was 2.8 mm; length was 1.8 and 2.2 mm; and porosity (grain size <5 μm) was 0.4 and 0.8%, respectively, for the two samples. For each of the propagation directions, a pair of faces was ground and polished flat and parallel within 0.2 μm with 1-μm diamond paste. Details are in G. D. Gwanmesia, R. C. Liebermann, F. Guyot, *Geophys. Res. Lett.* **17**, 1331 (1990).
9. Phase comparison allows for accurate determination of elastic wave travel times over a wide frequency range. Dispersion attributable to departures at low frequencies from conditions of infinite-medium plane wave propagation is recognized. Its influence upon measured travel times is minimized by use of an average travel time for the high-frequency interval (50 to 70 MHz) over which travel time is relatively frequency-insensitive. For details see I. Jackson, H. Niesler, D. J. Weidner, *J. Geophys. Res.* **86**, 3736 (1981).
10. The interfaces between the acoustic buffer rod, a thin (2 μm thick) gold foil, and the specimen are loaded by the confining pressure in order to provide good mechanical coupling. Minor corrections are applied for the influence of this bond (~+1 ns in ~460 ns for P waves and ~−1.5 ns in 780 ns for S waves).
11. For detailed discussion of the high-pressure technique and pressure calibration see H. Niesler and I. Jackson, *J. Acoust. Soc. Am.* **86**, 1573 (1989); S. M. Rigden *et al.*, *Geophys. Res. Lett.* **15**, 605 (1988); H. Niesler, I. Jackson, E. C. Morris, *High Temp. High Pressures* **20**, 495 (1988).
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13. Despite great care in polishing the ends of the specimens, the observed ratios of the buffer and sample echo amplitudes, and the low values of, and hysteresis among, the measured velocities indicate that the gold bond provides imperfect coupling between the buffer rod and specimen at pressures below ~1.4 GPa. See also (*11*).
14. Coefficients of Eulerian finite-strain Eqs. 1 and 2 are: $M_1 = G$; $L_1 = K + (4/3)G$; $M_2 = 5G - 3K(dG/dP)$; $M_2 = 5[K + (4/3)G] - 3K[dK/dP + (4/3)dG/dP]$.
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Methylation of an Immediate-Early Inducible Gene as a Mechanism for B Cell Tolerance Induction

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Stage-specific gene regulation is important in determining cell function during development. Immature B cells expressing membrane-bound immunoglobulin M (mIgM) are sensitive to antigen-induced tolerance, whereas mature B cells are activated by antigen. Previous studies have established an association between *Egr-1* gene induction and antigen receptor (mIgM)–mediated activation of mature B cells. Here it is shown that the immature B cell line WEHI-231 and tolerance-sensitive bone marrow–derived B cells do not express *Egr-1*. It is further shown that lack of inducible expression in these cells is due to specific methylation of the *Egr-1* gene. Thus, covalent inactivation of an activation-associated gene may explain tolerance sensitivity at specific stages of B cell development.

THE DEVELOPMENTAL PROGRAM LEADING to mature murine B cells can be divided into windows defined by antigen receptor and phenotypic marker expression on the cell surface and by cellular responses of the B cells. Immature murine B cells express mIgM only, whereas mature murine B cells express both mIgM and membrane-bound immunoglobulin D (mIgD) (*1*). Recognition and binding of antigen to mIgM on mature murine B cells results in activation as defined by entry into the cell cycle (*2*) or competence for subsequent T cell–derived progression signals (*3*). In contrast, signaling through mIgM on immature murine B cells results in a negative response manifested by a state of induced unresponsiveness or tolerance (*4*). This phenomenon is believed to be responsible for the deletion or functional inactivation of those cells that recognize self-antigens.

Differential expression of growth-related genes may account, at least in part, for the different growth responses observed in immature versus mature B cells. This hypothesis is supported by studies of the expression of *Egr-1*, an immediate-early gene that encodes a transcriptional regulatory factor (*5, 6*). *Egr-1* is inducible in mature murine B cells (*7*) and in the phenotypically mature murine B cell line, BAL-17 (*8–10*), after mIgM is cross-linked with antibodies to IgM (anti-μ) or after stimulation with TPA (12-*O*-tetradecanoyl phorbol-13-acetate) (*7*). Induction by these signals is associated with a positive growth response (*7*). In contrast, *Egr-1* induction does not accompany anti-μ or TPA stimulation of the murine B cell line, WEHI-231 (*7, 10*), which possesses a stable immature B cell phenotype (*10–14*). Furthermore, this lack of *Egr-1* expression is associated with a negative growth response in these cells to mIgM-generated signals, which is manifested by an inhibition of proliferation leading to eventual cell death (*7, 13*). These results demonstrate an association between *Egr-1* gene expression and the transduction of mIgM signals into positive B cell growth responses.

