Requirement of Type III TGF-β Receptor for Endocardial Cell Transformation in the Heart

Christopher B. Brown,[1] Angelique S. Boyer,[2] Raymond B. Runyan,[2] Joey V. Barnett[3]*

Transforming growth factor–β (TGF-β) signaling is mediated by a complex of type I (TBRI) and type II (TBRII) receptors. The type III receptor (TBRIII) lacks a recognizable signaling domain and has no clearly defined role in TGF-β signaling. Cardiac endothelial cells that undergo epithelial-mesenchymal transformation express TBRIII, and here TBRIII-specific antisera were found to inhibit mesenchyme formation and migration in atrioventricular cushion explants. Misexpression of TBRIII in nontransforming ventricular endothelial cells conferred transformation in response to TGF-β2. These results support a model where TBRIII localizes transformation in the heart and plays an essential, nonredundant role in TGF-β signaling.

Transforming growth factor–β controls cell growth and differentiation and regulates processes as diverse as development, wound healing, atherosclerosis, and tumor progression (*1*). TGF-β signaling is mediated by TBRI and TBRII, both of which contain a serine-threonine kinase domain (*2*). Binding of ligand to TBRII stimulates phosphorylation of TBRI by TBRII and the subsequent activation of members of the Smad family of transcription factors (*3*). TBRIII facilitates the binding of TGF-β2 to the TBRI-TBRII signaling complex (*4*), but TBRIII lacks a recognizable signaling domain (*5*) and has not previously been shown to be necessary for TGF-β signaling.

Here we used explanted chick atrioventricular (AV) cushions (*6*) as an in vitro model of epithelial-mesenchymal transformation to identify the role of TBRIII in TGF-β signal transduction during cardiac development. In the developing heart, endothelial cells that line the lumen in the regions of the AV cushion and outflow tract undergo epithelial-mesenchymal transformation and participate in the formation of the valves and membraneous septa (*7*). TGF-β stimulates AV cushion endothelial cells (*8*), but not ventricular endothelial cells, to transform in vitro. Restricted expression of TGF-β receptors in the endothelium of the heart is one conceivable mechanism to localize TGF-β responsiveness. However, TBRII is expressed by all endothelial cells in the heart and therefore cannot localize TGF-β responsiveness (*9*).

To investigate the role of TBRIII in AV cushion transformation, we generated several polyclonal antisera to the extracellular domain of TBRIII (*10*). Antisera specificity was determined by protein immunoblot analysis and by assays in which TGF-β ligand was cross-linked to overexpressed and native TBRIII proteins followed by immunoprecipitation. Protein immunoblot analysis (*10*) of chick TBRIII overexpressed in COS cells revealed a high molecular weight band of heterogenously glycosylated protein and a 100-kD band of unglycosylated protein core (Fig. 1A, lane 2) that increased after deglycosylation of the homogenate (Fig. 1A, lane 3). Similarly, only immune antisera precipitated a labeled protein from COS cell homogenates prepared from cells transfected with TBRIII cDNA and incubated with ^{125}I-labeled TGF-β1 followed by cross-linking (Fig. 1B, lane 2) (*10*). Cross-linking of ^{125}I–TGF-β1 to chick embryonic fibroblasts followed by immunoprecipitation yielded a similarly sized labeled protein (Fig. 1B, lane 4). The minor bands that coimmunoprecipitated with TBRIII comigrated with cross-linked TBRII and TBRI (*11, 12*). TBRIII antisera did not cross-react with overexpressed TBRII or TBRI protein in protein immunoblot or immunoprecipitation assays (*12*). Thus, the antisera specifically recognized both the overexpressed and native chick TBRIII.

To determine whether TBRIII antisera interfered with ligand binding, we incubated chick embryonic fibroblasts with various concentrations of antisera before the addition of human ^{125}I–TGF-β1 and cross-linking analysis (Fig. 1C) (*10*). Incubation with preimmune antisera had no effect on ligand binding (*10*), whereas immune antisera demonstrated a concentration-dependent decrease in ligand binding with a median effective concentration (EC_{50}) of 10 μg/ml (Fig. 1C). Thus, these antisera were used as a tool to further examine receptor function.

