Activities of the *Wnt*-1 Class of Secreted Signaling Factors Are Antagonized by the *Wnt*-5A Class and by a Dominant Negative Cadherin in Early *Xenopus* Development

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Abstract. When overexpressed in *Xenopus* embryos, *Xwnt*-1, -3A, -8, and -8b define a functional class of *Wnts* (the *Wnt*-1 class) that promotes duplication of the embryonic axis, whereas *Xwnt*-5A, -4, and -11 define a distinct class (the *Wnt*-5A class) that alters morphogenetic movements (Du, S., S. Purcell, J. Christian, L. McGrew, and R. Moon. 1995. *Mol. Cell. Biol.* 15:2625–2634). Since some embryonic cells may be exposed to signals from both functional classes of *Wnt* during vertebrate development, this raises the question of how the signaling pathways of these classes of *Wnts* might interact. To address this issue, we coexpressed various *Xwnts* and components of the *Wnt*-1 class signaling pathway in developing *Xenopus* embryos. Members of the *Xwnt*-5A class antagonized the ability of ectopic *Wnt*-1 class to induce *goosecoid* expression and a secondary axis. Interestingly, the *Wnt*-5A class did not block *goosecoid* expression or axis induction in response to overexpression of cytoplasmic components of the *Wnt*-1 signaling pathway, *β-catenin* or a kinase-dead *gsk-3*, or to the unrelated secreted factor, *BVg1*. The ability of the *Wnt*-5A class to block responses to the *Wnt*-1 class may involve decreases in cell adhesion, since ectopic expression of *Xwnt*-5A leads to decreased Ca^{2+}-dependent cell adhesion and the activity of *Xwnt*-5A to block *Wnt*-1 class signals is mimicked by a dominant negative *N-cadherin*. These data underscore the importance of cell adhesion in modulating the responses of embryonic cells to signaling molecules and suggest that the *Wnt*-5A functional class of signaling factors can interact with the *Wnt*-1 class in an antagonistic manner.

Wnt genes encode a family of secreted glycoproteins that are expressed in restricted temporal and spatial patterns during embryonic development and are involved in intercellular signaling (for review see McMahon, 1992; Nusse and Varmus, 1992; Moon, 1993). Recently, *Wnt* genes have been grouped into functional classes based on assays performed in *Xenopus* embryos (Du et al., 1995; Moon et al., 1993*b*) and transformation assays carried out with mammalian cell lines (Wong et al., 1994). Ectopic expression of *Xenopus Wnt* (*Xwnt*)[1]-1, -3A, -8, and 8b (the *Wnt*-1 class) (for review see Du et al., 1995; Moon et al., 1993*b*; Cui et al., 1995) induces a secondary axis and rescues Nieuwkoop center activity in embryos ventralized by UV irradiation. In contrast, ectopic expression of members of the *Wnt*-5A class, including *Xwnt*-5A, -4, and -11, inhibits morphogenetic movements during gastrulation without overtly altering cell fate (Du et al., 1995; Moon et al., 1993*a*, *b*). Mouse *Wnt* genes have been grouped in a similar manner, with *Wnt*-1, -3A, and -7A transforming mammary epithelial C57MG cells at a high frequency and *Wnt*-4 and -5A deficient in transforming activity (Wong et al., 1994). These independent studies support the existence of multiple functional classes of *Wnts*.

Much progress has been made in identifying downstream components of the signaling pathway stimulated by the products of *Wnt*-1 class, although no receptor has been reported. Epistasis studies in *Drosophila* have demonstrated that in response to signaling by *wingless*, the ortholog of vertebrate of *Wnt*-1 (Rijsewijk et al., 1987), activation of *dishevelled* (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994) leads to the inhibition of *zeste-white 3*, the ortholog of vertebrate *glycogen synthase kinase-3* (Siegfried et al., 1992). This results in activation of *armadillo*, the ortholog of vertebrate *β-catenin*

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1. *Abbreviations used in this paper*: *gsc*, *goosecoid*; MBT, midblastula transition; PI, phosphatidylinositol; RT, reverse transcription; *Xwnt*, *Xenopus Wnt*.

(Klingensmith et al., 1989; Peifer and Wieschaus, 1990; Riggleman et al., 1990), which is repressed by *zeste-white 3* in the absence of a *wingless* signal. Assays in vertebrates support the involvement of related genes in *Wnt*-1 class signaling, as injection of RNAs encoding vertebrate *dishevelled* (Sokol et al., 1995), *β-catenin* (Funayama et al., 1995; Guger and Gumbiner, 1995; Kelly et al., 1995), and kinase-dead, putative dominant negative forms of *gsk*-3 (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995) into developing embryos mimics the overexpression of members of the *Wnt*-1 class. Conversely, Heasman et al. (1994) demonstrated that the depletion of maternal *β-catenin* transcripts leads to the development of *Xenopus* embryos that lack dorsal structures and are nonresponsive to ectopic *Xwnt*-8. These results support the involvement of these molecules in a common signaling pathway.

Since *β-catenin* is both necessary (Heasman et al., 1994) and sufficient (Guger and Gumbiner, 1995; Kelly et al., 1995) for eliciting all embryonic responses previously reported for the *Wnt*-1 class (Moon et al., 1993*b*), considerable work has focused on this prospective signaling molecule. Besides its involvement in *Wnt* signaling, β-catenin is also a cadherin-associated cytoplasmic protein (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989). However, increases in cadherin-dependent cell adhesion without overexpression of *β-catenin* are not sufficient to induce *Wnt*-like responses in *Xenopus* embryos (Detrick et al., 1990; Fujimori et al., 1990; Levine et al., 1994; Guger and Gumbiner, 1995). Thus, β-catenin is likely to have signaling properties independent of its role in cell adhesion. Consistent with modulation of non–membrane-associated β-catenin by components of the *Wnt*-1 class signaling pathway, we have recently found that endogenous β-catenin in *Xenopus* embryos is present in nuclei as well as at the plasma membrane, and that the ratio of β-catenin in the nucleus relative to the membrane is increased by a dominant negative *Xgsk-3* (Yost, C., M. Torres, J.R. Miller, E. Huang, D. Kimelman, and R.T. Moon, manuscript submitted for publication).

Although increased cell adhesion does not elicit responses that mimic *Wnt* signaling, there have nevertheless been several indications that both the *Wnt*-1 class and the *Wnt*-5A class may lead to changes in cell adhesion. For example, transfection of *Wnt*-1 into PC12 pheochromocytoma cells elevates levels of E-cadherin, as well as the *β-catenin*–related protein plakoglobin, while increasing calcium-dependent cell adhesion (Bradley et al., 1993). Similarly, in both AtT20 and C57MG cells transfected with *Wnt*-1, increases were observed in the accumulation of β-catenin and plakoglobin and in calcium-dependent cell adhesion (Hinck et al., 1994). Finally, *Xwnt*-5A has been reported to reduce cell mixing in gastrula-stage embryos (Moon et al., 1993*b*), but these experiments do not directly address whether this involved changes in cell adhesion.

Although comparisons of the patterns of expression of

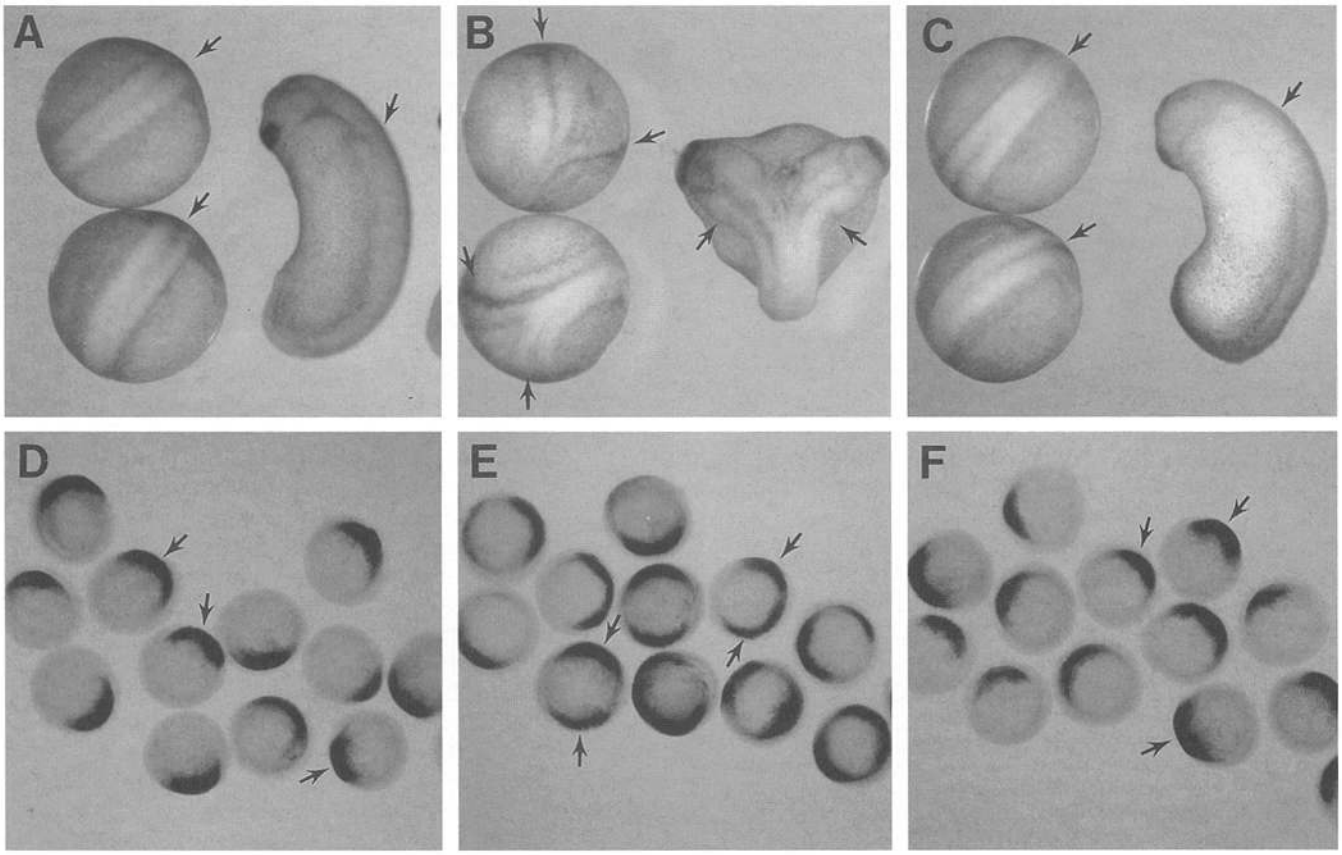

Figure 1. *Xwnt*-5A antagonizes *Wnt*-1 class activity. (*A*) Control embryos possess single axes at stage 25. (*B*) Embryos injected with *prolactin* followed by *Xwnt*-8 RNA have duplicated axes. (*C*) Embryos injected with *Xwnt*-5A followed by *Xwnt*-8 RNA have single axes. (*D*) Uninjected stage 10 control embryos possess a single site of *gsc* expression. (*E*) Embryos injected with *prolactin* followed by *Xwnt*-8 RNA display multiple sites of *gsc* expression. (*F*) Embryos injected with *Xwnt*-5A followed by *Xwnt*-8 RNA have a single site of *gsc* expression. (*Arrows*) Axes (*A*, *B*, and *C*) and sites of *gsc* expression (*D*, *E*, and *F*).