To determine if a nontransformed population of cells analogous to WEHI-231 cells exists in vivo, we examined *Egr-1* expression in mIgM-positive ($mIgM^+$) cells from adult mouse bone marrow. Because these cells were of the immature phenotype ($mIgM^+$, $mIgD^-$) and did not manifest a positive activation response to signaling through mIgM (*15*), we reasoned that they belonged to the tolerance-sensitive B cell population previously defined (*4*). We observed no detectable induction of *Egr-1* expression in $mIgM^+$ cells in response to TPA stimulation (*16*) (Fig. 1, A and B). This result contrasts with that observed in mature splenic B cells (Fig. 1, C and D). Identical results were obtained after stimulation with anti-μ (*17*). Thus, a population of cells analogous to WEHI-231 cells exists in adult mouse bone marrow. Further molecular studies aimed at defining the causative factors responsible for the lack of inducible *Egr-1* expression in immature B cells were hindered by difficulties in maintaining the cells in culture and the heterogeneity inherent in primary cell populations. Thus, we used the WEHI-231 cell line as a model system for studying this stage of B cell development.

We considered the following as explanations for the inability of anti-μ or TPA to

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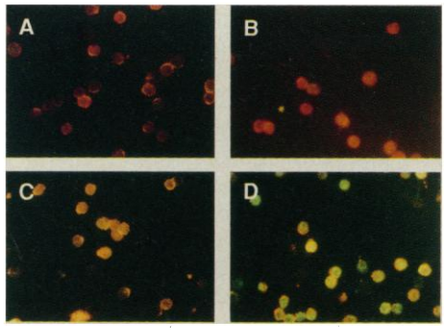

Fig. 1. *Egr-1* protein expression was not induced by TPA in mIgM$^+$ immature B cells. Two-color immunofluorescence (*16*) was used to detect expression of mIgM (red) and *Egr-1* (green). Immature B cells were isolated from unfractionated, erythrocyte-depleted bone marrow cells from 8-week-old adult mice and were either left unstimulated (**A**) or were stimulated for 4 hours with TPA (10 ng/ml) (**B**). Unstimulated (**C**) and TPA-stimulated (**D**) mature splenic B cells were similarly analyzed for comparison.

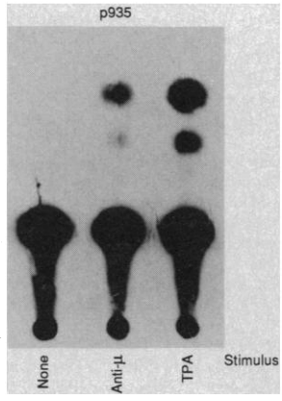

Fig. 2. The *Egr-1* promoter was transcriptionally active in WEHI-231 cells. WEHI-231 cells were transfected with 30 μg of p935 by the DEAE-dextran method (*21*) with dimethyl sulfoxide shock. After 24 hours in medium, the cells were stimulated with anti-μ (10 μg/ml) or TPA (10 ng/ml) for 24 hours and assayed for CAT activity (*18*).

induce *Egr-1* expression in WEHI-231 cells: (i) point mutations within *Egr-1*; (ii) lack of trans-activating factors necessary for the induction of *Egr-1* transcription; or (iii) trans-mediated repression of *Egr-1*. Point mutations within *Egr-1* or its promoter were unlikely to account for the lack of inducible *Egr-1* expression because such changes would most likely have to affect both alleles. To address whether WEHI-231 cells lacked trans-activating factors necessary for *Egr-1* induction by anti-μ or TPA, we assessed *Egr-1* promoter function in these cells with the use of the chloramphenicol acetyltransferase (CAT) gene as a reporter (*18*). The *Egr-1* promoter region, containing 935 bp to the 5′ side of the transcription start site and including the anti-μ and TPA response elements (*19, 20*), was inserted upstream of the CAT gene to make the plasmid p935. WEHI-231 cells (*10*) were transfected with p935 (*21*) and then either left unstimulated or stimulated with anti-μ or TPA for 24 hours. Stimulated cells showed increased CAT activity (*18*) compared to unstimulated cells (Fig. 2), demonstrating that WEHI-231 cells contain the factors necessary for trans-activation of *Egr-1*.