Immunohistochemical localization of TBRIII in the developing cardiovascular system revealed expression by a subpopulation of endothelial cells. In contrast to the readily detectable expression of TBRIII in endothelial and stromal cells of the extraembryonic vasculature (*13*), no intraembryonic endothelial cells outside the heart expressed TBRIII from stage 10 through stage 19. TBRII is expressed by endothelial cells in the extraembryonic vasculature (*13*), intersomitic vessels, and neural plexus (Fig. 2, A and C), whereas TBRIII is not detected in the latter two sites (Fig. 2, B and D). However, TBRIII is detected on the endocardial cells overlying the AV cushion (Fig. 2F) and on at least a subset of migrating mesenchymal cells. This pattern of expression is consistent with a role for TBRIII in localizing and mediating the effects of TGF-β on epithelial-mesenchymal transformation in the AV cushion in vivo.

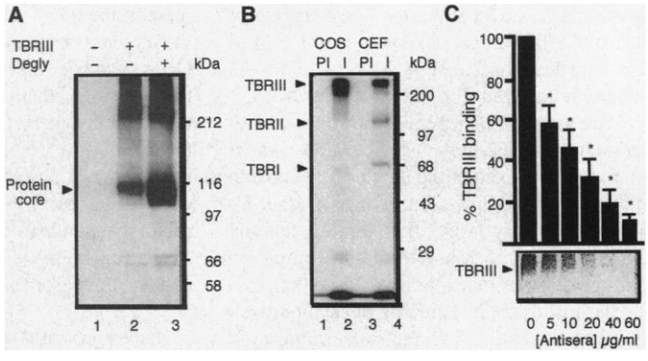

Fig. 1. Antisera to the TBRIII extracellular domain inhibit ligand binding. (**A**) Protein immunoblot analysis with antiserum κ to COS cell homogenates after transfection with vector alone (lane 1) or TBRIII without (lane 2) or with (lane 3) deglycosylation (degly). (**B**) Immunoprecipitations with preimmune (PI) or immune (I) κ of COS cells transfected with TBRIII (lanes 1 and 2) or chick embryonic fibroblasts (CEF) (lanes 3 and 4) after they were cross-linked with ^{125}I–TGF-β1. Relative molecular weights in kilodaltons are indicated to the right of each panel. (**C**) Effect of incubation with immune antisera on ligand binding in chick embryonic fibroblasts. The bar graph shows the concentration-dependent blockade of ^{125}I–TGF-β1 binding to TBRIII in chick embryonic fibroblasts by TBRIII antisera (*10*). The experiment depicted along the *x* axis was repeated three times, and the mean decrease in binding is depicted (*$P < 0.05$).

[1]Department of Pharmacology, Vanderbilt University Medical Center, 1161 21st Avenue South, Nashville, TN 37232–6600, USA. [2]Department of Cell Biology and Anatomy, University Heart Center, University of Arizona, 1501 North Campbell Avenue, Tucson, AZ 85724, USA. [3]Departments of Medicine (Division of Cardiovascular Medicine) and Pharmacology, Vanderbilt University Medical Center, 2200 Pierce Avenue, Nashville, TN 37232–6300, USA.

*To whom correspondence should be addressed. E-mail: joey.barnett@mcmail.vanderbilt.edu

To determine the functional role of TBRIII in AV cushion transformation, we tested the TBRIII antisera for their effects on mesenchyme formation as a measure of AV cushion transformation in vitro (*6*). AV cushion explants were obtained from stage 14, 16, and 18 chick embryos and incubated on three-dimensional, hydrated collagen gels with the addition of either preimmune or immune antisera (10 μg/ml) (*14*). In explants incubated with preimmune antisera, the endocardial cells formed a monolayer and, under the influence of the inductive AV cushion myocardium, underwent a specific activation, invasion of the matrix, and subsequent migration that together constitute epithelial-mesenchymal transformation. The activation step was characterized by a change from closely packed polygonal cells to a loose association of elongated cells (Fig. 3A).

In contrast, explants incubated with TBRIII antisera demonstrated fewer mesenchymal cells within the collagen matrix and an abundance of densely packed, polygonal or cobblestone-shaped endocardial cells on the matrix surface (Fig. 3B). Data from multiple explants demonstrate a greater than 70% decrease in mesenchyme formation at each stage examined (Fig. 3C). Further studies with stage 18 explants investigated whether blockade of TBRIII had an effect on the migration rate of mesenchymal cells. Analysis of the migration rate of mesenchymal cells in the collagen matrix indicated a 50% decrease in migration rate (Fig. 3D). These data indicate that TBRIII is necessary for mesenchyme formation and mesenchymal cell migration.

