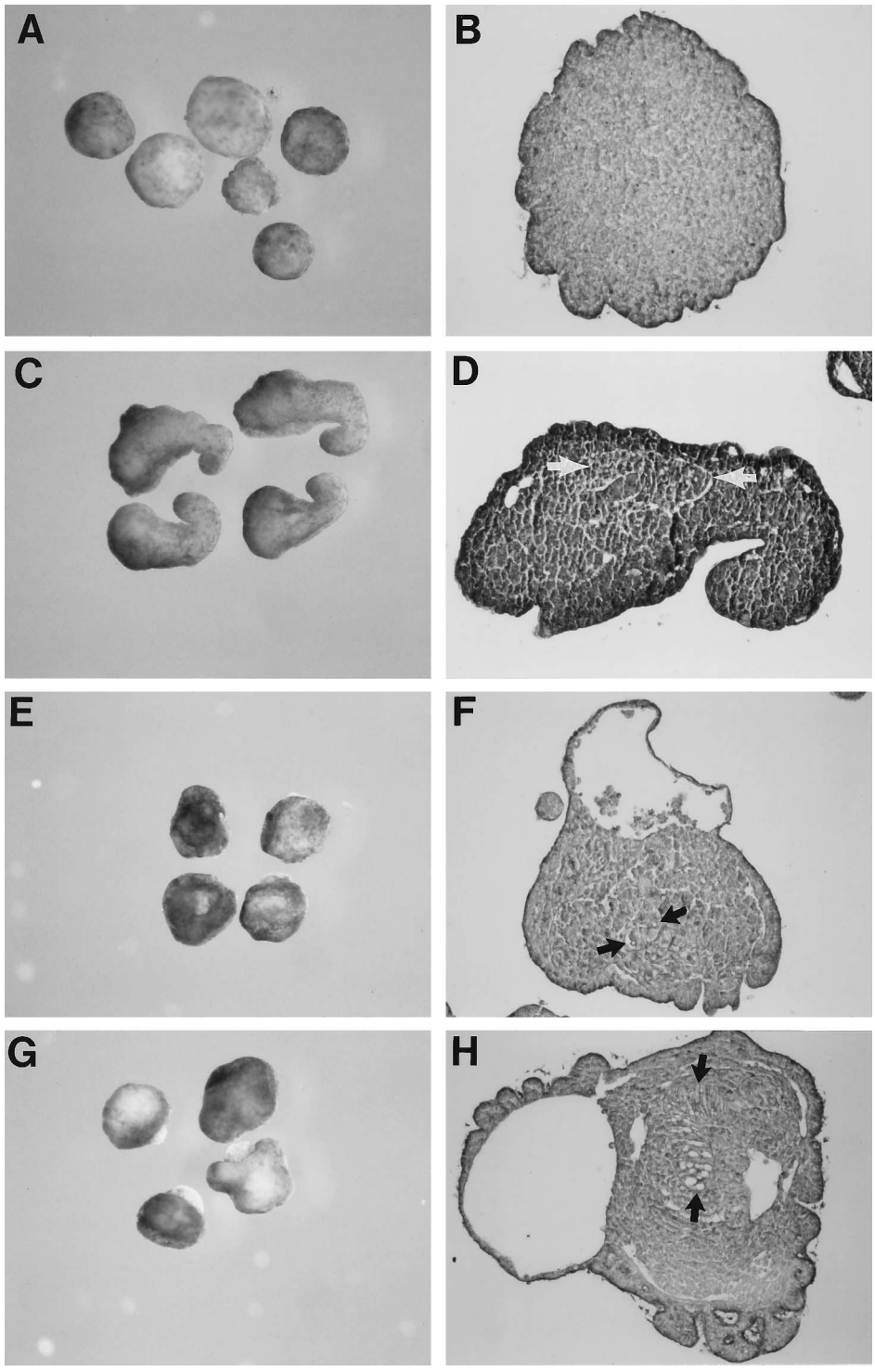

FIG. 1. Effects of overexpression of Xwnt-4 and Xwnt-5A on the differentiation and activin responsiveness of animal caps. (A and B) Control animal caps. (C and D) Control caps treated with activin. (E and F) Animal caps treated with activin from embryos injected with *Xwnt-4* RNA. (G and H) Animal caps treated with activin from embryos injected with *Xwnt-11* RNA. The arrows bracket the notochord. Untreated injected caps resemble untreated control caps.

notochordal tissue. None of these Xwnts directly induce any tissue types identifiable by histological analysis.

Overexpression of wild-type Xwnts in normal embryos. Xwnt-5A was previously found to inhibit the extension of the embryonic axis when injected dorsally and to have little effect when injected ventrally (17). Given the similarity between Xwnt-5A, -4, and -11 in the animal cap assay described above, we extended the comparison by injecting embryos with RNA encoding either Xwnt-4 or Xwnt-11 at the four-cell stage into both dorsal or ventral blastomeres. The amount of RNA in-

TABLE 1. Animal caps from embryos injected with wild-type *Xwnt* RNAs with and without activin treatment

Treatment	No. of caps[a]	Effect on caps	
		% Extended	% With notochord
Control	63	0	0
Control + activin	65	74	75
Xwnt-4	79	0	0
Xwnt-4 + activin	95	29	64
Xwnt-11	49	0	0
Xwnt-11 + activin	58	14	59
Xwnt-5A	10	0	0
Xwnt-5A + activin	10	0	40

[a] *Total number of caps in each group.*

jected, approximately 500 pg, is 50 times that required to induce an axis duplication with Xwnt-8 but still well below the dose at which toxic effects are seen. In dorsally injected embryos, severe shortening of the axis, identical to that reported for Xwnt-5A, was seen (Fig. 2). Occasionally, anterior neural structures such as the eyes are reduced but sections reveal that the amount of notochord is unaffected (data not shown). Ventral injections can sometimes produce a thickening of tissue near the ventral blastopore accompanied by increased pigmentation, but sectioning reveals no identifiable structures. In about 10% of ventrally injected embryos, the ventral thickening stains with 12/101 antibody, indicating some level of muscle differentiation (data not shown). Whether this result is due to disruption of convergence to the dorsal side of prospective somitic tissue or induction of new dorsal tissue is not known. We conclude that overexpression of Xwnt-4, overexpression of Xwnt-5A, and overexpression of Xwnt-11 have comparable effects on the phenotypes of embryos.