Wnts in both mouse (Parr et al., 1993; Takada et al., 1994) and *Xenopus* (for review see Moon, 1993) reveal that the two functional classes are generally expressed in distinct regions of the embryo, in situ hybridization data suggest that cells in some embryonic structures receive signals from both classes of *Wnts* (see Discussion). These overlapping patterns of expression, along with the ability of *Wnts* to signal in a paracrine manner beyond the cells in which they are expressed, raise the question of how the presence of both the *Wnt*-1 and *Wnt*-5A functional classes might affect responding cells.

In the present study we investigated whether there are synergistic or dominant interactions between the *Wnt*-5A and -1 class signaling pathways by coexpressing members of both classes together and with other signaling factors in embryos of *Xenopus laevis*. We report that injection of RNAs encoding members of the *Wnt*-5A class antagonizes the embryonic responses to ectopic expression of the *Wnt*-1 class, and that this may in part be mediated by reducing cell adhesion.

Materials and Methods

Expression Constructs

Xwnt-4 (Du et al., 1995; McGrew et al., 1992), *Xwnt*-5A (Moon et al., 1993*a*), *Xwnt*-8, *Xwnt-8myc* (Christian et al., 1991), *Xwnt*-11 (Du et al., 1995), *noggin* (generated by PCR from published sequence) (Lamb et al., 1993), and bovine *prolactin* (a gift of P. Walter, University of California at San Francisco) and β-galactosidase (a gift of D. Turner and R. Rupp, Fred Hutchinson Cancer Research Center, Seattle, WA) cDNAs were used in an SP64T expression vector (Krieg and Melton, 1984) as described previously. *BVg1* was used in the SP64T3 vector (Thomsen and Melton, 1993). *Xwnt*-8b (Cui et al., 1995), a kinase-dead *Xgsk-3* (Pierce and Kimelman, 1995), and *Xenopus β-catenin* cDNA (the latter generated by Yost, C., M. Torres, J.R. Miller, E. Huang, D. Kimelman, and R.T. Moon [manuscript submitted for publication] by PCR amplification from the published sequence of McCrea et al., 1991) were subcloned into the CS2$^+$ expression vector (a gift of D. Turner and R. Rupp, Fred Hutchinson Cancer Research Center, Seattle, WA). *N-cadherin* cDNA (a gift of C. Kintner, Salk Institute, La Jolla, CA) (Detrick et al., 1990) was inserted into the pT7TS expression vector (a gift of A. Johnson and P. Krieg, University of Texas, Austin), and we generated the clone *ΔN-cadherin* as described (Kintner, 1992). All cDNAs were transcribed using Message Machine in vitro transcription kits (Ambion Inc., Austin, TX).

Embryological Methods and RNA Injections

Eggs and embryos of *Xenopus laevis* were manipulated, microinjected, and cultured as previously described (Moon and Christian, 1989). Open-faced dorsal lip explants were dissected from stage 10 embryos (Keller, 1991) that had been injected with RNAs as listed in Table V and allowed to elongate in Danilchick's modified medium (Sater et al., 1993) for 6-8 h until sibling embryos reached stages 13 to 15. For the cell adhesion assays, 1–3 ng of *Xwnt*-5A, *Xwnt*-8, *N-cadherin*, or *ΔN-cadherin* RNA was injected into the marginal zone of both dorsal blastomeres of a four-cell embryo.

To test the effects of *Xwnt*-5A class members on signaling by other proteins, the marginal zone of both ventral blastomeres of four-cell embryos was first injected with 1–3 ng of RNAs encoding members of the *Xwnt*-5A class, wild-type *N-cadherin*, *ΔN-cadherin*, *prolactin*, or *β-galactosidase*.

Table I. Effects of the Wnt-5A Class and Dominant Negative N-cadherin on gsc Inducing and Axis Duplicating Activity by the Wnt-1 Class, β-Catenin, Kinase-dead Xgsk-3, noggin, and BVg1

RNAs injected*	Sites of *gsc* expression‡‖			Axis formation§‖			
	Single	Double	*n*	Single	Double	Dorsalized	*n*
Uninjected	100	0	150	100	0	0	456
Xwnt-8+*prolactin*	17	83	109	11	55	34	353
Xwnt-8+*Xwnt*-5A	100	0	53	77	20	3	173
Xwnt-8+*Xwnt*-4	–	–	–	88	12	0	59
Xwnt-8+*Xwnt*-11	98	2	47	80	20	0	45
Xwnt-8b+*prolactin*	–	–	–	65	34	1	76
Xwnt-8b+*Xwnt*-5A	–	–	–	83	8	9	64
k.d.*Xgsk*-3+*prolactin*	–	–	–	27	45	29	94
k.d.*Xgsk*-3+*Xwnt*-5A	–	–	–	38	51	11	88
β-catenin(1 ng)+*prolactin*	–	–	–	39	50	11	38
β-catenin+*Xwnt*-5A	–	–	–	17	61	22	18
Xwnt-8+*ΔNcad*	100	0	49	–	–	–	
Xwnt-8+*Ncad*	16	84	61	14	86	0	37
β-catenin(0.15 ng)+*prolactin*	64	36	67	–	–	–	–
β-catenin(0.15 ng)+*ΔNcad*	92	8	66	–	–	–	–
β-catenin(0.5 ng)+*prolactin*	60	40	68	–	–	–	–
β-catenin(0.5 ng)+*ΔNcad*	64	36	59	–	–	–	–
noggin+*prolactin*	18	82	49	–	–	–	–
noggin+*Xwnt*-5A	96	4	48	–	–	–	–
noggin+*ΔNcad*	100	0	39	–	–	–	–
BVg1+*prolactin*	25	75	55	70	14	16	63
BVg1+*Xwnt*-11	30	70	56	58	17	25	53
BVg1+*ΔNcad*	38	62	52	–	–	–	–

*RNA encoding *prolactin* (3 ng); *Xwnt*-5A, *Xwnt*-4, or *Xwnt*-11, (1 ng); wild-type *N-cadherin* (3 ng) or *ΔN-cadherin* (3 ng) were injected into the marginal zone of both ventral blastomeres of four-cell embryos. Subsequently, RNAs encoding *Xwnt*-8 (0.1 ng), *Xwnt*-8b (0.7 ng), *β-catenin* (0.15 ng, 0.5 ng, or 1 ng), kinase-dead *Xgsk-3* (2 ng), *noggin* (2.5 ng), or *BVg1* (0.1 ng) were injected into a single ventral marginal cell of 16-cell embryos. These latter doses were determined by arriving at the lowest threshold dose that elicits an axis duplication in the majority of embryos with that specific RNA.

‡Sites of *gsc* expression were monitored at the gastrula stage by whole mount in situ hybridization.

§Stage 25 embryos were scored by visual examination as having a single embryonic axis, duplicated axes, or a dorsalized phenotype, which occurs in response to high levels of *Wnt*-1 class signaling.

‖Numbers refer to percentages; *n*, number of embryos.

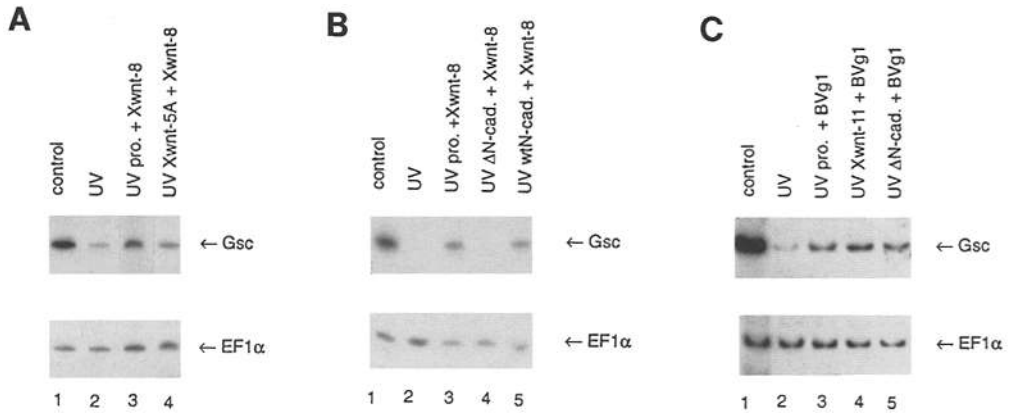

Figure 2. Detection of *gsc* transcripts by RT-PCR in stage 10 embryos ventralized by UV irradiation. (*A*) *Xwnt*-5A inhibits the rescue of *gsc* expression by *Xwnt*-8. (*B*) *ΔN-cadherin* blocks the rescue of *gsc* expression by *Xwnt*-8. (*C*) Neither *Xwnt*-11 nor *ΔN-cadherin* block the rescue of *gsc* expression by *BVg1*. (*A*, *B*, and *C*; lane *1*) Control embryos. (*A*, *B*, and *C*; lane *2*) Uninjected embryos UV irradiated to eliminate endogenous *gsc* expression. (Lane *3*) UV-irradiated embryos injected with *prolactin* followed by *Xwnt*-8 RNA (*A* and *B*) or *prolactin* followed by *BVg1* RNA (*C*). (Lane *4*) UV-irradiated embryos injected with *Xwnt*-5A (*A*), *ΔN-cadherin* (*B*), or *Xwnt*-11 (*C*) RNA followed by *Xwnt*-8 (*A* and *B*) or *BVg1* (*C*) RNA. (Lane *5*) UV-irradiated embryos injected with wild type *N-cadherin* (*B*) or *ΔN-cadherin* RNA (*C*) followed by *Xwnt*-8 (*B*) or *BVg1* RNA (*C*). All *EF1a* lanes serve as controls for RT-PCR (see Materials and Methods).

