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BRIEF COMMUNICATIONS

Pax3 and *Dach2* Positive Regulation in the Developing Somite

G. KARDON, T.A. HEANUE, AND C.J. TABIN*
Department of Genetics, Harvard Medical School, Boston, Massachusetts

ABSTRACT **In vertebrates, skeletal muscles of the body arise from cells of somitic origin. Recently, somite culture experiments have identified a set of genes, including *Pax3, Six1, Eya2*, and *Dach2*, that appear to play an important role in early myogenesis during somite development (Heanue et al. [1999] Genes Dev. 13:3231–3243). In somite culture *Pax3, Six1, Eya2*, and *Dach2* not only function to activate myogenesis, but they form a complex network regulating each other's transcription. We sought to examine whether this putative *Pax3/Six1/Eya2/Dach2* network of regulation actually functions in vivo. In particular, we tested whether *Pax3* and *Dach2* participate in a positive regulatory feedback loop in vivo as they do in culture. To test in vivo *Pax3/Dach2* interregulation, we took advantage of the known dependence of both factors on ectodermal signals. Somites isolated from the overlying ectoderm lose expression of *Pax3* and *Dach2*. Therefore, we attempted to rescue *Pax3* or *Dach2* expression in somites isolated from the ectoderm by retroviral misexpression of the complementary factor. Indeed misexpression of *Pax3* or *Dach2* resulted in rescue of *Dach2* or *Pax3*, respectively. These rescue experiments demonstrate that *Pax3* and *Dach2* positively regulate each other's expression in vivo and support the validity of the *Pax3/Six1/Eya2/Dach 2* network in regulating myogenesis.**

Key words: *Pax3*; *Dach2*; somite; myogenesis

INTRODUCTION

In vertebrates, all skeletal muscles of the body (trunk and limb) as well as some head muscles arise from somites, segmentally organized structures flanking the neural tube (reviewed in Brand-Saberi and Christ, 2000). During development, somites bud off from the cranial end of the presomitic mesoderm (psm) to form epithelial balls of tissue. In response to signals from the adjacent notochord and neural tube and overlying ectoderm, the somites become regionalized to form dorsal dermomyotome and ventral sclerotome (reviewed in Borycki and Emerson, 2000). The dermomyotome, in turn, gives rise to skeletal muscle. Cells in the medial dermomytome give rise to the epaxial (deep back) muscles, and lateral cells give rise to the hypaxial muscles, which form the body wall and limb muscles (Ordahl and Le Douarin, 1992). In both epaxial and hypaxial muscle, the expression of the muscle-specific helix-loop-helix transcription factors *Myf5* and *MyoD* marks the initiation of the myogenic differentiation program (reviewed in Buckingham, 2001).

Recently, several transcriptional regulators, including members of the *Pax, Six, Eya*, and *Dach* gene families, have been shown to play important roles in early myogenesis during somite development (reviewed in Relaix and Buckingham, 1999). Important clues to how these factors function together came from *Drosophila*, in which their homologues, *eyeless* (*ey*), *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*), are critical for eye development. A large body of work has now established that these genes form a complex regulatory network, including positive feedback regulation of each others' expression (reviewed in Wawersik and Maas, 2000). Therefore, when their respective homologues *Pax, Six, Eya*, and *Dach* genes were found expressed in overlapping patterns in vertebrates, this finding suggested that these factors might operate in a similar regulatory network. The expression of several gene family members in the somite suggested that these genes might function in vertebrate myogenesis. In particular, *Pax3* and *7*; *Six1* and *4*; *Eya1, 2*, and *4*; and *Dach1* and *2* are all expressed in the dorsal somite before the expression of *Myf5* and *MyoD* (Jostes et al., 1990; Williams and Ordahl, 1994; Oliver et al., 1995; Xu et al., 1997; Mishima and Tomarev, 1998; Borsani et al., 1999; Esteve and Bovolenta, 1999; Heanue et al.,

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Drs. Kardon and Heanue contributed equally to this work.
Dr. Heanue's present address is Department of Molecular Biology, National Institute of Medical Research, NW7 1AA London, England.
*Correspondence to: C.J. Tabin, Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115.
E-mail: tabin@rascal.med.harvard.edu
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1999). Preliminary studies indicate that these genes may indeed form a regulatory network similar to that found in *Drosophila* (Heanue et al., 1999).