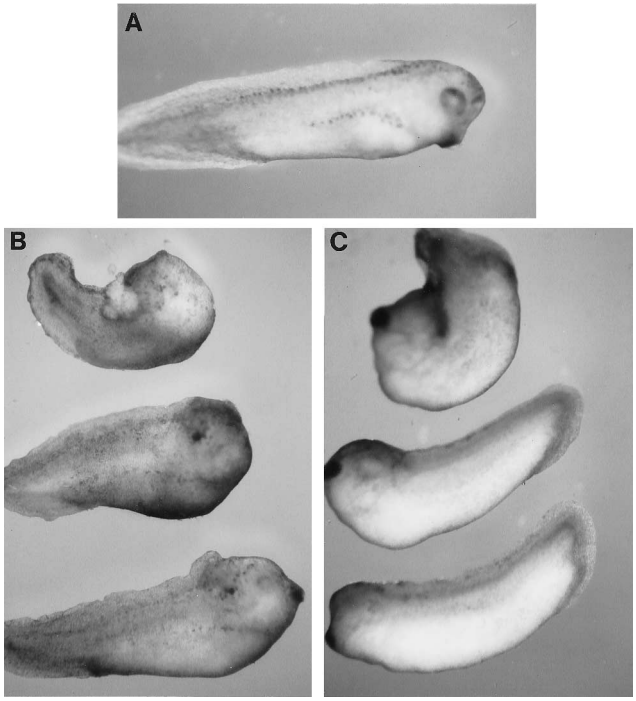

FIG. 2. Effects of overexpression of Xwnt-4 and Xwnt-11 on normal development. (A) Control embryo. (B) *Xwnt-4* RNA-injected embryos. (C) *Xwnt-11* RNA-injected embryos.

Expression of wild-type Xwnts in ventralized embryos. UV light can be used to prevent the formation of dorsal axial structures (12, 25). These ventralized embryos can be rescued by several methods, including injection of *Xwnt-8* RNA. Xwnt-8 completely rescues the axis, including all dorsal and anterior structures. Xwnt-11 has been reported to cause a partial rescue of the axis when injected at a higher dose (11), without induction of notochord or head structures. Xwnt-5A, scored by different molecular markers, and Xwnt-4, encoded by transcripts with substantial untranslated regions, were previously reported not to have axis-rescuing ability (14, 17). We retested Xwnt-5A and Xwnt-4, after modification of the latter to remove the untranslated flanking regions, for axis-rescuing ability, using doses similar to that needed to induce partial rescue with Xwnt-11. Embryos were treated with UV light during the first cell cycle, and then a single blastomere was injected with either Xwnt-4, Xwnt-5A, or Xwnt-11 at the four-cell stage. Embryos were fixed for whole-mount antibody staining for the muscle and neural markers analyzed for Xwnt-11 (11). Comparable results were obtained with all three constructs, and we conclude that each can partially rescue axial structures in embryos, as was evident by the presence of muscle and neural staining (Fig. 3). Histological analyses demonstrate that none of these Xwnts fully rescue UV-irradiated embryos as Xwnt-8 does, nor do they restore formation of a notochord (data not shown). The average rescue score is low: 0.2 to 0.3 on the dorsoanterior index scale. Five to fifteen percent of the embryos showed a substantial degree of rescue, including visibly segmented somites, and most of the embryos showed no rescue of dorsal structures.

Phenotypes of embryos expressing chimeric Xwnts. Given the above evidence that all available full-length *Xenopus* Wnts display one of two distinct activities, we turned to investigating whether representative members of each class of activity could be dissected in order to identify putative functional domains that activate either of the two Wnt responses observed in embryos. Six cDNAs encoding Xwnt-5A and Xwnt-8 chimeras were generated as shown in Fig. 4. In the chimeras, homologous regions of *Xwnt-5A* and *Xwnt-8* were swapped by using conserved restriction sites or a PCR-based methodology. This homologous swapping minimized the likelihood of drastic changes in the protein conformation of the chimeras. Chimeric cDNAs generated by PCR were partially sequenced to ensure that the proper reading frame was conserved across the splice junction. In this process, a 1-nucleotide error in the originally reported sequence of *Xwnt-8* was discovered. The correct DNA sequence, which has been submitted to GenBank, introduces an earlier stop codon, such that the predicted translation product is 29 amino acids shorter than that originally reported by Christian et al. (4).

To test which chimeric Xwnts displayed the activities of either Xwnt-5A or Xwnt-8, mRNA transcribed from these constructs was injected into the marginal zone of both dorsal or both ventral blastomeres of four-cell embryos. The injected embryos were cultured to hatching stage 35/36 (21) and were scored for phenotype (Table 2; Fig. 5). Chimera 2 (Fig. 5A), with the carboxy-terminal half of Xwnt-8; chimera 4 (Fig. 5B), with relatively less of Xwnt-8 in the carboxy-terminal region; and chimera 6 (Fig. 5A), with an Xwnt-8 amino terminus, a middle region of Xwnt-5A, and a carboxy terminus of Xwnt-8 the same as that of chimera 4 were all able to induce the formation of a second axis when injected into the ventral marginal zone. As noted for injection of native *Xwnt-8* RNA, the axial duplication occurs after injection into the ventral but not