This was followed by injection of 0.1–2 ng of *Xwnt*-8, *Xwnt-8myc*, *Xwnt*-8b, kinase-dead *Xgsk-3*, *β-catenin*, *noggin*, or *BVg1* RNAs into a single ventral marginal cell of sixteen-cell embryos. We titrated levels of RNAs encoding *Xwnt*-1 class members, kinase-dead *Xgsk-3*, *β-catenin*, *noggin*, and *BVg1* to give comparable embryonic responses such as the induction of secondary embryonic axes. Moreover, we used doses of RNA to give responses in under 100% of embryos to insure that the levels of ectopic expression of these proteins were just sufficient to elicit comparable embryonic responses. Identical procedures were carried out in embryos exposed to UV irradiation for 2.5 min to block the formation of endogenous dorsal structures (Du et al., 1995).

We tested whether the block of the *Xwnt*-1 class by the *Xwnt*-5A class required their expression in the same cells by injecting 0.1 ng *Xwnt*-8 RNA into a single ventral marginal blastomere at the 16-cell stage, followed by 1 ng *Xwnt*-5A or control *prolactin* RNA at the 32-cell stage into each blastomere adjacent to the *Xwnt*-8–injected one. We also investigated whether the effect of *Xwnt*-5A on the elongation of gastrula open face dorsal lip explants required *Xwnt*-5A to be overexpressed in all cells by injecting rhodamine dextran ($>10^6$ M_r) (Molecular Probes, Eugene, OR), mixed with either *β*-galactosidase RNA as a control or with *Xwnt*-5A RNA, into a single dorsal cell at the four-cell stage. The entire dorsal lip was then explanted as above and analyzed by fluorescence microscopy using a cooled CCD camera (Hamamatsu Corp., Middlesex, NJ) on a microscope (FXA; Nikon Inc., Garden City, NY), with image analysis using Metamorph 2.0 software (Universal Imaging Corp., West Chester, PA).

To examine the interaction of *Xwnt*-4 and *Xwnt*-8 expressed after midblastula transition (MBT), 0.2 ng of *Xwnt*-4 RNA and 0.1 ng of *CSKA-Xwnt*-8 DNA (Christian and Moon, 1993) were injected into both dorsal blastomeres of a four-cell–stage embryo.

Cell Adhesion Assays

Dorsal lip explants were excised from stage 10 embryos and dissociated to a single cell suspension in 0.1× Ca^{2+}/Mg^{2+}-free Modified Barth's Solution (MBS) (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes, pH 7.5) for 2 h in 35-mm petri dishes coated with 1% agarose in the same buffer. Cells were subsequently reaggregated at room temperature in MBS with 4 mM Ca^{2+} and horizontal rocking for 15 and 30 min, followed by fixation with 3.7% formaldehyde in 0.1× MBS. Cell reaggregation was analyzed by photographing random cell populations from each treatment and scoring blind for cell clumping. Cell adhesion is operationally defined in this study as the ability of dissociated cells to reaggregate in the presence of Ca^{2+}. We assayed for decreased cell adhesion by analyzing the relative number of large (greater than eight cells) and small (five to eight cells) cell clumps formed in the reaggregation assays relative to control experiments.

In situ Hybridization and Immunocytochemistry

Stage 10 gastrula embryos were processed for in situ hybridization as described (Harland, 1991) using probes for *goosecoid* (*gsc*) (Cho et al., 1991), *Xbra* (Smith et al., 1991), and *F-spondin* (a gift from A. Klar and T. Jessel, Columbia University, New York). Stage 10 gastrula embryos injected with *Xwnt-8myc* RNA and stage 25 tailbud embryos injected with *Xwnt*-4 RNA and *CSKA-Xwnt*-8 DNA were processed for whole mount immunostaining to detect the myc and the Tor 70 epitopes, respectively (Christian and Moon, 1993). Whole mount X-gal staining was performed as described in Sanes et al. (1986).

Analysis of RNA by Reverse Transcription (RT) PCR

RNA was extracted from stage 10 gastrula embryos as described in Chomczynski and Sacchi (1987). 5 μg RNA was used to generate cDNA with first strand synthesis kits (Life Sciences, Inc., St. Petersburg, FL). To control for DNA contamination, we omitted reverse transcriptase from the synthesis reactions. RT-PCR was used as described to detect *gsc*, *EF1a* (Hemmati-Brivanlou et al., 1994), and *histone 4* (Cui et al., 1995) transcripts. Control *EF1a* and *histone 4* cDNAs were amplified 20 cycles, and exponential amplification was verified by taking an aliquot of the PCR amplification reaction every third cycle (data not shown). *gsc* was amplified 24 cycles under conditions that generally yield exponential amplification (Hemmati-Brivanlou et al., 1994; unpublished observations). The RT-PCR data presented are qualitative, and any nonexponential amplification would result in reducing any reported differences between samples. All RT-PCR experiments were reproducible in completely independent experiments.

Results

The Wnt-5A Class Antagonizes Embryonic Responses to the Wnt-1 Class

We first injected RNAs encoding members of both classes

Table II. Ectopic Xwnt-5A Antagonizes Ectopic Wnt-1 Class Activity after Injections into Distinct Cells‡

RNAs injected*	Single axis	Double axis	Dorsalized	*n*
Uninjected	99	1	0	205
Xwnt-8	5	74	21	170
Xwnt-8+*prolactin*	2	91	7	58
Xwnt-8+*Xwnt*-5A	50	47	3	90

*Single blastomeres at the ventral marginal zone of 16-cell–stage embryos were injected with 0.1 ng of *Xwnt*-8 mRNA, followed by 1 ng of *prolactin* or *Xwnt*-5A mRNA into each of the surrounding ventral marginal blastomeres at the 32-cell stage.
‡Numbers refer to percentages; *n*, number of embryos.

of *Wnts* to determine the effect of coexpression in *Xenopus* embryos. As a control, *prolactin* RNA was injected into the marginal zone of the two ventral blastomeres at the four-cell stage, followed by injection of RNA encoding *Xwnt*-8 or *Xwnt*-8b into a single ventral marginal blastomere at the 16-cell stage. As expected, this resulted in the induction of a secondary axis (Fig. 1 *B* and Table I), since members of the *Wnt*-1 class mimic the Nieuwkoop center activity (Cui et al., 1995; Smith and Harland, 1991; Sokol et al., 1991). When RNAs encoding *Xwnt*-5A, -4, or -11 (members of the *Wnt*-5A class) (Du et al., 1995) were injected instead of the *prolactin* RNA, induction of a secondary axis by subsequent injection of *Xwnt*-8 RNA was blocked in most embryos (Fig. 1 *C* and Table I). These results suggest that the *Wnt*-5A class antagonizes the ability of the *Wnt*-1 class to induce a secondary axis in *Xenopus* embryos.

Since ectopic sites of *gsc* expression are induced by the *Wnt*-1 class (Christian and Moon, 1993), we next asked whether this response to *Wnt*-1 class signaling was also blocked by the *Wnt*-5A class. Whole mount in situ hybridization analysis showed that members of the *Wnt*-5A class greatly reduced the incidence of ectopic induction of *gsc* expression in the ventral marginal zone (Fig. 1 *F* and Table I), whereas injection of *prolactin* RNA did not interfere with this activity of the *Wnt*-1 class (Fig. 1 *E* and Table I). Similarly, the *Wnt*-5A class inhibited the induction of *gsc* by the *Wnt*-1 class in UV-irradiated embryos lacking endogenous *gsc* expression, as monitored by RT-PCR (Fig. 2 *A*). Fig. 2, *B* and *C*, is described in subsequent sections and addresses questions related to how *Xwnt*-5A might reduce *gsc* expression and whether *Xwnts* can affect *gsc* expression induced by *BVg1*.

Additional control experiments support the specificity of the above results. We insured that the *Wnt*-5A class RNAs injected at the four-cell stage were not competing for translation with X*wnt*-8 by injecting RNA encoding *Xwnt*-11 or *prolactin* at the four-cell stage, followed by RNA encoding *Xwnt*-8 with a c-*myc* epitope tag at the 16-cell stage. After fixing embryos at the gastrula stage and processing them for whole mount immunostaining for the c-*myc* epitope, we found that overexpression of neither *prolactin* nor *Xwnt*-11 had an appreciable effect on *Xwnt-8-myc* expression (data not shown). To control for mosaic expression of exogenous RNAs, we coinjected *Xwnt*-5A and *β-galactosidase* RNAs at the four-cell stage, followed by *Xwnt-8-myc* at the 16-cell stage. We found that the expression of the proteins encoded by the injected RNAs overlap in 99% of the embryos, as determined by Xgal and anti-myc antibody staining (data not shown). Finally, we coinjected *Xwnt*-5A and *Xwnt*-8 RNA into the marginal zone of a single ventral blastomere at the four-cell stage to test whether expression at the same time, as opposed to the ~40-min delay in the above experiments, would still block the *Xwnt*-8 induction of gsc. We found that at a 1:1 or 10:1 RNA ratio, *Xwnt*-5A was unable to antagonize the *Xwnt*-8–mediated induction of ectopic *gsc*, as scored by in situ hybridization (data not shown). This suggests that for *Xwnt*-5A to antagonize induction of *gsc* in response to *Xwnt*-8, it may need to be expressed before the *Xwnt*-8 signal, although in assays described below, coinjection of both *Xwnt*-5A and *Xwnt*-8 yields an *Xwnt*-5A–like response.