The best understood of these factors is *Pax3*. *Pax3* is necessary for the migration of muscle precursor cells from the somites. Homozygous *splotch* mice, which carry a mutation in *Pax3*, lack migrating muscle populations, resulting in the absence of limb, diaphragm, tongue, ventral body wall, and some shoulder muscles (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994; Tajbakhsh et al., 1997). Moreover, analysis of mice doubly homozygous mutant for *Pax3* and *Myf5* suggests that *Pax3* is necessary for the activation of *MyoD* (Tajbakhsh et al. 1997; but see Mankoo et al., 1999). Not only is *Pax3* necessary but it is sufficient to induce myogenesis when ectopically expressed in several explanted tissues, including somites (Maroto et al., 1997).

Recent experiments have revealed that *Six1, Eya2*, and *Dach2* synergistically regulate myogenesis. In somite cultures, ectopic expression of *Dach2* with *Eya2* or *Eya2* with *Six1* is able to induce *MyoD* and *Myosin Heavy Chain*, whereas ectopic expression of *Dach2, Eya2*, or *Six1* alone is unable to induce these myogenic markers (Heanue et al., 1999). This synergistic regulation of myogenesis by *Dach2* with *Eya2* and by *Eya2* with *Six1* is underlain by protein-protein interactions (Heanue et al., 1999). Furthermore, particular combinations of Six and Eya proteins have been found to synergistically activate another myogenic regulatory gene, *Myogenin*, by means of binding to its MEF3 binding site (Spitz et al., 1998; Ohto et al., 1999).

Not only do the *Pax, Six, Eya*, and *Dach* genes regulate myogenesis, but they regulate each others' expression in somites cultured in vitro. In somite culture experiments (Heanue et al., 1999), ectopic expression of *Pax3* leads to the up-regulation of *Dach2* and *Eya2* expression. In turn, ectopic expression of *Six1, Eya2*, or *Dach2* is able to induce *Pax3*. Moreover, ectopic expression of *Dach2* with *Eya2* or *Eya2* with *Six1* in somite culture is able to strongly up-regulate *Pax3*. Thus *Dach2* with *Eya2* or *Eya2* with *Six1* synergistically regulate *Pax3* expression. These somite culture experiments have led to the model of the regulatory relationships between *Pax, Six, Eya*, and *Dach* genes shown in Figure 1.

Despite this in vitro evidence, there is reason to question whether these regulatory networks actually play a role in vivo in controlling myogenesis. Members of the *Pax, Six, Eya*, and *Dach* families have been shown to be coexpressed in other regions of the vertebrate embryo, such as the developing eye and ear, but genetic experiments failed to demonstrate any interdependence in their expression (Heanue et al., 2002). Moreover, in the case of myogenesis in the somites, neither misexpression of *Pax3* or *Dach2* in vivo (Heanue et al., 1999) has any effect on myogenic cell fates or on the expression of *Pax, Six, Eya*, or *Dach* genes. The lack of ectopic myogenesis could be explained by the strong influence of other factors produced by the surrounding tissues that limit myogenesis to the proper dermomytomal domains in vivo (reviewed in Borycki and Emerson, 2000). Although this explanation seems reasonable, it remains critical to demonstrate that the *Pax3*/*Six1*/*Eya2*/*Dach2* network functions in vivo during myogenesis in the somite.

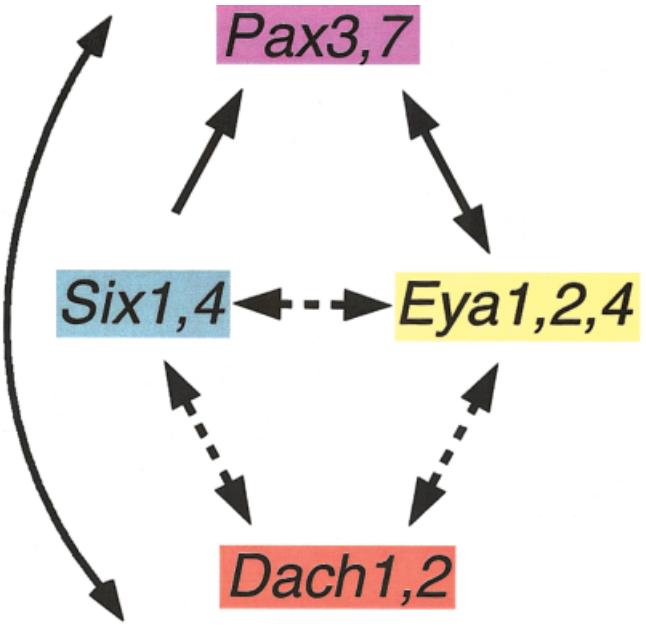

Fig. 1. Model of regulatory relationships between *Pax, Six, Eya*, and *Dach* genes in the developing vertebrate somite based on somite culture experiments (Heanue et al., 1999). This model is based on ectopic misexpression experiments in chick with *Pax3, Six1, Eya2*, and *Dach2*. *Pax7, Eya1* and *4*, and *Dach1* are expressed in the developing somite, but their regulatory relationships have not been explicitly tested. Solid arrows represent regulatory relationships between *Pax3, Six1, Eya2*, and *Dach2* that have been verified experimentally. Dashed arrows represent relationships not yet tested but theorized on the basis of data from *Drosophila*.