Our results, together with the immature phenotype of these cells, led us to consider DNA methylation as a possible mechanism for inactivation of *Egr-1* in WEHI-231 cells because DNA hypermethylation can inactivate genes in developing cells (*22*). The methylation status of *Egr-1* in WEHI-231 cells (*10*) and the developmentally mature BAL-17 cells was analyzed at 5′-CCGG-3′ sequences with the restriction enzyme isoschizomers Hpa II (an enzyme sensitive to cytosine methylation) (*23*) and Msp I (which is methylation-insensitive) (*23*) (Fig. 3). Digestion of BAL-17 genomic DNA with either Hpa II or Msp I yielded the same restriction pattern (Fig. 3), thus showing that *Egr-1* is not methylated at these sites. Isolated mature splenic B cells from adult BALB/c mice gave results consistent with a lack of methylation at these sites (*17*). Genomic digests of WEHI-231 DNA with Msp I revealed a pattern similar to that seen with BAL-17 DNA; however, Hpa II digestion of WEHI-231 DNA produced a different pattern, mainly consisting of larger fragments than those of the Msp I digest. Thus, *Egr-1* in WEHI-231 cells is methylated to a greater extent than in BAL-17 cells. The larger (2.0-kb) fragment observed in the Msp I digest of BAL-17 but not WEHI-231 DNA may reflect a polymorphism of *Egr-1* in cell lines derived from distinct inbred strains of mice.

We evaluated the relevance of the hypermethylation of *Egr-1* to its lack of anti-μ or TPA inducibility in WEHI-231 cells by studying the effect of culturing these cells for 48 hours in the presence of 3 μM 5′-azacytidine—a nonspecific inhibitor of DNA methylation (*24*)—before stimulation with anti-μ or TPA (Fig. 4A). Analysis of the methylation status of *Egr-1* in the 5′-azacytidine–treated cells indicated that the gene was hypomethylated compared to untreated cells (*17*). As depicted, both anti-μ and TPA induced the expression of *Egr-1* in the 5′-azacytidine–treated cells. Furthermore, induced *Egr-1* expression in 5′-azacytidine–treated WEHI-231 cells was independent of de novo protein synthesis because induction was observed in the continued presence of cycloheximide (Fig. 4B). Therefore, the effect of 5′-azacytidine on inducible *Egr-1* expression is on *Egr-1* itself rather than another gene encoding an inducible factor necessary for *Egr-1* induction. *Egr-1* mRNA was also observed (*17*) after cycloheximide treatment of the 5′-azacytidine cells in the absence of anti-μ; however, the mRNA levels in this case were lower than those observed in the presence of anti-μ. These results showed the functional importance of DNA methylation in the regulation of *Egr-1* in WEHI-231 cells and, furthermore, established that cis-acting repressor elements were unlikely to be responsible for the silencing of *Egr-1* transcription in immature B cells.

The GC-rich nature of the *Egr-1* promoter and its correspondingly large number of restriction sites for methylation-sensitive restriction enzymes (Fig. 5A) made it difficult to determine the relative methylation of the *Egr-1* promoter in WEHI-231 and BAL-17 cells by Southern (DNA) analysis. Rather, we assessed the effect of methylation of the *Egr-1* promoter by examining the effect of in vitro cytosine methylation on inducible *Egr-1* promoter activity. The plasmid p935 was methylated at its Hpa II or Hha I sites (*25*) and transfected into WEHI-231 cells; methylation at these sites was verified by digestion of the methylase-treated plasmids and resolution of the bands by polyacrylamide gel electrophoresis. In both cases where