To determine whether the misexpression of TBRIII was sufficient to confer TGF-β–mediated transformation on non–AV cushion endothelial cells, a retroviral vector encoding either TBRIII (rTBRIII) or alkaline phosphatase (rAP) was used to infect ventricular explants in vitro. Ventricular endothelial cells express TBRII (*9*) and TBRI, but do not express TBRIII (*13*) or transform in response to TGF-β1, TGF-β2, or AV cushion myocardium (*6*). Retrovirally infected cells were identified and scored as having an endothelial or mesenchymal morphology. Analysis of infected explants after the addition of vehicle or TGF-β2 demonstrated that 40% of the total cells infected with rAP were mesenchymal (Fig. 4) and most likely represented AV cushion endothelial cells present in the explant that had been infected with virus. In control experiments, the addition of TGF-β1 or TGF-β2 to uninfected ventricular explants did not increase mesenchyme formation (*15*). Similarly, explants infected with rTBRIII did not demonstrate an increase in infected cells that were mesenchymal when compared with rAP (Fig. 4). However, explants infected with rTBRIII and incubated with TGF-β2 demonstrated a dramatic increase in the percentage of infected cells that were mesenchymal (Fig. 4). TGF-β2 was added because ventricular myocardium alone does not support transformation (*6*). Thus the expression of TBRIII in normally unresponsive endothelial cells results in mesenchyme formation in response to TGF-β2 and supports an essential role for TBRIII in mediating AV cushion transformation.

Because both TBRIII and TBRII (*9*) are colocalized in AV cushion endothelial cells, and each is required for transformation, we propose that a complex of at least TBRIII and TBRII mediates transformation and that the restricted expression of TBRIII localizes this response. TBRIII has been reported to present ligand to the TBRI-TBRII signaling complex

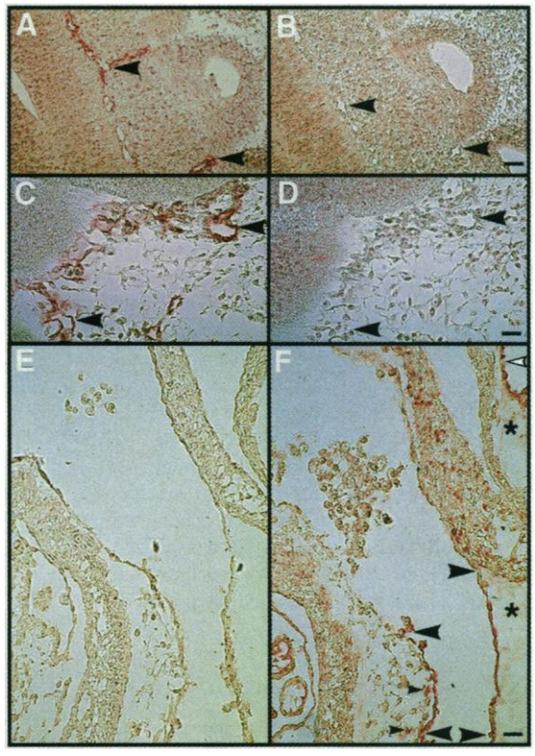

Fig. 2. Immunolocalization of TBRIII in the developing cardiovascular system. Expression of TBRII or TBRIII by endothelial cells (arrows) of the intersomitic vessels (**A** and **B**) and neural plexus (**C** and **D**), respectively. Endothelial cells lack TBRIII expression. (**E** and **F**) Adjacent sections of the AV cushion region of a stage 18 embryo incubated with preimmune or immune antiserum κ. TBRIII is expressed by endothelial cells overlying the AV cushion (demarcated by black arrows), migrating mesenchymal cells (small black arrows), and endocardial cells of the outflow tract (white arrow). Extracellular matrix is indicated by asterisks. For immunostaining of the sections, embryos were fixed in Bouin's solution or 4% paraformaldehyde, embedded in paraffin, and cut in 5-μm-thick sections (*11*). Primary antibody concentration was a 1:100 dilution. Protein was visualized with a 1:1000 dilution of alkaline phosphatase–coupled anti–rabbit immunoglobulin G (Sigma) and Fast Red Naphthol color substrate (Sigma). Sections were photographed with brightfield optics on a Zeiss Axiophot microscope. Bars depict 25 μm.