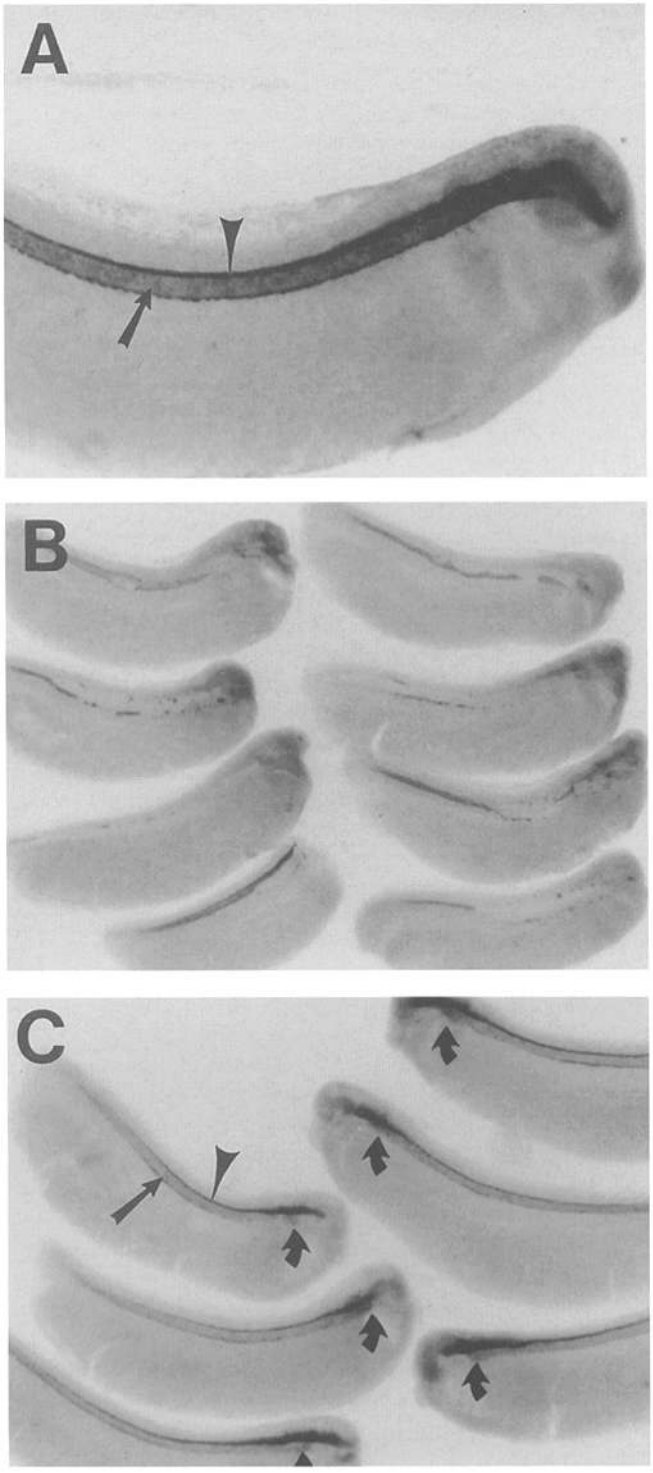

Figure 3. *Xwnt*-4 antagonizes the activity of *Xwnt*-8 after MBT. The ability of *CSKA-Xwnt*-8 to interfere with notochord formation (Christian and Moon, 1993) was scored in stage 25 embryos by anti-Tor 70 whole mount immunocytochemistry to detect the notochord and by *F-spondin* in situ hybridization to detect the floorplate. (*A*) Uninjected embryos possess a normal notochord and floorplate. (*B*) Transcription of *Xwnt*-8 from the cytoskeletal actin promoter vector after MBT leads to loss of notochord and floorplate staining. (*C*) *Xwnt*-4 interferes with the *Xwnt*-8 activity, restoring the formation of the notochord and the floorplate. (*Arrowheads*) *F-spondin* floorplate staining. (*Straight arrows*) Tor 70 notochord staining. (*Curved arrows*) anterior limit of the notochord.

Xwnt-5A Does Not Antagonize Embryonic Responses to β-Catenin or Kinase-dead Xgsk-3

We next investigated whether *Xwnt*-5A could antagonize the activities of *Xgsk*-3 and *β-catenin*, both of which are cytoplasmic elements of the *Wnt*-1 class pathway (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995; Heasman et al., 1994). Ectopic *Xwnt*-5A did not prevent axis duplication or ectopic *gsc* induction mediated by injection of RNA encoding *β-catenin* or a kinase-dead *Xgsk*-3 (Table I). The kinase-dead version of *Xgsk*-3 was employed since it mimics the *Wnt*-1 class response in embryos (Pierce and Kimelman, 1995).

Xwnt-5A Antagonizes Embryonic Responses to Xwnt-8 Even after Ectopic Expression in Distinct Cell Populations

We then tested whether the ability of *Xwnt*-5A to antagonize embryonic responses to the *Wnt*-1 class required that both functional classes of *Wnts* be expressed in the same cells. *Xwnt*-8 RNA was injected into a single ventral marginal blastomere at the 16-cell stage, followed by the injection at the 32-cell stage of RNAs encoding *prolactin*, as a control, or *Xwnt*-5A. The *prolactin* and *Xwnt*-5A RNAs were specifically targeted to all ventral marginal blastomeres adjacent to the *Xwnt*-8–injected blastomere, thus insuring that the two functional classes of *Wnts* would be expressed in adjacent yet different cell populations (confirmed by lineage tracing experiments, not shown). We found that *Xwnt*-5A secreted from neighboring cells reduced axis duplication by ectopic *Xwnt*-8, while *prolactin* had no effect (Table II). These data demonstrate that the *Xwnt*-5A antagonism of embryonic responses to the *Wnt*-1 class can occur even if the two functional classes are expressed in distinct cells.

A Member of the Wnt-5A Class Is Able to Antagonize the Effects of Xwnt-8 Expressed after Midblastula Transition

We also examined the ability of a member of the *Wnt*-5A class, *Xwnt*-4 (Du et al., 1995), to block the effects of overexpressing *Xwnt*-8 later in development. In this experiment, expression of *Xwnt*-8 was driven by a cytoskeletal actin promoter, which leads to overexpression of *Xwnt*-8 after MBT when endogenous *Xwnt*-8 is expressed in ventral-lateral regions (Christian and Moon, 1993). Overexpression of *Xwnt*-8 in the dorsal marginal zone at this time in development diverts the fate of presumptive notochord cells to more lateral fates, as scored histologically by lineage tracing of *Xwnt*-expressing cells, and by the reduction in staining with the notochord-specific Tor 70 antibody (Christian and Moon, 1993). We hypothesized that overexpression of members of the *Wnt*-5A class, such as *Xwnt*-4 that is normally expressed in the floor plate (McGrew et al., 1992), might interfere with the ability of *Xwnt*-8 to divert the developing notochord to more ventral-lateral cell fates.

Table III. Xwnt-4 Interferes with Embryonic Responses to Xwnt-8 Expressed after Midblastula Transition‖

Injection*	Tor expression‡	F-spondin expression§	*n*
Untreated	100	100	10
CSKA-*Xwnt*-8	19	10	47
Xwnt-4+CSKA-*Xwnt*-8	56	60	84

*Both dorsal blastomeres of four-cell–stage embryos were injected with either CSKA-*Xwnt*-8 DNA (0.1 ng) or *Xwnt*-4 RNA (0.2 ng) mixed CSKA-*Xwnt*-8 DNA (0.1 ng).
‡Notochord formation was assayed by staining with the mAb Tor 70.
§Floorplate formation was monitored by in situ hybridization of *F-spondin*.
‖Numbers refer to percentages; *n*, number of embryos.

Consistent with data reported in Christian and Moon (1993), overexpression of *CSKA-Xwnt*-8 in both dorsal cells of four-cell embryos reduced the formation of the notochord, as scored by staining with the Tor 70 antibody (Fig. 3 *B* and Table III) relative to control staining (Fig. 3 *A*). As loss of the notochord would be expected to preclude formation of the floor plate, an in situ hybridization marker for the floorplate, *F-spondin* (Klar et al., 1992) was also used. We observed a reduction of F-spondin expression in response to *CSKA-Xwnt*-8 (Fig. 3 *B*) relative to controls (Fig. 3 *A*). In contrast, coinjection of *Xwnt*-4 RNA with the *CSKA-Xwnt*-8 DNA resulted in normal formation of the notochord and floor plate in a greater percentage of embryos (Fig. 3 *C* and Table III). Coinjection of *Xwnt*-4 RNA with *CSKA-Xwnt*-8 DNA increased the number of embryos staining normally with Tor 70 and *F-spondin* approximately threefold relative to embryos injected with *CSKA-Xwnt*-8 DNA alone (Table III). Injection of *Xwnt*-4 RNA alone did not prevent the formation of the notochord (data not shown) (Ungar et al., 1995). These data demonstrate that the effects of ectopic expression of *Xwnt*-8 after MBT, diverting the fate of the notochord to a more ventral-lateral fate, can be antagonized by a member of the *Wnt*-5A class. These data support the hypothesis that the *Wnt*-5A class reduces embryonic responses to the *Wnt*-1 class throughout embryonic development, although we have only tested one member of the *Xwnt*-5A class for its ability to block the activities of *CSKA-Xwnt*-8 DNA.

Xwnt-5A Decreases Ca^{2+}-dependent Cell Adhesion and Inhibits Morphogenetic Movements In Vitro

Overexpression of *Xwnt*-5A leads to the inhibition of cell mixing and perturbation of morphogenetic movements in

Figure 4. Overexpression of *Xwnt*-5A inhibits open face dorsal lip explant elongation and decreases Ca^{2+}-dependent cell reaggregation. (*A*) Uninjected control dorsal lip explants elongate normally. (*B*) Dorsal lip explants overexpressing *Xwnt*-5A RNA do not elongate. (*C*) *Xwnt*-8 does not inhibit dorsal lip explant elongation. (*D*) *Xwnt*-5A blocks dorsal lip explant elongation when coexpressed with *Xwnt*-8 at a 1:1 ratio of injected RNAs. (*E*) Dissociated control dorsal lip explants reaggregate in a Ca^{2+}-dependent manner. In the absence of Ca^{2+}, reaggregation is inhibited (*inset*). (*F*) *Xwnt*-5A inhibits Ca^{2+}-dependent cell reaggregation, which can be rescued by co-expressing *Xwnt*-5A with *N-cadherin (inset)*. (*G*) *Xwnt*-8 has no appreciable effect on Ca^{2+}-dependent cell reaggregation. (*H*) *Xwnt*-5A blocks Ca^{2+}-dependent cell reaggregation when coexpressed with *Xwnt*-8 at a 1:1 ratio of injected RNAs. *ΔN-cadherin* blocks Ca^{2+}-dependent cell reaggregation in a manner similar to *Xwnt*-5A (*inset*).