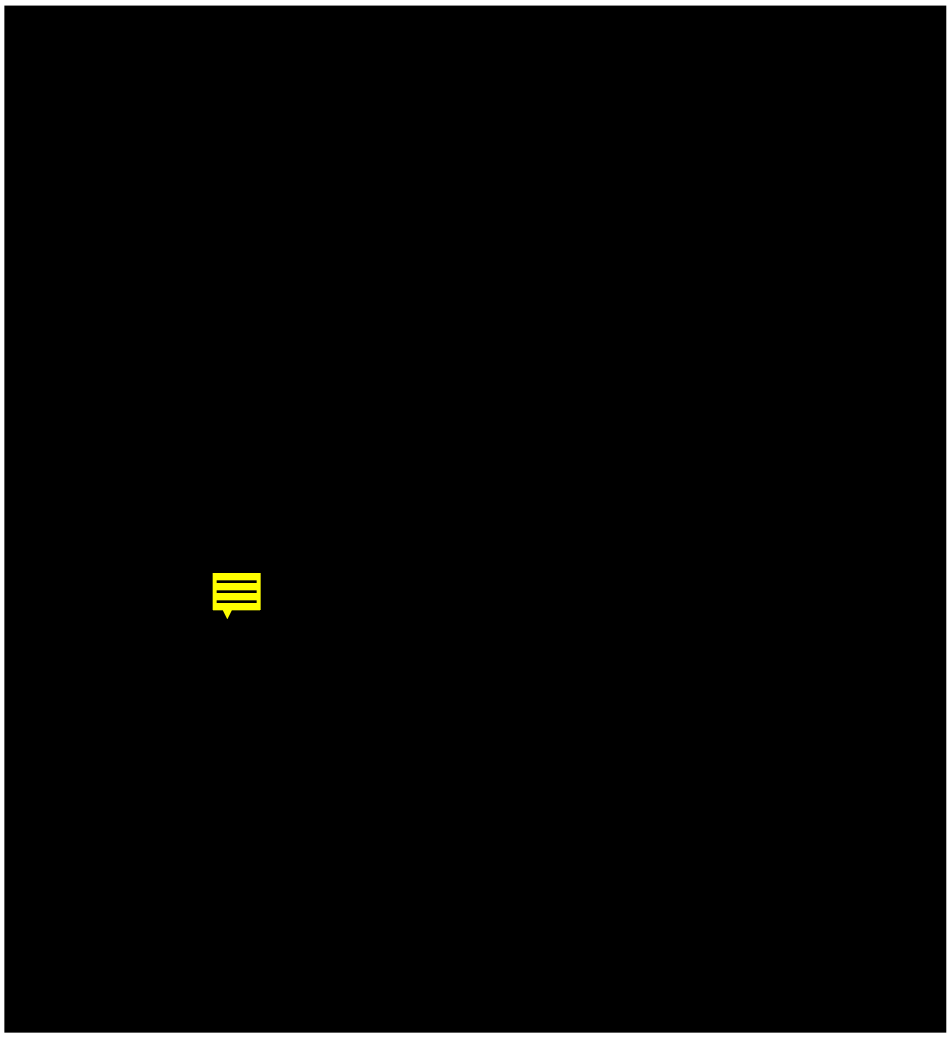

FIG. 3. Effects of overexpression of Xwnt-4, Xwnt-5A, and Xwnt-11 on the development of UV-irradiated embryos. (A) Control (top) and UV-irradiated (bottom) embryos labeled with the muscle-specific antibody 12/101 (black) and the neural tissue-specific NCAM antibody (brown). (B) *Xwnt-4* RNA-injected UV-irradiated embryos. (C) *Xwnt-5A* RNA-injected embryos. (D) *Xwnt-11* RNA-injected embryos. n, neural tissue; s, somitic tissue.

into the dorsal marginal zone (27) (Table 2). The few axis duplications observed following dorsal injection are most likely due to an inability to assign prospective dorsal and ventral sides with 100% accuracy. In contrast, chimeras 1 and 5, with Xwnt-5A in the carboxy terminus, could not induce a second axis after injection into the ventral marginal zone. Embryos injected ventrally with these chimeras had a small thickening of tissue near the ventral blastopore but otherwise were indistinguishable from uninjected controls (Fig. 5C). Injection of these chimeras into the dorsal marginal zone produced a phenotype similar to that observed following injection of wild-type Xwnt-5A (17) (Fig. 5D). Interestingly, the phenotype of embryos injected with chimera 3, without the extreme carboxy terminus of Xwnt-8, differed from those obtained with other chimeras. Histological analysis of these embryos revealed that they developed additional notochords (data not shown), suggestive of an Xwnt-8-like activity.

Although chimeras 2, 3, 4, and 6 each contained some Xwnt-8 in the carboxy half and led to duplication of the embryonic axis after injection into the ventral marginal zone, there were nevertheless differences in the activities of these chimeras, even after each chimera was tested over a wide range of RNA concentrations. Chimeras 2 and 6 were as active as intact Xwnt-8 in inducing an anteriorly complete second axis. In contrast, chimera 4 could induce only a partial axis, which consisted of mainly posterior structures and lacked anterior structures (Fig. 5B). For chimera 4, axial induction required injection of approximately 100 times more RNA than that required for induction by chimera 2, 3, 6, or wild-type Xwnt-8. As these data indicated that parts of the carboxy-terminal region of Xwnt-8 are necessary to induce a secondary axis, we turned to addressing whether the carboxy half was sufficient for this activity in isolation. A construct (construct 7) consisting of the initiation methionine, the signal peptide, and the C-terminal half of Xwnt-8 was prepared and tested as described above. We found that this construct did not have any activity (Table 2), indicating that the carboxy-terminal half of Xwnt-8 was by itself insufficient for eliciting a duplication of the axis.

Effects of chimeric Xwnts on ventralized embryos. As injection of *Xwnt-8* but not *Xwnt-5A* RNA can mimic the Nieuw-