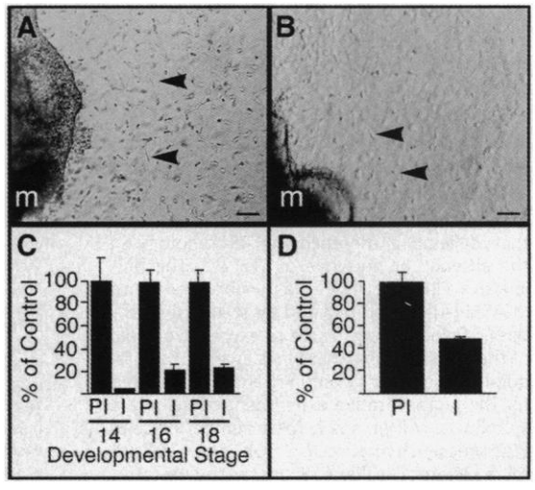

Fig. 3. Blockade of transformation by antisera to TBRIII. (**A**) An explant incubated with preimmune (PI) antiserum. (**B**) Explant incubated with immune (I) antiserum κ. The myocardial portion of the explant is labeled (m); endothelial cells form a monolayer on the gel surface. Each explant was optically sectioned and the number of mesenchymal cells determined. In all panels arrows indicate representative mesenchymal cells. Bars in (A) and (B) depict 15 μm. (**C**) Quantification of mesenchymal cells in κ-incubated explants expressed as a percent of the number of mesenchymal cells in PI-incubated explants. Error bars represent mean ± SEM, and all comparisons were significant when compared with PI ($P < 0.05$). The number of explants in each group and the mean number of mesenchymal cells in I-incubated (antiserum κ) explants, expressed as a percent of the mean number of mesenchymal cells in PI-incubated explants, were as follows: (stage 14; PI, $n = 9$; I, $n = 8$; 1%), (stage 16; PI, $n = 17$; I, $n = 19$; 19%), and (stage 18; PI, $n = 16$; I, $n = 14$; 19%). (**D**) Average lateral migration rate of mesenchymal cells for stage 18 explants. Representative experiment is of the lateral migration rates within a field of 10 (I) or 11 (PI) cells. Error bars represent mean ± SEM and were significant when compared with PI controls ($P < 0.05$).

(*4*) and is essential for the high-affinity binding of TGF-β2 (*16*) by the TBRI-TBRII signaling complex. If TBRIII solely functions to present TGF-β2 to the signaling complex, it is unclear why the addition of TGF-β1, which will directly activate the TBRI-TBRII signaling complex (*2*), does not result in transformation of ventricular endothelial cells. This suggests a distinct role for TBRIII signaling in endothelial cell transformation.

TBRIII may signal in response to the ligand TGF-β2. In support of this idea, TGF-β2 has a restricted pattern of expression in the AV cushion and outflow tract of both the chick and mouse heart during AV cushion morphogenesis (*17*), and homozygous tgf-β2 null mice (*18*) display a phenotype that includes cardiac defects that does not overlap with the phenotype of either tgf-β1 or tgf-β3 null mice (*19*). We propose that the binding of TGF-β2 to TBRIII alters the composition or activity of the TBRI-TBRII signaling complex to activate a unique set of downstream mediators that results in AV cushion transformation. The guanosine triphosphate–binding protein Ras and the transcription factor NF-ATc have recently been demonstrated to play a role in valvulogenesis. In mouse AV cushion explants, activated Ras potentiates transformation and inactive Ras mutants block transformation in a pathway modulated by neurofibromin (*20*). NF-ATc is localized to endothelial cells of the AV cushion and outflow tract. After the targeted disruption of NF-ATc, AV cushion transformation occurs with subsequent defects in the outflow tract valves and the ventricular septum (*21*). Whether alterations in TBRIII underlie specific cardiac defects is unknown, but defects in human AV cushion morphogenesis have recently been linked to a region of chromosome 1 near the gene encoding TBRIII (*22*).

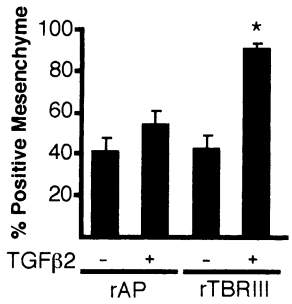

Fig. 4. Misexpression of TBRIII in ventricular endothelial cells promotes transformation. The graph shows the percent of virally infected cells that develop a mesenchymal morphology in the absence or presence of TGF-β2. The full-length TBRIII cDNA was subcloned into RCASBP(A) and propagated by standard methods (*24*) and demonstrated to express a protein of the predicted size that binds ligand. The total number of explants examined and cells counted for the representative experiment depicted are as follows: rAP (n = 23; total number of cells, 236; mesenchymal cells, 96); rAP+TGF-β2 (n = 26; total cells, 678; mesenchymal cells, 319); rTBRIII (n = 25; total cells, 636; mesenchymal cells, 227); and rTBRIII+TGF-β2 (n = 26; total cells, 677; mesenchymal cells, 623). Error bars represent mean ± SEM and only the TBRIII + TGF-β2 group was significant (P < 0.05) when compared with TBRIII alone. Representative of three experiments.