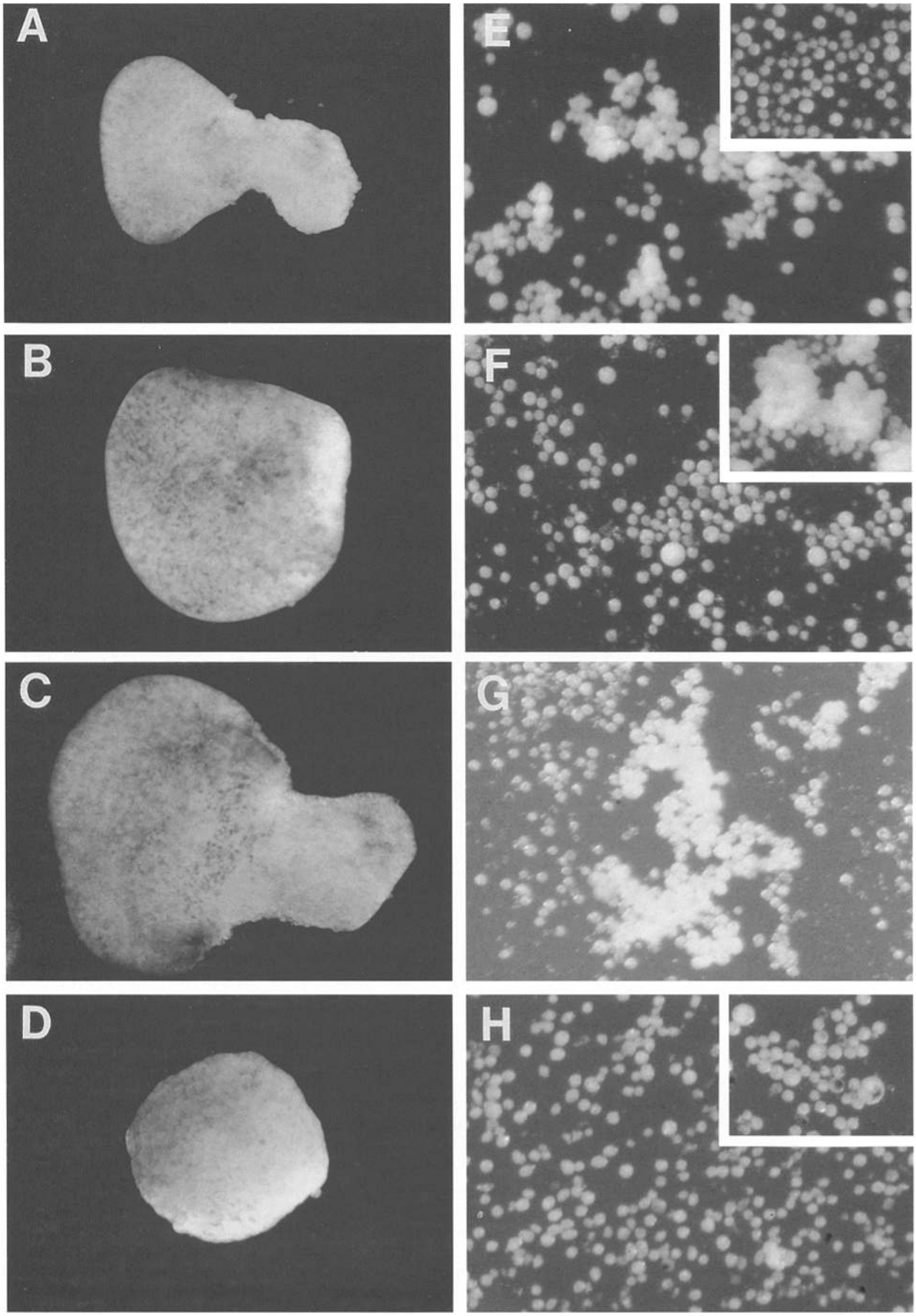
A
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Table IV. Cell Aggregation in Calcium-dependent Cell Adhesion Assays‡

RNAs injected*	Five to eight cell clumps	Greater than eight cell clumps	*n*
Pre-Ca^{2+}	89	11	19
Control	42	58	209
Xwnt-5A	99	1	78
Xwnt-8	70	30	71
Xwnt-5A+*Xwnt*-8	98	2	81
Xwnt-5A+wt *N-cadherin*	42	58	106
ΔN-cadherin	94	6	34

*Both dorsal blastomeres of four-cell embryos were injected with a total of 1 ng of RNAs encoding *Xwnt*-5A or *Xwnt*-8, or 3 ng of RNAs encoding wild-type N-cadherin and *ΔN-cadherin*. In experiments with mixed RNAs, 1 ng of each *Wnt*, or 1 ng of a Wnt with 3 ng of a cadherin were injected.
‡Numbers refer to percentages of clumps of the indicated sizes; *n*, number of cell clumps counted in five seperate samples of each cell adhesion assay.

Table V. Dorsal Lip Explant Elongation

RNAs injected*	Elongation‡ None	Normal	Partial	*n*
Untreated	8	45	46	71
Xwnt-8	0	62	38	13
Xwnt-5A	37	24	39	51
Xwnt-11	14	14	71	7
N-cadherin	12	32	56	25
ΔN-cadherin	32	26	42	19
Xwnt-8+*ΔN-cadherin*	100	0	0	4
Xwnt-8+*Xwnt*-5A	80	10	10	20
Xwnt-5A+*N-cadherin*	29	33	38	49

*Both dorsal blastomeres of four-cell embryos were injected with a total of 1 ng of RNAs encoding *Xwnt*-5A, *Xwnt*-11, *Xwnt*-8, or 3 ng of RNA encoding wild-type *N-cadherin* or *ΔN-cadherin*. In experiments with mixed RNAs, 1 ng of each *Wnt*, or 1 ng of a *Wnt* with 3 ng of a cadherin were injected.
‡Numbers refer to percentages; *n*, number of dorsal lip explants.

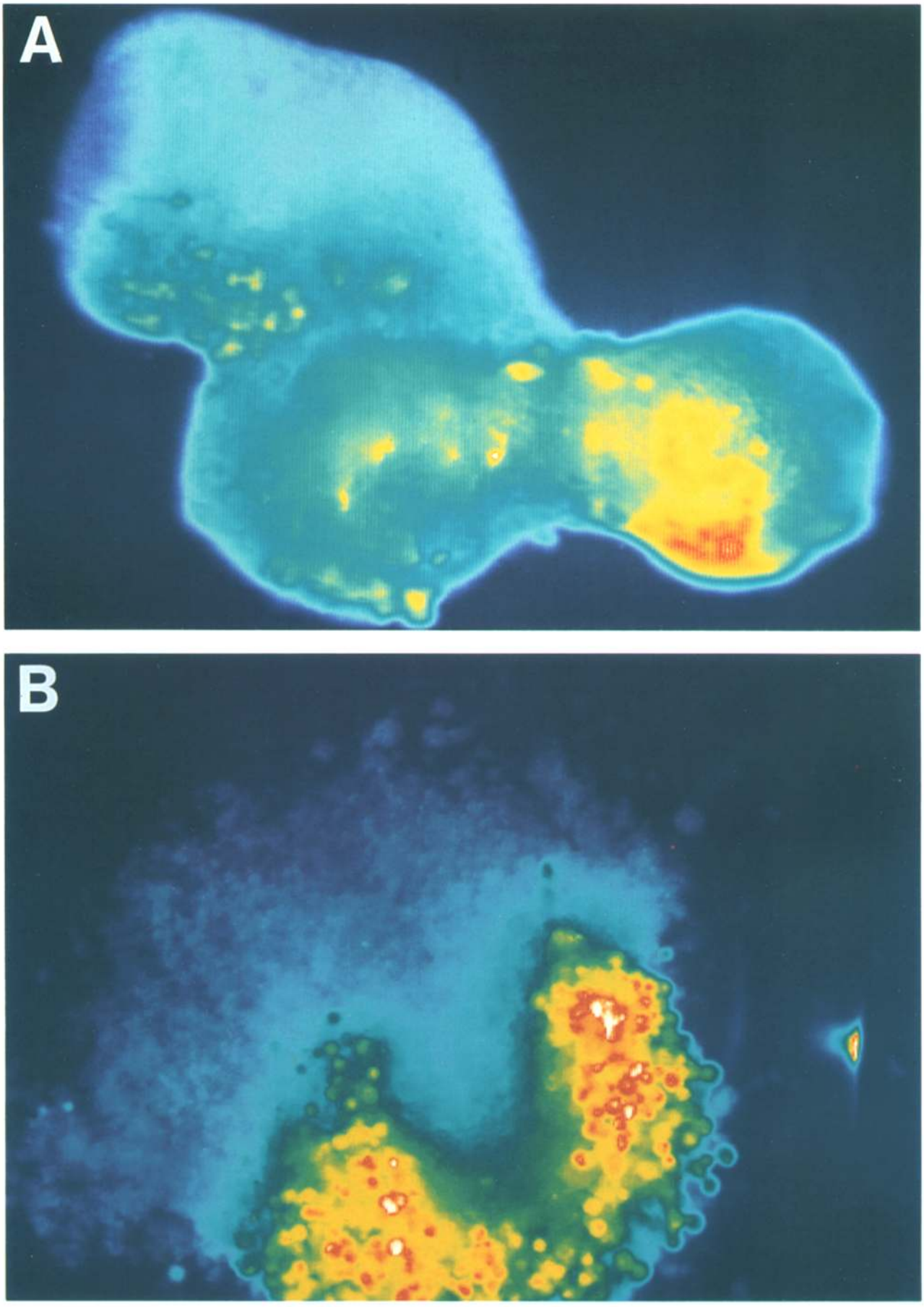
Xenopus embryos (Moon et al., 1993*a*,*b*), raising the possibility that this class of *Wnts* modulates cell adhesion. To test this hypothesis, dorsal marginal explants were dissected from control and *Xwnt*-injected embryos. These explants were dissociated in Ca^{2+}-free medium and then allowed to reaggregate in medium containing 4 mM Ca^{2+}. In embryos injected with RNA encoding bright green fluorescent protein as a control for mosaicism of expression from injected RNAs, the vast majority of cells were fluorescent (data not shown), indicating that almost all cells being scored for cell adhesion either expressed protein from the injected RNAs or were in contact with such cells. While cells from untreated explants often reaggregated into large clumps of more than eight cells in a Ca^{2+}-dependent cell reaggregation assay (Fig. 4 *E* and Table IV), only 1% of clumps from cells expressing *Xwnt*-5A were in this size range (Fig. 4 *F* and Table IV). Intriguingly, coinjection of *Xwnt*-5A and -8 at a 1:1 ratio of RNAs also resulted in cells that did not reaggregate (Fig. 4 *H* and Table IV), and resembled those injected only with *Xwnt*-5A. Similarly, cells expressing *ΔN-cadherin* did not reaggregate into large cell clumps (Fig. 4 *H*, inset; Table IV). As the effect of *Xwnt*-5A to decrease Ca^{2+}-dependent cell adhesion was overcome by coexpressing *N-cadherin* RNA (Fig. 4 *F*, inset; Table IV), this precluded the *Xwnt*-5A effect from being a consequence of toxicity and indicated that *Xwnt*-5A is likely to decrease cell adhesion. Although cells expressing *Xwnt*-8 did not reaggregate as efficiently as control cells (see Fig. 7 *G* and Table IV), they were able to form clumps of eight or more cells in 30% of clumps (vs 58% for control cells), whereas *Xwnt*-5A expressing cells formed these large clumps only 1% of the time (Fig. 4, *F* and *H*; Table IV). Finally, stable transfection of *Xwnt*-5A into C57MG mammary epithelial cells decreases cell clumping relative to mock transfectants, or *Wnt*-1 transfectants, confirming that *Xwnt*-5A decreases cell–cell interactions (Papkoff, J., and R.T. Moon, unpublished data).