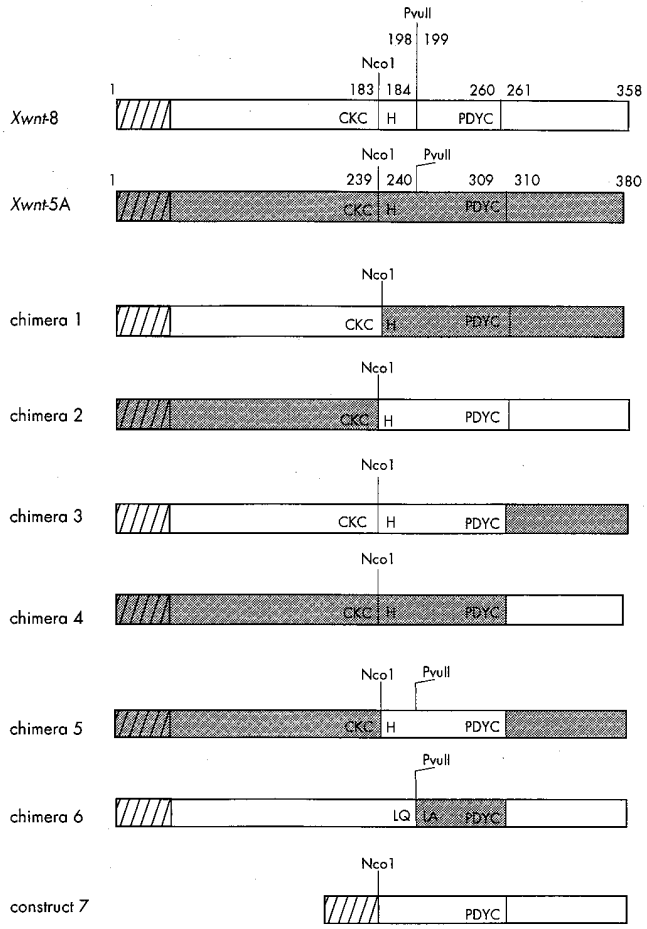

FIG. 4. Structures of the *Xwnt-5A*/*Xwnt-8* chimeric constructs. ▨, signal peptide of Xwnt-8; ▨, signal peptide of Xwnt-5A; □, mature protein of Xwnt-8; ▨, mature protein of Xwnt-5A. The numbers above each construct indicate the total number of amino acids in Xwnt-5A and Xwnt-8, and the labeled restriction enzyme sites indicate some junction sites. The amino acid sequences (CKCH and PDYC) at the two conserved homologous regions are indicated. The amino acid sequences LQ and LA in chimera 6 flank the *Pvu*II site.

TABLE 2. Axis induction in normal embryos by chimeric *Xwnt* mRNAs[a]

Chimera and injection site	No. of embryos scored	Embryos with phenotype (%)	
		Xwnt-8	Xwnt-5A
1 DMZ	128	0	46
1 VMZ	112	0	8
2 DMZ	25	8	0
2 VMZ	22	77	0
3 DMZ	ND	ND	ND
3 VMZ	125	96	0
4 DMZ	33	0	0
4 VMZ	26	50	0
5 DMZ	92	0	62
5 VMZ	97	0	6
6 DMZ	26	4	0
6 VMZ	140	65	0
7 DMZ	43	0	0
7 VMZ	37	0	0

[a] Normal embryos were injected with mRNAs encoding Wnt chimeras. RNA was injected into the marginal zone of either the dorsal or ventral two cells of four-cell-stage embryos. Tailbud embryos (stage 35/36) from these injected embryos were scored for the Xwnt-8 phenotype (4) or the Xwnt-5A phenotype (17). DMZ, dorsal marginal zone; VMZ, ventral marginal zone; ND, not determined.

koop center activity and restore normal embryonic development to UV-irradiated embryos (19), we employed this assay as a further test of whether the carboxy half of an Xwnt is sufficient for its activity. RNAs transcribed from chimeras 1 to 6, as well as Xwnt-8 or Xwnt-5A, were injected into UV-irradiated embryos (Fig. 6). As summarized in Table 3, chimeras 2 (Fig. 6B), 3 (Fig. 6C), 4 (Fig. 6D), and 6 (Fig. 6F) (all with some Xwnt-8 sequence in the carboxy-terminal half) rescued dorsal axial structures in the UV-ventralized embryos to different degrees, while chimeras 1 (Fig. 6A) and 5 (Fig. 6E), like Xwnt-5A, did so to only a small extent in some of the injected embryos. Embryos injected with chimeras 2 and 6 had normal axes (Fig. 6B and F), and histological examination revealed well-developed dorsal structures, including auditory vesicle, neural tube, and notochord in some of these embryos (Fig. 7B and F). Chimera 4 could partially rescue UV-ventralized embryos insofar as the embryos develop a normal posterior dorsal axis, but these embryos do not develop anterior dorsal structures such as eyes or cement glands (Fig. 6D). Histological analysis confirmed that these embryos had notochord, neural tube, and somite but lack eyes, prosencephalon, and mesencephalon (Fig. 7D). Interestingly, embryos injected with chimera 3 clearly had an axial structure with both notochord and neural tube (Fig. 6C and 7C). However, these embryos usually died after neurulation over a wide range of RNA doses in multiple experiments.

These results are consistent with the data from the normal embryos injected with these chimeras. Chimeras 2 and 6, which can induce a second axis in normal embryos, can restore the axis in UV-irradiated embryos. While chimera 4, which could induce only a partial axis in UV-irradiated embryos, could induce only a partial second axis in normal embryos. The partial axis induced by chimera 4 was mainly the posterior axis, containing neural tube and notochord but lacking anterior structures. Stewart and Gerhart (29) have shown that the extent of anterior development in *Xenopus* embryos depends on the strength of the gastrula organizer. Therefore, it may be that chimera 4 can induce only a small organizer, possibly due to poor stability of the expressed chimera, or to low-affinity binding to the putative receptor. In support of this speculation, we found that there was no activity of chimera 4 when a low dose of mRNA was injected, while the same dose of *Xwnt-8* RNA was very effective. The partial axis induction activity of chimera 4 could be produced only when high doses of mRNA were injected, while this amount of *Xwnt-8* RNA is two orders of magnitude greater than that producing a threshold effect yielding a complete axis.

Effects of chimeric Xwnts on expression of *goosecoid* in ventralized embryos. It has previously been noted that UV irradiation of *Xenopus* embryos blocks the normal expression of zygotically transcribed *goosecoid* and that injection of *Xwnt-8* (27) or *Xwnt-1* (19) RNA mimics the Nieuwkoop center in restoring expression of *goosecoid*. We exploited these observations to provide an independent assay of whether the carboxy-terminal region of Xwnt-8 was necessary for Xwnt-8 activity. UV-ventralized embryos were injected with different chimeras, and RNA was isolated at gastrula stage 10.5, blotted, and probed for *goosecoid* transcripts. As shown in Fig. 8, chimeras 2, 3, and 6 increased the expression of *goosecoid*, while chimera 4 induced much less, if any, *goosecoid* expression. As predicted, chimeras 1 and 5, which could not rescue UV-ventralized embryos, did not induce *goosecoid* expression.