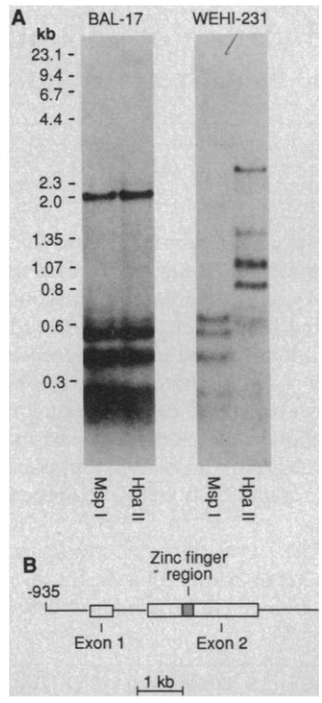

Fig. 3. DNA methylation analysis of *Egr-1* in WEHI-231 and BAL-17 cells. (**A**) Southern blots of genomic DNA (20 μg per lane) isolated from BAL-17 and WEHI-231 cells and digested with either Msp I or Hpa II. The blot was probed with the *Egr-1* genomic clone, p357 (*19*), shown in (**B**).

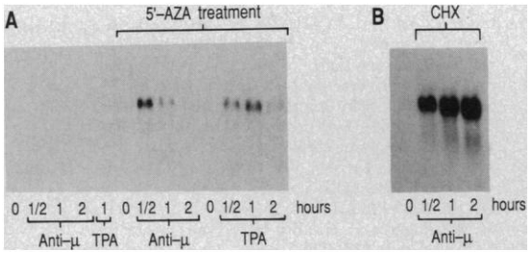

Fig. 4. Anti-μ or TPA induction of *Egr-1* in WEHI-231 cells treated with 5′-azacytidine. (**A**) Northern blot of total cellular RNA (20 μg per lane) from untreated WEHI-231 cells that were either unstimulated, stimulated with anti-μ (5 μg/ml), or stimulated with TPA (10 ng/ml) for the indicated times. On the same blot is total cellular RNA (20 μg per lane) from WEHI-231 cells treated for 48 hours with 3 μM 5′-azacytidine (5′-AZA) and then left unstimulated or stimulated with anti-μ (5 μg/ml), or TPA (10 ng/ml). The blot was probed with an *Egr-1* cDNA clone OC68 (*5*). The molecular size of the complementary transcript is indicated on the right. (**B**) Northern blot analysis of total cellular RNA (20 μg per lane) isolated from WEHI-231 cells cultured for 48 hours with 3 μM 5′-azacytidine, treated with cycloheximide (CHX) (5 μg/ml) (except the zero time lane) for 15 min, and then stimulated with anti-μ (5 μg/ml) for the indicated times. The blot was probed as in (A).

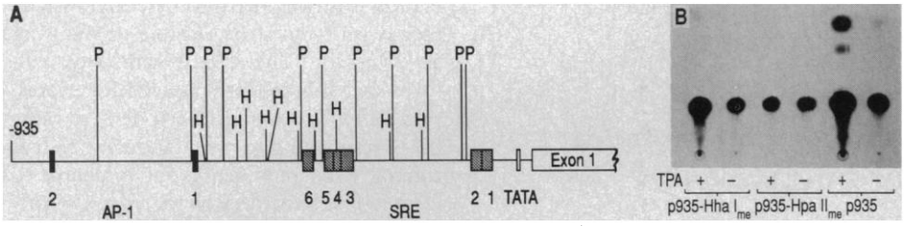

Fig. 5. In vitro methylation of the *Egr-1* promoter by Hha I and Hpa II bacterial methylases blocked inducible transcriptional activity. (**A**) Hha I (H) and Hpa II (P) sites within the 5′ flanking region of *Egr-1* and selected putative regulatory elements determined by consensus homologies (*19*). Solid bars, AP-1 binding regions; stipled bars, serum-response elements (SRE); open bar, TATA box. (**B**) The 5′ flanking region (up to −935 bp) depicted in (A) was used to make plasmid p935 (Fig. 2). Bottom panel shows CAT activity of TPA (10 ng/ml; 24 hours)-stimulated WEHI-231 cells transfected as in Fig. 2 with unmethylated p935 or Hha I or Hpa II methylase–treated p935 (p935–Hha I_{me} and p935–Hpa II_{me}, respectively).

methylated p935 was tested, TPA-inducible CAT activity was reduced as compared to the unmethylated p935 (Fig. 5B). Identical results were obtained after stimulation with anti-μ (*17*). The lack of CAT activity with methylated p935 was not due to methylation of the CAT gene itself, because it has been shown (*26*) that methylation of the CAT gene coding region does not block transcription of the gene. Methylation of p935 also abrogated CAT activity in the permissive BAL-17 transfectants (*17*). Thus, methylation is capable of blocking transcription mediated through the *Egr-1* promoter.