As the movements of gastrulation have been reported to involve alterations in cadherin-mediated cell adhesion (Brieher and Gumbiner, 1994), we next studied convergence and extension movements in dorsal open face explants after overexpression of various *Xwnts*. Whereas explants from uninjected (Fig. 4 *A*) and *Xwnt*-8–injected embryos (Fig. 4 *C*) elongated normally, overexpression of members of the *Wnt*-5A class (Fig. 4 *B*), or a 1:1 ratio of mixed *Xwnt*-5A and *Xwnt*-8 RNAs (Fig. 4 *D*), resulted in the inhibition of explant elongation (Table V). Thus, experimental treatments that resulted in the inability to form large cell aggregates in a reaggregation assay (Fig. 4, *F* and *H*; Table IV) also resulted in the inhibition of elongation of dorsal open face explants (Fig. 4, *B* and *D*; Table V). Explants derived from embryos injected with *N-cadherin* or *ΔN-cadherin* also exhibited blocked elongation (Table V), suggesting that convergence and extension movements are sensitive to either increases or decreases in cell adhesion. Collectively, the reaggregation assay and the open face explant elongation data support the hypothesis that the *Wnt*-5A class signal decreases Ca^{2+}-dependent cell adhesion, thereby affecting morphogenetic movements in embryos. Interestingly, the ability of *Xwnt*-5A to interfere with convergence and extension movements also occurs when only half of the gastrula explant expresses *Xwnt*-5A (Fig. 5 *B* vs control in 5 *A*). Also, the explants expressing *Xwnt*-5A often display cells dissociating from the edges of the explant (Fig. 5 *B* vs control in 5 *A*), consistent with *Xwnt*-5A acting to decrease cell adhesion.

Overexpression of Dominant Negative N-cadherin Mimics the Activity of the Wnt-5A Class in Inhibiting Responses to the Wnt-1 Class

As overexpression of *Xwnt*-5A RNA results in decreasing Ca^{2+}-dependent cell adhesion, as well as reducing the induction of *gsc* and ectopic axes in response to the *Wnt*-1

Figure 5. The inhibition of elongation of the gastrula organizer explants by *Xwnt*-5A does not require *Xwnt*-5A to be expressed in all cells of the explant. Embryos were injected into the marginal zone of one dorsal cell at the four-cell stage with either *β-galactosidase* RNA mixed with rhodamine dextran (*A*), or with *Xwnt*-5A RNA mixed with rhodamine dextran (*B*). Open face explants of the entire dorsal marginal zone were prepared at stage 10 and visualized by fluorescence microscopy after control embryos had developed to stage 13–15. Control explants (*A*) elongate extensively (6 of 8 explants), while explants expressing *Xwnt*-5A (*B*) display no convergence and extension movements (10 of 15 explants), or reduced elongation (3 of 15 explants). In both panels, pseudocolor imaging reveals that the injected dextran and RNAs were restricted to the yellow-red cells.


A
B

class, we hypothesized that a decrease in cadherin-dependent cell adhesion may be sufficient to account for this *Wnt*-5A class activity. We tested this hypothesis by injecting four-cell embryos with RNAs encoding *prolactin* as a control (Fig. 6 *B*) or dominant negative *N-cadherin* (*ΔN-cadherin*) (Fig. 6 *C*) (Kintner, 1992), followed by the injection of *Xwnt*-8 RNA into a single ventral marginal blastomere at the 16-cell stage. As determined by in situ hybridization, *ΔN-cadherin* (Fig. 6 *C*) but not *prolactin* (Fig. 6 *B*) blocked the induction of ectopic *gsc* by *Xwnt*-8 (Table I). *ΔN-cadherin* did not alter the expression of a pan-mesodermal gene, *Xbra* (data not shown). *ΔN-cadherin* also prevented *Xwnt*-8 (Fig. 2 *B*) and *Xwnt*-8b (data not shown) from rescuing *gsc* expression in embryos ventralized by UV irradiation, further supporting the hypothesis that one mechanism by which the *Wnt*-5A class antagonizes the activity of the *Wnt*-1 class may involve decreases in cell adhesion. Again, the effects of *ΔN-cadherin* were not an artifact of inhibiting the translation of *Xwnt*-8, as *c-myc* epitope-tagged Xwnt-8 was detected by anti–*c-myc* immunostaining in the presence of *ΔN-cadherin* (data not shown). Importantly, the overexpression of full-length *N-cadherin* did not an-

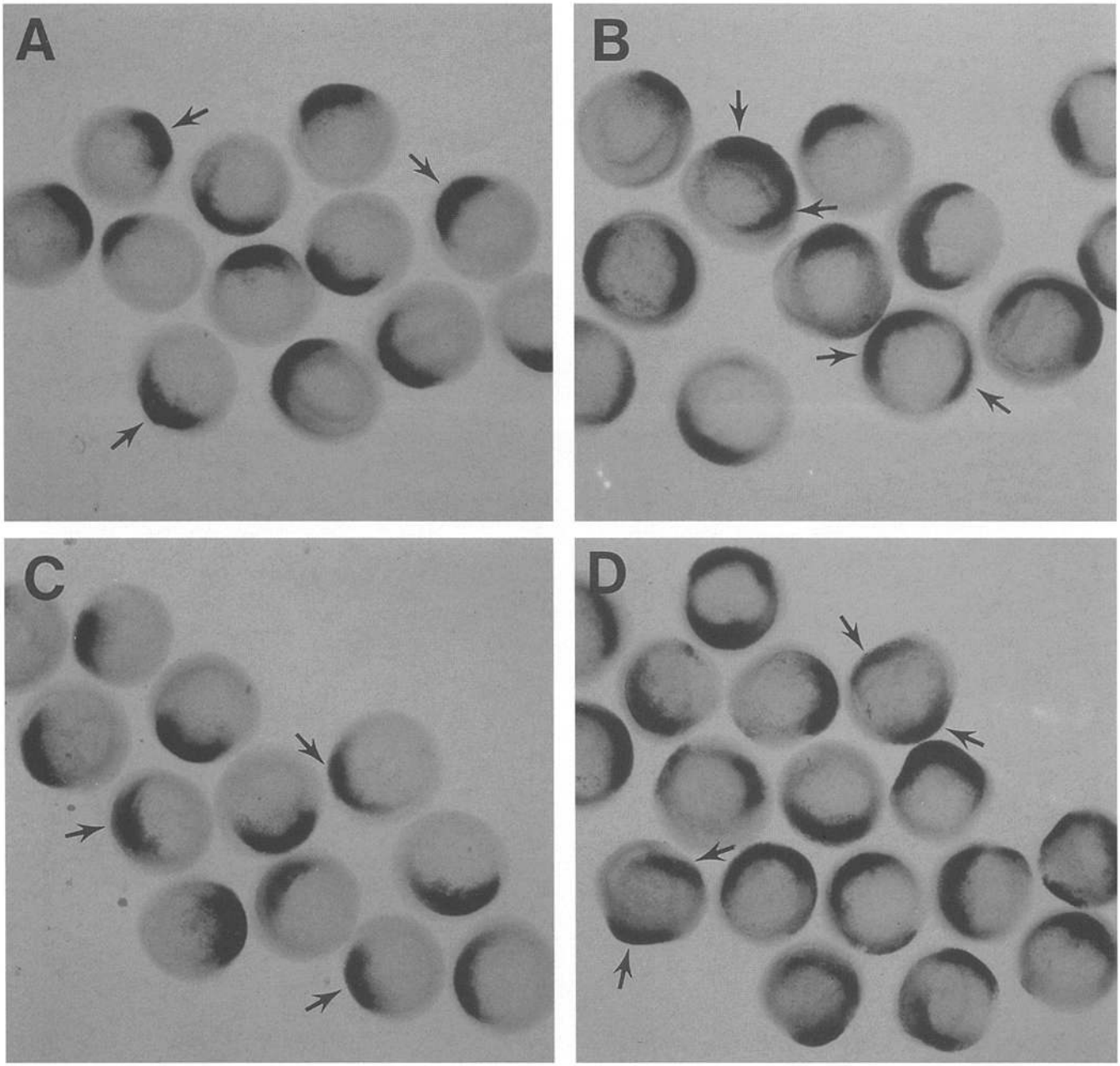


Figure 6. *ΔN-cadherin* but not *N-cadherin* inhibits the induction of ectopic *gsc* expression by *Xwnt*-8 as assayed by in situ hybridization for *gsc* in stage 10 gastrula embryos. (*A*) Uninjected control embryos possess a single site of *gsc* expression. (*B*) Embryos injected with *prolactin* followed by *Xwnt*-8 RNA possess two sites of *gsc* expression. (*C*) Embryos injected with *ΔN-cadherin* followed by *Xwnt*-8 RNA possess a single site of *gsc* expression. (*D*) Embryos injected with *N-cadherin* followed by *Xwnt*-8 RNA possess two sites of *gsc* expression. (*Arrows*) Representative sites of *gsc* expression.

tagonize ectopic expression of *gsc* or axis duplication in response to subsequent injection of *Xwnt*-8 RNA (Figs. 2 *B*, lane 5, and 6 *D*; Table I), indicating that neither changes in cell adhesion, per se, nor increasing the availability of β-catenin binding sites (identical in *N-cadherin* and *ΔN-cadherin*) are sufficient to antagonize ectopic *gsc* induction.