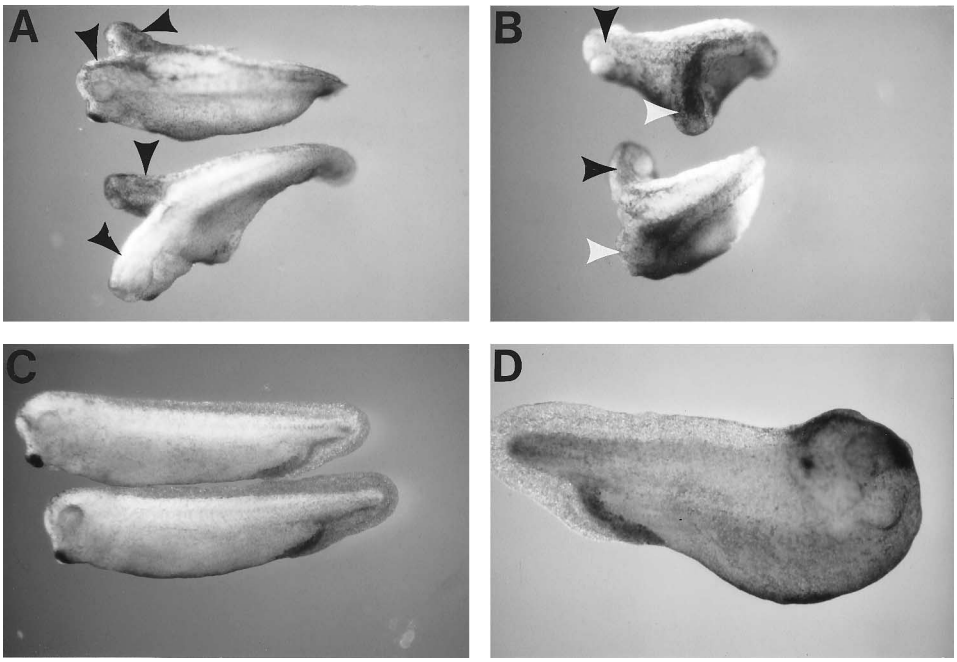

FIG. 5. Representative phenotypes of embryos injected with chimeric *Xwnt* mRNAs. Normal embryos at the four-cell stage were injected in the dorsal or ventral marginal zones with chimeric *Xwnt* mRNAs. (A) Ventral injection of Xwnt-8 or chimera 2 or 6. (B) Ventral injection of chimera 4. (C) Wild-type embryos, dorsal injection of Xwnt-8, dorsal injection of chimera 2, 4, or 6, ventral injection of Xwnt-5A, or ventral injection of chimera 1 or 5. (D) Dorsal injection of Xwnt-5A or chimera 1 or 5. Chimera 3 is not shown (see text). A complete axis with eyes is indicated by a black arrowhead, while an incomplete axis is denoted by a white arrowhead.

DISCUSSION

Wnts are developmental signaling molecules likely to act in autocrine and paracrine pathways (22), which may involve *Notch* signaling (5). Although *Wnt*s constitute a sizable multigene family (22), there has been little evidence of whether many or all of these genes are functionally interchangeable. On the basis of the results reported here, at least two classes of Wnt activity exist in vertebrates. In the most comprehensive comparison of murine Wnt activities to date, Wong et al. (32) have shown that Wnts fall into three categories defined according to their ability to transform mammary epithelial cells. Wnt-4, Wnt-5A, and Wnt-6 failed to induce transformation, while Wnt-1, Wnt-3A, and Wnt-7A were highly transforming. Our *Xenopus* assays show that Xwnt-1, -3A, and -8 all elicit the same responses when overexpressed in embryos (19), indicating agreement between the cell transformation assay and the embryonic assay for Wnt-1 and Wnt-3A. However, the lack of demonstrable activity for Wnt-4 and Wnt-5A in the cell transformation assay (32) does not define a class based on demonstrable activity and it is noteworthy that both of these Wnts, as well as Xwnt-11, are members of an active functional class in *Xenopus laevis*. All three Xwnts block the elongation of blastula caps treated with activin without interfering with the induction of mesodermal cell types, interfere with morphogenetic movements when overexpressed in normal embryos, and have a limited ability to rescue muscle and neural tissue (but not notochord) in UV-irradiated embryos. Wong et al. (32) identified a third group of Wnts (Wnt-2, Wnt-5B, and Wnt-7B) that induce transformation of mammary epithelial cells at a low frequency. Since we have not tested any of the *Xenopus* homologs of these Wnts, we cannot rule out the possibility of a third class of activity in *Xenopus* embryos.

On the basis of our prior evidence that overexpression of Xwnt-8 and Xwnt-5A elicits different responses in developing embryos (reviewed in the introduction), we exploited these differences to determine whether a specific region of Xwnt-8 was necessary for eliciting various responses in embryos. We prepared chimeras of Xwnt-8 and Xwnt-5A, injected RNAs encoding these chimeras into normal or UV-irradiated *Xenopus* embryos, and scored the embryos on the basis of phenotypes, histology, and induction of *goosecoid* expression. These experiments revealed that the N-terminal half of Xwnt-8 could be replaced with that of Xwnt-5A while the chimeric protein still maintained all the activities of Xwnt-8. The reciprocal chimeras, though scored subjectively, indicate that chimeras with the amino terminus of Xwnt-8 and the carboxy terminus of Xwnt-5A behave like Xwnt-5A. These data, in addition to RNA blot analyses of *goosecoid* induction, suggest that the C-terminal regions of these Xwnts determine the activity of Xwnt in the embryos.