Our results suggest that methylation of *Egr-1*, particularly of its promoter, is a mechanism by which this gene is silenced in WEHI-231 cells. Our findings are consistent with the general premise that DNA methylation is a means for silencing genes during development and differentiation, because *Egr-1* is hypermethylated in immature B cells compared to mature B cells. These results are also consistent with those of others (*26–28*) showing (i) an association between increased methylation of cytosine residues and gene inactivation and (ii) hypomethylation associated with transcriptional activation of a gene (*22, 27, 29*).

A phenomenon observed in B cells and related to the growth response to mIgM stimulation is that of immunological tolerance (*4*). This process is thought to be responsible for deleting, or rendering inactive, self-reactive B cells during the early stages of development (*4*). Whereas signaling differences at the membrane or in the cytoplasm of the immature versus mature B cells may account for tolerance versus activation, respectively, alterations in gene expression may also be involved in the mechanisms accounting for these different responses. In fact, it is plausible that B cells use more than one mechanism to ensure the tolerant state because they are a heterogeneous population of cells and because both clonal deletion (*30*) and clonal anergy (*31*) have been experimentally supported as two functionally different strategies for B cell tolerance. In any case, it is crucial for a B cell to establish a mechanism for maintaining a negative growth response to signals from the antigen receptor, mIgM, during its immature stage of development. If *Egr-1* expression is important for initiating a positive growth response to mIgM stimulation in mature B cells (*7*), then a mechanism would be required for maintaining its inactivity in immature B cells. Our results suggest that hypermethylation serves to ensure the inactivation of *Egr-1* and possibly other growth response genes in immature B cells.

Finally our results suggest that hypermethylation of *Egr-1* may be indicative of the immature stage of B cell development. As appropriate phenotypic markers are developed and separation techniques become more refined, it will be interesting to evaluate the extent of methylation of *Egr-1* in nontransformed B cells at different stages in their development.

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16. mIgM$^+$, immature B cells were isolated from bone marrow of adult (8-week-old) BALB/c mouse femurs. The cell preparation was depleted of erythrocytes and then treated with a mixture of antibodies to Thy-1 and δ chain and with rabbit complement to remove T cells and mature B cells, respectively. The remaining cells were typically 5 to 10% mIgM$^+$ cells, as determined by fluorescence-activated cell sorting (FACS). Isolated cells were equilibrated in medium at 37°C for 3 hours and then stimulated with TPA (10 ng/ml) for 4 hours at 37°C. The cells were centrifuged onto glass slides, fixed in 3% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Slides were then washed three times in PBS and incubated for 90 min with biotin-conjugated goat antibodies to mouse μ chain (anti-μ) and rabbit antibodies to mouse *Egr-1* diluted in PBS containing 2% bovine serum albumin (BSA) and 1% NaN_3. Slides were then washed five times in the PBS-BSA-NaN_3 and incubated for 20 min with the secondary antibody reagents (fluorescein-conjugated goat antibodies to rabbit immunoglobulin and rhodamine-conjugated streptavidin). The slides were finally washed five times in PBS-BSA-NaN_3, air-dried, and mounted with glycerol and 0.2 M DABCO (1,4-diazabicyclo[2.2.2]octane) (pH 8.6) (Sigma). Cell-associated immunofluorescence was visualized by fluorescence microscopy and recorded on Kodak Ektachrome 400 film.

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Region-Specific Neural Induction of an *engrailed* Protein by Anterior Notochord in *Xenopus*

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Anterior-specific neural induction can be assayed by means of an antibody that recognizes the *Xenopus* homeobox-containing protein En-2. The En-2 antigen is an excellent early marker, since it is present as a discrete band in the anterior neural plate of neurula embryos. Regional induction was assayed by combining dorsal mesoderm with competent ectoderm. Anterior notochord from the early neurula induced En-2 frequently, while posterior notochord induced En-2 less frequently. Presumptive somitic mesoderm and presumptive head mesoderm, though they induced neural tissue, were not strong inducers of En-2. Thus, anterior notochord may be the primary mesodermal tissue responsible for the patterning of the anterior neural plate.