To further rule out β-catenin sequestration as a mechanism by which *ΔN-cadherin* inhibits responses to the *Wnt*-1 class signal, we attempted to block ectopic induction of *gsc* resulting from injection of *β-catenin* RNA at the 32-cell stage, by prior injection of RNA encoding *ΔN-cadherin* at the four-cell stage. When low doses of *β-catenin* RNA (0.15 ng) are injected at the 32-cell stage, prior injection of *ΔN-cadherin* RNA at the four-cell stage prevents ectopic *gsc* induction, possibly by sequestering β-catenin polypeptides (Table I). However, *ΔN-cadherin* RNA is unable to inhibit the *gsc*-inducing activity of *β-catenin* at higher doses of *β-catenin* RNA (0.5 ng) (Table I), even though the dose of *β-catenin* used in these experiments is below the level required to induce ectopic sites of *gsc* expression in all injected embryos. As *ΔN-cadherin* is able to antagonize the activity of members of the *Wnt*-1 class at doses that induce ectopic *gsc* expression or axis duplication in nearly all of the embryos when coinjected with control RNAs, but is unable to antagonize functionally equivalent

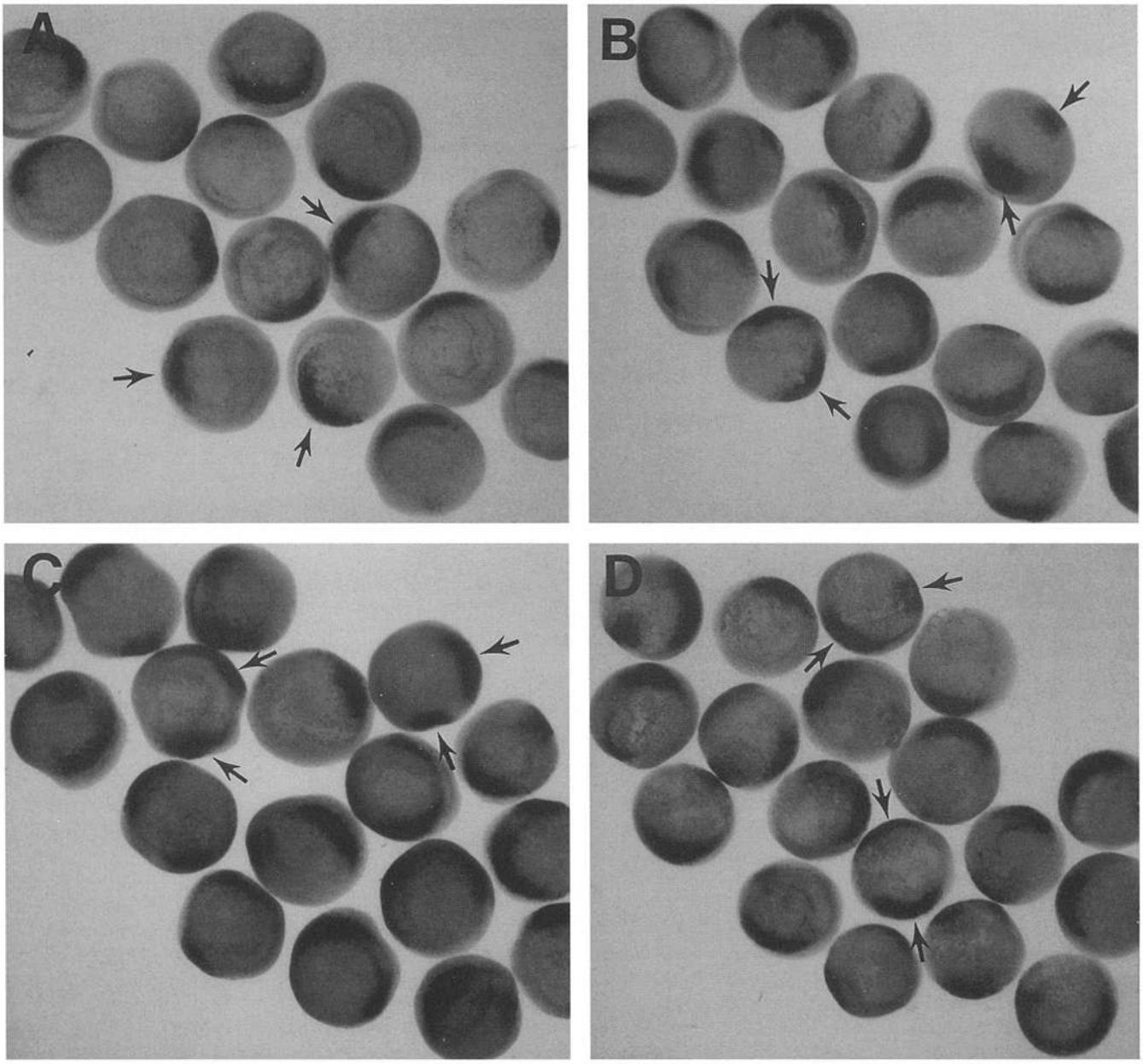


Figure 7. Members of the *Wnt*-5A class and *ΔN-cadherin* do not block the induction of ectopic *gsc* expression by *BVg1*, as assayed by in situ hybridization for *gsc* in stage 10 gastrula embryos. (*A*) Uninjected control embryos possess a single site of *gsc* expression. (*B*) Embryos injected with *prolactin* followed by *BVg1* RNA possess two sites of *gsc* expression. (*C*) Embryos injected with *Xwnt*-11 or *ΔN-cadherin* (*D*) RNA followed by *BVg1* RNA also possess multiple sites of *gsc* expression. (*Arrows*) Sites of *gsc* expression.

doses of *β-catenin*, we believe that *ΔN-cadherin* inhibits *Wnt*-1 class activity specifically by decreasing cadherin-dependent cell adhesion.

Finally, we were unable to prevent the antagonism of the *Wnt*-1 class by the *Wnt*-5A class by overexpression of *N-cadherin* to increase cell adhesion (data not shown). However, Holt et al. (1994) have demonstrated that overexpression of *N-cadherin* is not as effective as other cadherins at rescuing the effects of *ΔN-cadherin* in *Xenopus* embryos. Thus, it is possible that the *N-cadherin* in our rescue experiments did not sufficiently reverse the *Xwnt*-5A–mediated decrease in cell adhesion.

Members of the Wnt-5A Class and ΔN-Cadherin Inhibit Ectopic Induction of gsc and Embryonic Axes by Noggin but Not BVg1

We next asked whether the ability of the *Wnt*-5A class and *ΔN-cadherin* to inhibit embryonic responses to *Wnt*-1 class signals is *Wnt* specific, or whether they may also interfere with the dorsalizing activities of other secreted factors. We first tested the ability of *Xwnt*-5A and *ΔN-cadherin* to antagonize the reported abilities of ectopic *noggin* (for review see Lamb et al., 1993) to induce expression of *gsc* in the ventral marginal zone. Injection of RNAs encoding either *Xwnt*-5A or *ΔN-cadherin* blocked the ectopic induction of *gsc* expression by *noggin* in both untreated and UV-irradiated embryos, as assayed by *gsc* in situ hybridization (Table I) and by RT-PCR (data not shown), respectively.

We also asked whether a member of the *Wnt*-5A class or *ΔN-cadherin* would interfere with the induction of *gsc* by *BVg1* (Thomsen and Melton, 1993). In parallel experiments, neither *Xwnt*-11 (Fig. 7 *C*) (Ku and Melton, 1993), a member of the *Wnt*-5A class whose expression pattern overlaps with that of *BVg1* (for review see Kessler and Melton, 1995), nor *ΔN-cadherin* (Fig. 7 *D*) was able to block induction of *gsc* or ectopic axes by *BVg1* (Table I). Moreover, neither *Xwnt*-11 nor *ΔN-cadherin* blocked the ability of *BVg1* to induce *gsc* expression in UV-irradiated embryos (Fig. 2 *C*, lanes *4* and *5*). Therefore, it appears that embryonic responses to ectopic *BVg1* are relatively independent of cadherin-dependent cell adhesion, making an important distinction from the cadherin-dependent induction of *gsc* by ectopic *Wnt*-1 class and *noggin* signals.

Discussion

Antagonism Between Functional Classes of Wnts

Wnts are secreted signaling proteins that can be grouped by functional assays into at least two distinct classes (Wong et al., 1994; Du et al., 1995). We report here that members of the *Wnt*-5A class antagonize the responses of *Xenopus laevis* embryos to ectopic expression of members of the *Wnt*-1 class. Specifically, ectopic expression of the *Wnt*-1 class in cleavage stage embryos leads to induction of *gsc* and to the subsequent duplication of the embryonic axes. Both responses are blocked by prior expression of members of the *Wnt*-5A class. In addition, we observed that ectopic expression of *Xwnt*-4 (a member of the *Wnt*-5A class) antagonizes the ability of *Xwnt*-8 expressed after MBT to divert the differentiation of prospective notochord cells to a more lateral fate (Christian and Moon, 1993). Thus, members of the *Wnt*-5A class interfere with embryonic responses to ectopic expression of the *Wnt*-1 class before and after MBT.

We speculate that this antagonism requires secretion of the Wnts, as *Xwnt*-5A is able to antagonize *Wnt*-1 class signals without needing to be expressed in the same cells. Interestingly, members of the *Wnt*-5A class were unable to inhibit *gsc* induction or axis duplication mediated by a cytoplasmic component of the *Wnt*-1 class signaling pathway, *β-catenin* (Heasman et al., 1994; Guger and Gumbiner, 1995), or by cytoplasmic kinase-dead *Xgsk*-3 (He et al., 1995; Pierce and Kimelman, 1995), a dominant negative protein that mimics activation of this pathway. There are several possible interpretations of this result, one of which is that both functional classes of *Wnts* impinge on the same signaling pathway, but the antagonism between the two classes occurs upstream of *Xgsk*-3 and *β-catenin*. However, it remains possible that the *Wnt*-5A class activates a distinct signaling pathway that indirectly, perhaps through decreasing cell adhesion (see below), antagonizes the *Wnt*-1 class.

These data have several implications for *Wnt* signaling in embryos. While embryos generally express *Wnts* in unique patterns (for review see McMahon, 1992; Nusse and Varmus, 1992; Moon et al., 1993*b*), there are areas of overlap between different *Wnts* and different functional classes of *Wnts*. First, there is some overlap between the patterns of expression of *Wnt*-3A (a member of the *Wnt*-1 class) and *Wnt*-5A in the late streak–stage egg cylinder of the mouse (Takada et al., 1994). Second, the *Xenopus* homologs of both of these genes overlap in expression in the posterior regions of the *Xenopus* neurula and tailbud embryos (Wolda et al., 1993; Moon et al., 1993). Third, *Xwnt*-1 and *Xwnt*-3A (Wolda et al., 1993) overlap in expression with *Xwnt*-4 in the dorsal midline of the mesencephalon until the tail bud stage (McGrew et al., 1992). Moreover, embryonic cells may receive signals from both classes of *Wnts* since Wnts are secreted and can act in a paracrine manner (for review see Nusse and Varmus, 1992), and our data on gastrula explants demonstrate that cell behavior can be affected even though not all cells are expressing the ectopic *Xwnt*.