Does the amino-terminal half of a Wnt play a role in observed Wnt activity? Mason et al. (13) reported that mutating the cysteine residue at position 143 in the amino-terminal half of Wnt-1 destroyed its transformation activity. However, in chimeras 1 and 2, we did not observe the amino-terminal half of a chimeric Xwnt acting in a dominant manner to determine the activity of the Xwnt. However, the amino-terminal half of an Xwnt is still necessary for Xwnt activity, since a construct of the initiation methionine, leader sequence, and carboxy-terminal half was translatable in vitro yet was inactive in vivo. Moreover, since the amino-terminal half in chimera 3 (Xwnt-8) converts the phenotype of chimera 5 (Xwnt-5A phenotype) to a Xwnt-8 phenotype, provided that some of the carboxy-terminal half is also derived from Xwnt-8, apparently the amino-terminal half can exert some influence on activity. It is therefore likely that the amino-terminal region is required for the stability or for folding of Wnts. Since several conserved cysteine residues are located within the amino-terminal region, these may participate in disulfide bond formation with cysteine

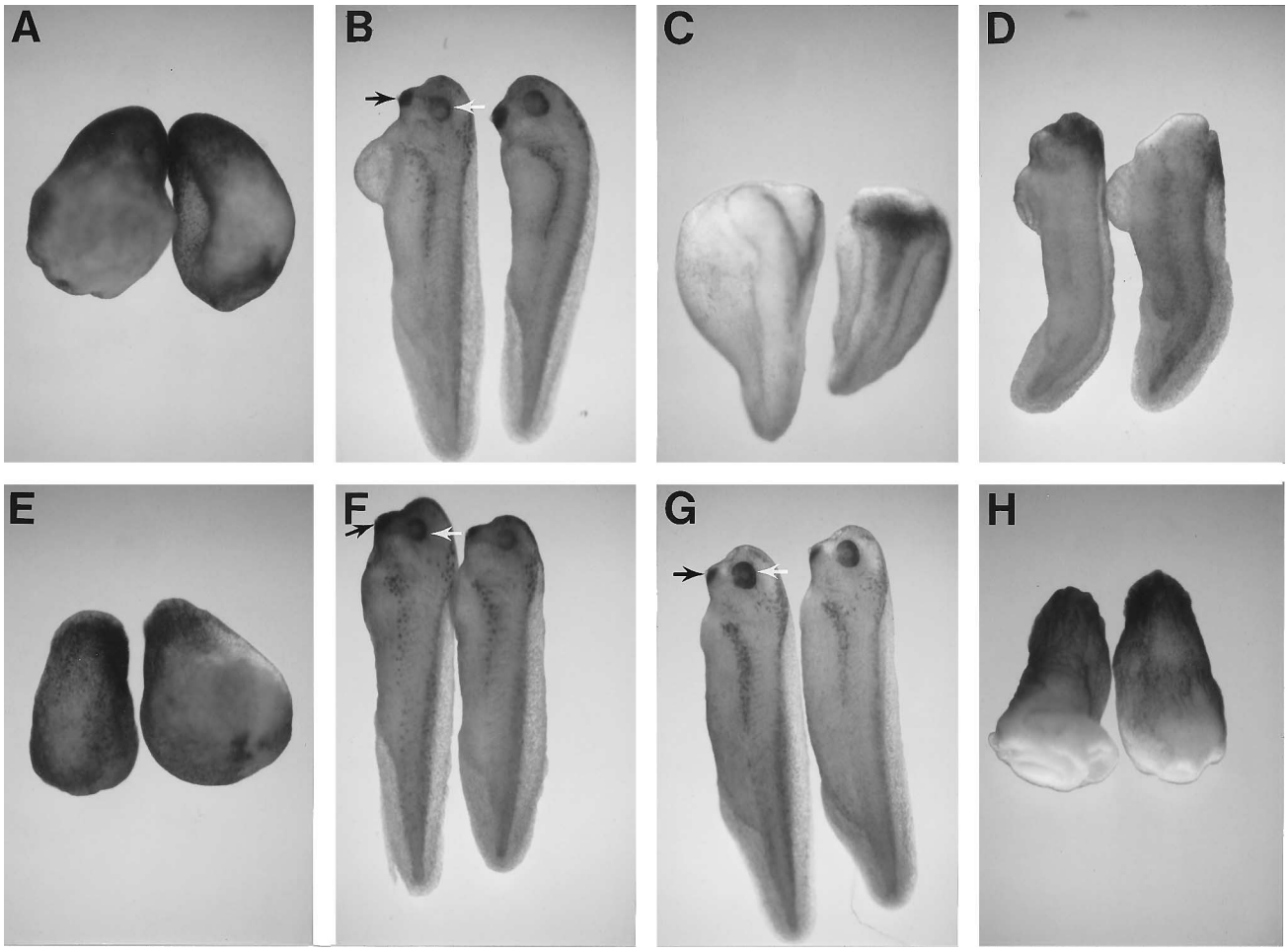

FIG. 6. Representative phenotypes of UV-irradiated embryos injected with chimeric *Xwnt* mRNA. Embryos were irradiated with UV light and were then injected with chimeric *Xwnt* mRNAs. UV-irradiated embryos were injected with chimera 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), or 6 (F). (G) Normal embryos (no UV treatment and no injection). (H) Uninjected UV-irradiated embryos. The cement gland is indicated by a black arrow, and the eye is indicated by a white arrow.

residues in the C-terminal region, which would be consistent with the significance of cysteine 143 in Wnt-1 (13).

Our finding that the carboxy half of Xwnt protein conveys specificity to Wnt responses is consistent with that of McMahon and Moon (16), who employed deletions to demonstrate that carboxy-terminal regions of murine Wnt-1 are required for activity. Our findings are also pertinent to the data of Parkin et al. (24), who reported that Wnt-1 could function as a transmembrane protein. These investigators tethered Wnt-1 to the cell membrane by fusing it to transmembrane protein CD4 or CD8. They found that Wnt-1 located at the cell surface could still transform cells in an autocrine and paracrine fashion. However, the activity could only be maintained if there was a large space between the Wnt-1 C terminus and the transmembrane domain of CD4 or CD8. The transforming activity of the membrane-bound Wnt-1 was reduced dramatically when the Wnt-1 carboxy terminus was directly linked to the transmembrane domain of CD4 or CD8. Thus, our data showing that the response-activating region of Wnts is largely an activity of the carboxy-terminal half suggests that some of the chimeras of Parkin et al. (24) may have interfered with the Wnt activities they scored.