References and Notes

1. A. B. Roberts and M. B. Sporn, in *Peptide Growth Factors and their Receptors*, M. B. Sporn and A. B. Roberts, Eds. (Springer-Verlag, New York, 1990), vol. 1, pp. 419–472.
2. J. L. Wrana, L. Attisano, R. Wieser, F. Ventura, J. Massague, *Nature* **370**, 341 (1994); C. H. Bassing *et al.*, *Science* **263**, 87 (1994).
3. M. Kretzschmar and J. Massague, *Curr. Opin. Genet. Dev.* **8**, 103 (1998).
4. F. Lopez-Casillas, J. L. Wrana, J. Massague, *Cell* **73**, 1435 (1993).
5. F. Lopez-Casillas *et al.*, *ibid.* **67**, 785 (1991); X.-F. Wang *et al.*, *ibid.*, p. 797.
6. R. B. Runyan and R. R. Markwald, *Dev. Biol.* **95**, 108 (1983).
7. L. M. Eisenberg and R. R. Markwald, *Circ. Res.* **77**, 1 (1995); M. C. Fishman and K. R. Chien, *Development* **124**, 2099 (1997).
8. J. D. Potts and R. B. Runyan, *Dev. Biol.* **134**, 392 (1989).
9. C. B. Brown, A. S, Boyer, R. B. Runyan, J. V. Barnett, *ibid.* **174**, 248 (1996).
10. The cDNA coding regions from amino acid 154 to 439, termed a, and 31 to 150, termed b, of the TBRIII extracellular domain (*17*) were subcloned into pGEX-3X (Pharmacia) for glutathione S-transferase fusion protein production. Antisera were generated as previously described (*9*) and designated as follows: a, κ and λ; b, 5681 and 5682. In all assays equal amounts of either purified preimmune (PI) immunoglobulin G or purified immune (I) antisera were used and each antiserum gave indistinguishable results when compared. For protein immunoblot analysis, COS cells were transfected with chick TBRIII (*17*), and analysis was performed on l00 μg of total protein per lane (*9*). Cell lysis buffer contained 10 mM tris, 50 mM sodium acetate, pH 7.5, 1% Triton X-100, 0.2 mM EDTA, 100 μg/ml each of soybean trypsin inhibitor, benzamidine HCl, and leupeptin, and 1 mM phenylmethylsulfonyl fluoride. For deglycosylation, samples were diluted 1:5 with Triton X-100–free lysis buffer and incubated overnight with chondroitinase ABC (2 U/ml), heparitinase (2 U/ml), and *N*-glycosidase F (5 U/ml). Primary antibody dilution was 1:100. Secondary antibody coupled to horseradish peroxidase was diluted 1:1000 and visualized by chemiluminescence (Renaissance, NEN-DuPont). For immunoprecipitations, cells were lysed in 1% Triton X-100, 10 mM tris, pH 7.4, and 1 mM EDTA. Equal volume samples of cellular extracts were incubated with 1 μg of PI or I antisera for 1 hour at 4°C before the addition of 100 μl of 10% protein A–Sepharose beads and incubated overnight with mixing at 4°C. Beads were pelleted, washed six times with cold lysis buffer, and boiled in SDS loading buffer. Samples were resolved on 6% SDS-polyacrylamide gels, dried, and subjected to autoradiography. For cross-linking, COS cells transiently transfected with TBRIII or chick embryonic fibroblasts were cross-linked with 50 pM ^{125}I–TGF-β1 (NEN-DuPont) as described previously (*17*) after incubation with PI or I antisera (5 to 60 μg/ml). Specific binding was determined by the addition of 500 pM TGF-β1 or TGF-β2. Equal volumes of cell lysates were added per gel lane for cross-linking experiments or per sample for immunoprecipitation studies. For cross-linking experiments, the resultant banding patterns were examined by autoradiography and quantified by Phosphorimage analysis. Preimmune antisera had no effect on ligand binding.
11. A. Moustakas *et al.*, *J. Biol. Chem.* **268**, 22215 (1993); S. Sankar, N. Mahooti-Brooks, M. Centrella, T. L. McCarthy, J. A. Madri, *ibid.* **270**, 13567 (1995).
12. C. B. Brown and J. V. Barnett, unpublished data.
13. Supplementary material is available at http://www.sciencemag.org/feature/data/985904.shl.