RECENT STUDIES ON *Xenopus* HAVE focused on general neural induction without addressing formation of an anterior-posterior pattern within the neural plate. In amphibian embryos, the presumptive neurectoderm is induced by the underlying mesoderm to become neural tissue (*1–6*). Experiments in urodeles indicate that the anterior-posterior pattern is due to induction by dorsal mesoderm (*7*). In this report, we use an anterior-specific neural marker to study the source of pattern in the neuroectoderm of *Xenopus*.

The 4D9 antibody recognizes the *Drosophila engrailed* protein as well as a *Xenopus* homolog, En-2 (*8, 9*). Early neurula (stage 12.5) *Xenopus* embryos do not express *En-2* RNA (*10*) or protein (*9*) until stage 14, when the protein is expressed in 600 cells in the anterior neural plate in a band perpendicular to the notochord (*9*). This antigen therefore provides an excellent early marker of anterior neural differentiation. The sequence of a cDNA clone confirmed that the *Xenopus engrailed* protein detected by the antibody is homologous to the murine En-2 protein, and we therefore refer to it as En-2 (*10*). Previous evidence from the examination of the distribution of En-2 in embryos where the mesoderm has been experimentally altered to be either exclusively ventral or dorsal in character suggests that dorsal mesoderm is involved in the induction of En-2 (*9*). The anterior notochord, the presumptive anterior somites, and head mesoderm underlie the En-2 expressing cells of the stage 14 embryo (*11, 12*). Any or all of these could be responsible for inducing En-2 expression.

In this study, we recombined various portions of the dorsal mesoderm of the early neurula with ectoderm to determine which mesodermal types are capable of inducing *En-2* (Fig. 1). The recombinants were cultured and subsequently assayed for expression of *En-2*. Competent ectoderm is capable of responding to neural inducing signals coming from mesoderm, but is unable to form neural structures on its own. Competent ectoderm was obtained as animal caps from stage 9 (late blastula) embryos derived from eggs irradiated with ultraviolet (UV) light during the first cell cycle. Eggs irradiated with UV light during the first cell cycle form no dorsal mesoderm and develop as ventralized embryos (*13–15*). Thus, the use of late blastula animal caps derived from eggs irradiated with UV light reduces the chance that the ectoderm is contaminated with dorsal mesoderm. Competent ectoderm almost never expressed the En-2 antigen (2 of 40 cases) when cultured in isolation to control stage 28 (tailbud) (see Fig. 2A). In contrast, ectoderm wrapped around stage 12.5 anterior notochord expressed En-2 in a high percentage of recombinants (81%) (see Figs. 2B and 3). Ectoderm wrapped around posterior notochord expressed En-2 less frequently (36% overall) and less strongly (two of five positives had fewer than 50 nuclei stained with En-2 (Fig.

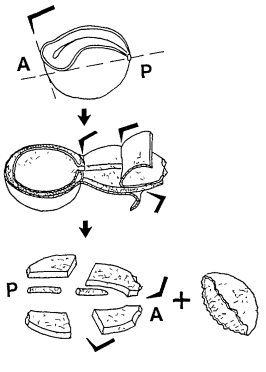

Fig. 1. Schematic of the operations used to generate different recombinants. Stage 12.5 embryos (*26*) were cut with eyebrow knives and hairloops in low calcium and magnesium modified Ringer solution (LCMR) (*15*) so that a "tongue" of dorsal mesoderm, neural plate, and archenteron roof was generated (A, anterior; P, posterior). The cut embryos were then placed in collagenase (0.4 mg/ml) (Cooper Biochemicals) in LCMR to allow the peeling away of the archenteron roof and neural plate. Isolated dorsal mesoderm was placed in 43 mM NaCl, 0.85 mM KCl, 0.37 mM $CaCl_2$, 0.19 mM $MgCl_2$, 5 mM Hepes, pH 7.2, and gentamycin (50 μg/ml) and cut into anterior notochord, posterior notochord, presumptive posterior somites, and presumptive head mesoderm with anterior somites. Each piece was then wrapped in an animal cap from a stage 9 embryo irradiated with UV light [sibling control irradiated embryos displayed ventralized phenotypes, average dorsoanterior index of less than 0.5 (*14, 15*)]. Recombinants were cultured until controls reached stage 28 then fixed and processed for immunohistochemistry (*9*). Results presented are from three independent experiments, where ectoderms from an albino spawning were combined with notochords from a pigmented embryo spawning. Each expeirment yielded the same conclusion, and the numbers from each experiment have been combined for presentation here.

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