In a range of assays, it was apparent that chimeras based on amino- versus carboxy-terminal halves of Xwnts displayed the

TABLE 3. Axis rescue in UV-irradiated embryos by chimeric *Xwnt* mRNAs[a]

Chimera	No. of embryos scored	No. of embryos with DAI score (stage 35/36) of:					Avg DAI score
		0	1	2	3	4–5	
1	60	23	28	9			0.77
Control	57	46	5	6			0.30
2	43	2	1	4	16	20	3.42
Control	42	34	6	2			0.24
3[b]	55						
Control	117	107	7	3			0.11
4	104	3	17	58	18	8	2.10
Control	120	102	5	5	8		0.33
5	59	47	6	4	2		0.34
Control	60	38	15	7			0.48
6	20	0	1	1	8	10	3.65
Control	31	23	8				0.26

[a] UV-irradiated embryos were injected with mRNAs encoding Wnt chimeras before the first cleavage. The DAI (dorsoanterior index) scores of stage 35/36 embryos were calculated by the method of Kao and Elinson (9). Uninjected UV-irradiated embryos were used as controls.

[b] Lethal treatment (embryos died at the neurula stage).

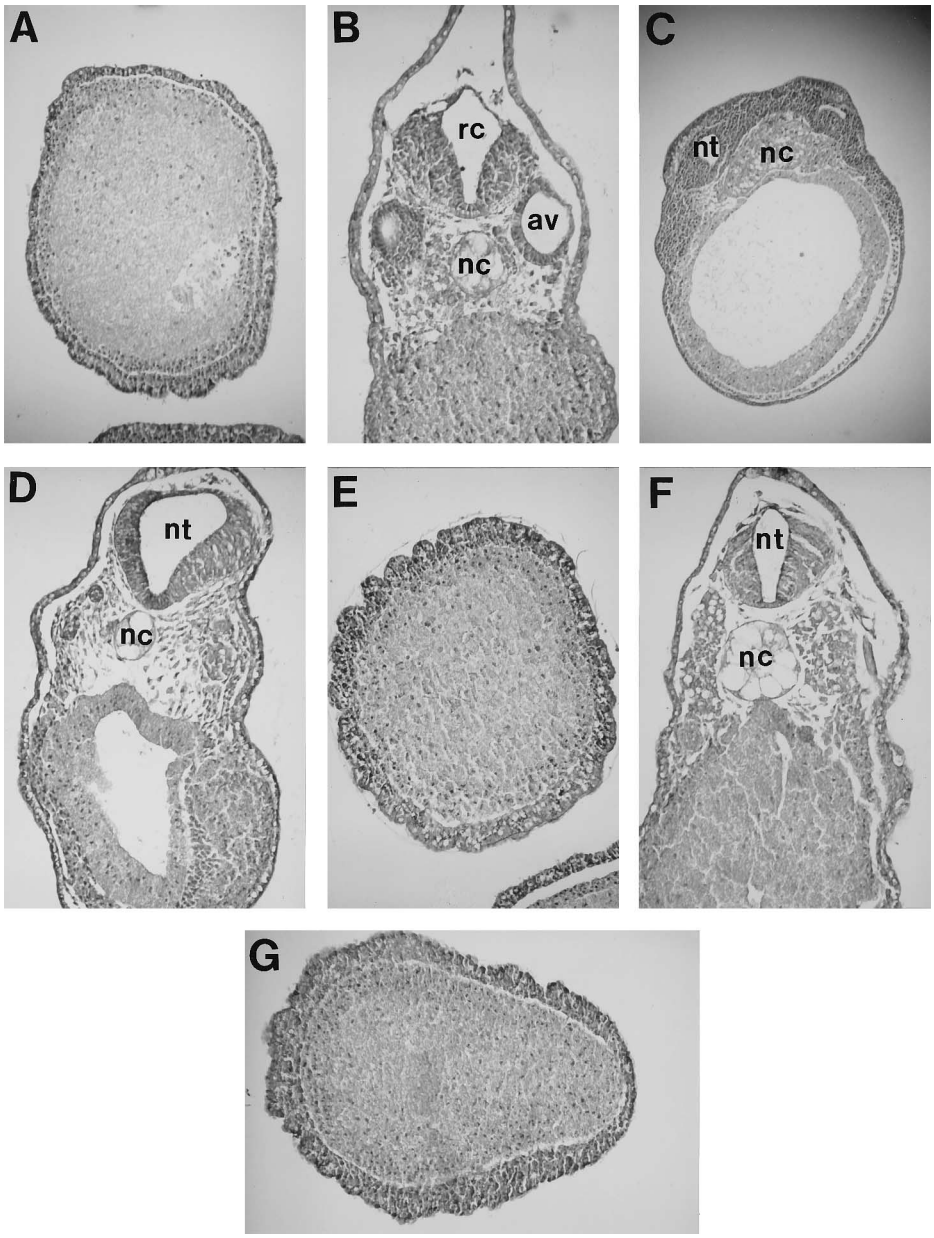

FIG. 7. Histological analysis of UV-irradiated embryos injected with different chimeric *Xwnt* RNAs. All transverse sections shown were from stage 35/36 embryos except for chimera 3, which was sectioned at the neurula stage. UV-irradiated embryos were injected with chimera 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), or 6 (F). (G) Uninjected UV-irradiated embryo. The rhombencephalon (rc), the neural tube (nt), the notochord (nc), and the auditory vesicle (av) are indicated.

activities of the carboxy-terminal half. As we used multiple assays for Xwnt-8 activity, we employed chimeras 3 to 6 in an attempt to define the position of putative receptor-activating domains within the carboxy-terminal half of Xwnt-8. While this may have been a useful goal in principle, the results were inconclusive. Chimera 4, which contains the last 98 amino acids of Xwnt-8 (260 to 358), could only partially rescue axis formation in UV-ventralized embryos and unlike Xwnt-8 could not rescue *goosecoid* induction in these embryos. Nevertheless, analysis of chimera 4 and the fact that Xwnt-5A rescues no notochord in UV-irradiated embryos suggest that the carboxy third of a Wnt predominantly determined its activity. However, results with the reciprocal construct of Xwnt-5A and Xwnt-8, chimera 3, containing the amino-terminal two-thirds of Xwnt-8