14. AV cushion explants from staged chick embryos (*23*) were cultured as described (*8, 9*). Six hours after explantation either PI or I antisera (10 μg/ml) was added, the incubation continued for 16 to 18 hours, and the number of mesenchymal cells in the collagen gel determined for each explant. The percentages reported are the mean number of cells in the gel in I-incubated explants divided by the mean number of cells in the gel in PI-incubated explants at each stage. The mean number of cells in each group, the SEM, and the number of explants examined from each stage is as follows: stage 14 (PI 53 ± 18, n = 9; I 1 ± 2, n = 8), stage 16 (PI 172 ± 23, n = 17; I 33 ± 8, n = 18), stage 18 (PI 131 ± 19, n = 16; I 25 ± 4, n = 14); P < 0.05. To determine cell migration rate, we placed stage 18 explants on the collagen gel without the addition of antiserum for 18 to 24 hours. Next, individual mesenchymal cells were video recorded for a 2-hour period, beginning 45 min after the addition of antiserum, and the migration distance of individual cells subjected to Bioquant (Rand M Biometrics, Nashville, TN) analysis. For retroviral experiments, stage 14 ventricular explants were harvested and incubated for 1 hour with concentrated retrovirus (titer 10^8 to 10^9 plaque-forming units per milliliter) (*24*) and placed on collagen gels. After 12 hours vehicle or 200 pM TGF-β2 was added and each explant analyzed as above after 24 hours. Cells were identified as virally infected by AMV3C2 antibody staining or alkaline phosphatase activity (*25*). The number of infected mesenchymal cells was divided by the total number of infected cells. All mean comparisons by Student's t test.
15. Ventricular explants were assayed for mesenchyme production, and no significant difference was noted between groups. The number of explants examined were as follows: vehicle addition (n = 26), TGF-β1 (n = 19), and TGF-β2 (n = 22).
16. F. Lopez-Casillas, H. M. Payne, J. L. Andres, J. Massague, *J. Cell Biol.* **124**, 557 (1994); H .Y. Lin *et al.*, *J. Biol. Chem.* **270**, 2747 (1995).
17. M. C. Dickson, H. G. Slager, E. Duffie, C. L. Mummery, R. J. Akhurst, *Development* **117**, 625 (1993); J. V. Barnett *et al.*, *Dev. Dyn.* **199**, 12 (1994).
18. L. P. Sanford *et al.*, *Development* **124**, 2659 (1997).
19. M. M. Shull *et al.*, *Nature* **359**, 693 (1992); A. B. Kulkarni *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 770 (1993); G. Proetzel *et al.*, *Nature Genet.* **11**, 409 (1995).
20. M. H. Lakkis and J. A. Epstein, *Development* **125**, 4359 (1998).
21. J. L. de la Pompa *et al.*, *Nature* **392**, 182 (1998); A. M. Ranger *et al.*, *ibid.*, p. 186.
22. D. W. Johnson, M. Qumsiyeh, M. Benkhalifa, D. A. Marchuk, *Genomics* **28**, 356 (1995); V. C. Sheffield *et al.*, *Hum. Mol. Genet.* **6**, 117 (1997).
23. V. Hamburger and H. L. Hamilton, *J. Morphol.* **88**, 49 (1951).
24. B. A. Morgan and D. M. Fekete, *Methods Cell Biol.* **51**, 185 (1996).
25. W. M. Potts, M. Olsen, D. Boettiger, V. Vogt, *J. Gen. Virol.* **68**, 3177 (1987); supplied by the Developmental Studies Hybridoma Bank, University of Iowa.

26. We thank R. Emeson, A. George, L. Limbird, B. Hogan, C. Wright, and members of the Barnett laboratory for critical reading of the manuscript, M. Cleggett for technical assistance, C. Cepko for RCASBP containing alkaline phosphatase, and H. Moses for thoughtful discussion. Supported by grants HL52922 (J.V.B.), 38649 (R.B.R.), 42266 (R.B.R.), and FY98-0446 from the March of Dimes Birth Defects Foundation (J.V.B.). C.B.B. was supported by an institutional training grant from the NIH General Medical Sciences number GM07628. J.V.B. and R.B.R. are Established Investigators of the American Heart Association. J.V.B. wishes to acknowledge the support of the Vanderbilt Cancer Center, NCI CA68485.

14 October 1998; accepted 22 February 1999