Our data suggest that cells receiving signals from both the *Wnt*-1 class and the *Wnt*-5A class may respond in a class-specific manner, although this would likely be modulated by the relative levels of the prospective ligands as well as their relative timing of expression. A class-specific effect in the embryo may help define functional domains and spatial borders within embryonic structures, which is in keeping with observed polarized expression of the two classes in some structures. For example, the anterior vertebrate neural tube expresses *Wnt*-1 and *Wnt*-3A (members of the *Wnt*-1 class) in the dorsal midline, while the floorplate expresses *Wnt*-4 (a member of the *Wnt*-5A class) (Parr et al., 1993; McGrew et al., 1994; Moon et al., 1993*b*; Ungar and Moon, 1995). Similarly, the dorsal otic vesicle expresses *Xwnt*-3A, while the ventral otic vesicle expresses *Xwnt*-4 (for review see Moon, 1993).

However, our data do not support the dominant effect of the *Wnt*-5A class over the *Wnt*-1 class in all circumstances, as sequential expression of *Xwnt*-5A then *Xwnt*-8

blocks *gsc* induction by *Xwnt*-8, but coexpression does not. Similarly, it is worth noting that in the developing chick limb bud, ectopic *Wnt*-7A (which belongs to the *Wnt*-1 class) (Wong et al., 1994) is capable of inducing the *Lim* homeobox gene *Lmx1* (Riddle et al., 1995) in a manner that may be dependent on the presence of *Wnt*-5A (Dealy et al., 1993), raising the question of synergy rather than antagonism. Finally, *Xwnt*-5C, tentatively a member of the *Wnt*-5A class, acts indistinguishably from *Xwnt*-1 in inducing *engrailed*-1 (Koster et al., 1996), despite the *Wnt*-1 and *Wnt*-5A classes having no similar activities in other assays (Du et al., 1995). Despite such questions, which arise due to the fact that the functional classes of *Wnts* are only now being recognized, the current data suggest that studies of *Wnts* should take into account that some *Wnts* may have indistinguishable activities, whereas *Wnts* belonging to another functional class may modulate or oppose that activity. This is of some interest in considering *Wnts* in human breast cancer (e.g., Huguet et al., 1994, 1995).

Wnts and Cell Adhesion

As overexpression of members of the *Wnt*-5A class leads to the inhibition of morphogenetic movements during gastrulation (Moon et al., 1993*a*), we investigated whether this effect may be due to changes in cell adhesion. We found that *Xwnt*-5A decreased Ca^{2+}-dependent cell adhesion in a manner similar to *ΔN-cadherin*, which lacks its extracellular domain and inhibits cadherin-mediated cell adhesion (Kintner, 1992). A possible mechanism by which members of the *Wnt*-5A class might decrease cadherin-mediated cell adhesion is via stimulation of an *src*-like tyrosine kinase, since *src*-mediated tyrosine phosphorylation of cadherin-associated β-catenin can result in decreased cadherin-dependent cell adhesion (Behrens et al., 1993; Hamaguchi et al., 1993; Matsuyoshi et al., 1992). In support of this hypothesis, we have observed subtle increases in tyrosine phosphorylation of β-catenin in response to the injection of RNA encoding *Xwnt*-5A but not *Xwnt*-8 (data not shown).

Although cultured cells overexpressing *Wnt*-1 accumulate β-catenin and exhibit increased cadherin-dependent cell adhesion (Bradley et al., 1993; Hinck et al., 1994), we did not observe increases in Ca^{2+}-dependent cell reaggregation of dissociated dorsal lip explant cells overexpressing *Xwnt*-8, a member of the *Wnt*-1 functional class (Du et al., 1995). Nevertheless, our data reproducibly demonstrate that the *Wnt*-5A class, which can antagonize the *Wnt*-1 class, decreases cell adhesion.

Antagonism of Wnt-1 Class and Noggin Activity by the Wnt-5A Class Is Mimicked by Decreasing Cadherin-mediated Cell Adhesion

Since overexpression of *Xwnt*-5A inhibits cadherin-dependent cell adhesion, we asked whether decreased cell adhesion alone might be capable of antagonizing the induction of ectopic *gsc* and axis duplication by the *Wnt*-1 class, *noggin*, or *BVg1*. Our results show that a dominant negative cadherin, *ΔN-cadherin*, was able to act like the *Wnt*-5A class and thus antagonize the induction of *gsc* and axis duplication by members of the *Wnt*-1 class as well as by *noggin*, but not by *BVg1*. In contrast, overexpression of *N-cadherin* had no effect on *Wnt*-1 class activity in similar assays. The fact that overexpression of *ΔN-cadherin*, but not *N-cadherin*, mimics the *Wnt*-5A block of embryonic responses to the *Wnt*-1 class and *noggin* further suggests that decreased cadherin-mediated cell adhesion is involved in the embryonic responses to the *Wnt*-5A class. Evidence that factors that regulate cell adhesion may be important during embryogenesis is supported by data showing that the maintenance of cell adhesion in *Xenopus* embryos is required for proper expression of MyoD (Holt et al., 1994).

How might decreased cell adhesion in response to either *ΔN-cadherin* or the *Wnt*-5A class interfere with cellular responses to several putative signaling factors? With respect to the effects of *ΔN-cadherin*, at least two mechanisms need to be considered. The first mechanism could involve the artifactual sequestration of β-catenin, which is required for *Wnt* signaling (Heasman et al., 1994), simply through the overexpression of a cadherin domain that contains available β-catenin binding sites. Thus, sequestering β-catenin on the cytoplasmic domains of ectopic cadherins could conceivably interfere with its signaling role. Our data are inconsistent with this β-catenin sequestration model because *ΔN-cadherin* can block *Wnt*-1 class signals at doses of *Xwnt*-8 RNA that induce ectopic *gsc* expression or axis duplication in nearly all injected embryos, but the *ΔN-cadherin* is unable to block the activity of functionally equivalent doses of ectopic *β-catenin*. Furthermore, ectopic *N-cadherin*, which possesses identical intracellular catenin binding domains as *ΔN-cadherin* (Kintner, 1992), did not affect embryonic responses to the *Wnt*-1 class.

The second mechanism addressing the ability of *ΔN-cadherin* and *Wnt*-5A class members to antagonize embryonic responses to *Wnt*-1 class members involves decreasing cadherin-dependent cell adhesion. It is conceivable that the *Wnt*-5A class leads to decreased cell adhesion, which interferes with the responses of embryos to a *Wnt*-1 class ligand independently of whether the complete *Wnt*-1 signaling pathway has been activated. If this were the case, one would predict that the *Wnt*-5A class would be able to prevent induction of secondary axes in response to a cytoplasmic component of the *Wnt*-1 signaling pathway, *β-catenin*, or a cytoplasmic activator of this pathway, a kinase-dead *gsk-3*. Importantly, we observed that the duplication of the embryonic axis in response to ectopic expression of these downstream elements was not blocked. A working hypothesis based on these observations is that a threshold level of cell adhesion is required for, and perhaps involved in, receptor-mediated signal transduction by the *Wnt*-1 class, but once intracellular components of the *Wnt* signal transduction pathway have been activated (or bypassed by injection of RNAs), cellular and embryonic responses are less sensitive to decreases in cell adhesion in response to the *Wnt*-5A class.

What then of the observation that members of the *Wnt*-5A class and *ΔN-cadherin* block embryonic responses to *noggin* but not to *BVg1*? It is possible that the *Wnt*-1 class and *noggin* can operate in a shared pathway, which would explain how interference with this pathway blocks responses to both types of signaling molecules. Alternatively, *noggin* may act in an independent pathway which is itself sensitive

at some level to decreases in cell adhesion. The observation that responses to *BVg1* are not antagonized by the *Wnt*-5A class supports the hypothesis that *BVg1* activates a distinct pathway. The observation that *ΔN-cadherin* does not interfere with embryonic responses to *BVg1* suggests that the pathway stimulated by this factor is less sensitive than the *Wnt*-1 class pathway to decreases in cell adhesion.

Additional experimentation will be required to rigorously determine how the *Wnt*-5A class and *ΔN-cadherin* block embryonic responses to the *Wnt*-1 class and to *noggin*. However, previous studies and recent work suggest further consideration of the phosphatidylinositol (PI) cycle as a target for Wnt action by both functional classes. Previously it has been shown that lithium, a potent inhibitor of PI turnover, yields a phenotype indistinguishable from that obtained from the overexpression of *Xwnt*-1 class RNAs (for review see Christian and Moon, 1993). However, recent work suggests that the *Wnt*-5A activities may be a consequence of activating, not inhibiting, the PI cycle. First, stimulating the PI cycle and calcium release during early *Xenopus* development, by activation of ectopic serotonin receptors, leads to a phenotype similar to that of the *Wnt*-5A class (Ault, K.T., G. Durmowicz, A. Galione, P.L. Harger, and W.B. Busa, manuscript submitted for publication). Second, expression of *Xwnt*-5A leads to increases in the release of calcium from intracellular stores (Yang-Snyder, J., D.C. Slusarski, R.T. Moon, and W.B. Busa, manuscript submitted for publication), as also reported for the ectopic serotonin receptor (Ault, K.T., G. Durmowicz, A. Galione, P.L. Harger, and W.B. Busa, manuscript submitted for publication). Third, activation of the PI cycle by ectopic expression of serotonin receptors antagonizes the induction of *gsc* and secondary axes by *Xwnt*-8 (Yang-Snyder, J., D.C. Slusarski, R.T. Moon, and W.B. Busa, manuscript submitted for publication), indistinguishable from the antagonism of *Xwnt*-8 by *Xwnt*-5A in the present study. Therefore, we hypothesize that the *Wnt*-5A class antagonism of the *Wnt*-1 class may occur at the level of the PI cycle, or through the PI cycle, modulating cell adhesion.

In conclusion, we have demonstrated that members of the *Wnt*-5A class decrease cadherin-dependent cell adhesion and antagonize the embryonic responses to *Wnt*-1 class members expressed ectopically before and after midblastula transition. These activities of the *Wnt*-5A class are mimicked by a dominant negative cadherin that decreases cell adhesion. Therefore, *Wnt*-5A class antagonism of the *Wnt*-1 class may be mediated in part by decreasing cell adhesion below a threshold level necessary for embryonic responses to signaling by the *Wnt*-1 class.

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