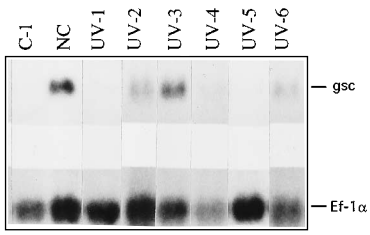

FIG. 8. Northern blot analysis indicating *goosecoid* expression in UV-irradiated embryos injected with chimeric *Xwnt* RNAs. UV-irradiated embryos were injected at the one-cell stage, and total RNA was extracted from stage 10.5 embryos. RNA from three embryos was used for Northern analysis, probed with a *goosecoid* (gsc) riboprobe, and then reprobed for a control RNA, EF-1α. RNA samples of UV-irradiated embryos injected with chimeras 1 to 6 are labeled UV-1 to UV-6, while a representative uninjected, UV-irradiated control, is indicated by C-1 and normal control embryos are denoted NC.

and the carboxy third of Xwnt-5A dispel this idea. Before the embryos died at the neurula stage, this chimera, like Xwnt-8, led to supernumerary notochords in both normal and UV-irradiated embryos and to increased *goosecoid* levels in UV-irradiated embryos. It is tenable that the activity of chimera 2 restricts Xwnt-8 activity to between Xwnt-8 amino acids 184 and 358 and that the activity of chimera 3 further restricts it to between 184 and 260, since the remainder of the carboxy-terminal region of chimera 3 consists of Xwnt-5A. Chimera 5 directly tests Xwnt-8 amino acids 184 to 260 in an Xwnt-5A background, but this chimera, like Xwnt-5A, does not rescue an axis or *goosecoid* expression in UV-irradiated embryos. In comparing the activities of chimeras 3 and 5, we observed that the amino-terminal region can indeed influence the effects of the chimera on axis formation and on *goosecoid* expression. Chimera 6 is the reciprocal of chimera 5, and it behaves like Xwnt-8.

Our comparison of the activities of Xwnt-5A, Xwnt-4, and Xwnt-11, clearly shows that all three belong to the same functional class, which is distinct from the type of activity observed with Xwnt-8, Xwnt-3A, and Xwnt-1. We conclude from the analysis of all the chimeras that elements in the carboxy-terminal halves of Xwnt-8 and Xwnt-5A are necessary for eliciting the respective Xwnt-8 or Xwnt-5A activities. In some chimeras, the amino-terminal region can still influence the overall activity of the protein but not in a dominant manner if the entire carboxy-terminal half is derived from one Wnt. The identification of the carboxy-terminal half of Wnts as being necessary but not sufficient for eliciting distinct Wnt responses in *Xenopus* embryos should be useful in further dissecting the mechanisms by which cells and embryos respond to Wnt signals.

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The first three authors contributed equally to this work.

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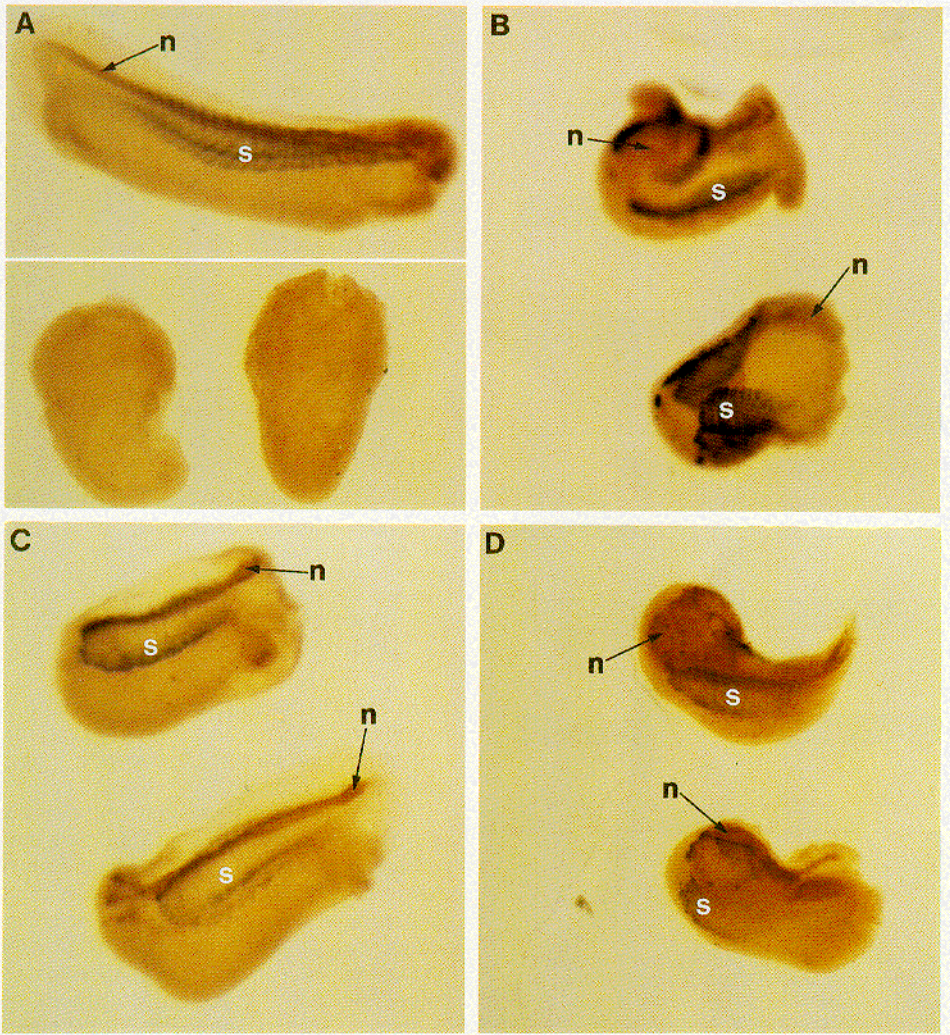
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