In this study, we test whether one component of the network, the positive regulatory relationship between *Pax3* and *Dach2*, functions in vivo in the developing somite. We took advantage of previous studies (Dietrich et al., 1997, 1998; Heanue et al., 1999) that have shown that lateral dermomyotomal expression of *Pax3* and *Dach2* depends on signals from the ectoderm. We blocked contact of the somites with the ectoderm and attempted to rescue endogenous *Pax3* or *Dach2* expression by retroviral misexpression of *Dach2* or *Pax3*. Indeed misexpression of *Pax3* or *Dach2* resulted in rescue of *Dach2* or *Pax3*, respectively. These experiments demonstrate that *Pax3* and *Dach2* can positively regulate each other's expression in vivo.

RESULTS AND DISCUSSION

Misexpression of *Pax3* or *Dach2* in Presomitic Mesoderm or Somites Does not Alter *Dach2* or *Pax3* Expression

We first attempted to test for in vivo *Pax3*/*Dach2* regulation by simply misexpressing *Pax3* or *Dach2* by

means of injection of replication-competent retroviral vectors (RCAS) transducing these genes into psm or recently formed somites. Embryos harvested 36 hr after injection and analyzed by in situ hybridization showed no alteration in *Dach2* or *Pax3* expression (Fig. 2A,B). The lack of *Pax3* or *Dach2* up-regulation in the ventral result is agreement with previous observations (Heanue et al., 1999). Such a result may be due to the strong influence of *Shh* from the notochord and floorplate that inhibits myogenesis in the ventral somite (reviewed in Borycki and Emerson, 2000).

Signals from the Ectoderm Are Required for Normal *Pax3* and *Dach2* Expression in the Dermomyotome

One successful experimental approach to avoiding the normally tight regulation of somite development is to manipulate somites in culture; however, this strategy leaves open the question of in vivo relevance. Here, we present an in vivo approach to dissecting this complex system that takes advantage of knowledge of the factors influencing somite differentiation. The ectoderm overlying the developing somite has been shown to regulate patterning of the dorsal somite (reviewed in Borycki and Emerson, 2000). In the absence of ectodermal signals (perhaps members of the Wnt family; see Fan et al., 1997, and review in Borycki and Emerson, 2000), *Pax3* and *Dach2* are down-regulated in the dermomyotome (Fan and Tessier-Lavigne, 1994; Dietrich et al., 1997, 1998; Heanue et al., 1999). In agreement with these previous studies, we found that dermomyotomal expression of *Pax3* and *Dach2* depends on signals from the ectoderm. A barrier inserted between the ectoderm and the psm or recently formed somites had no gross morphologic effect on somite growth. Indeed when harvested, the somites of these embryos appear indistinguishable on the experimental and control sides. However, in situ hybridization revealed that this manipulation results in down-regulation of both *Pax3* and *Dach2* (Fig. 2C,D), although somite growth was otherwise normal. In our experiments, we frequently found that both lateral and medial *Pax3* and *Dach2* expression were lost due the barrier inhibition of signals from both the ectoderm (leading to loss of lateral expression, in agreement with Dietrich et al., 1997, 1998; Heanue et al. 1999) and the neural tube (leading to loss of medial expression, in agreement with Dietrich et al. 1997).

In the Absence of Ectodermal Signals, Ectopic *Pax3* and *Dach2* Can Rescue Each Other's Expression in Somites

Isolation of the somite from the overlying ectoderm and the resulting down-regulation of *Pax3* and *Dach2* gene expression allowed us to test whether misexpression of one gene was capable of rescuing expression of the other, by using RCAS vectors to ectopically express *Pax3* or *Dach2*. It proved technically demanding to target the infections to the regions blocked by the barriers. In experiments where *Dach2* was misexpressed but the infection as visualized by staining by 3C2 missed the targeted region (brown staining, Fig. 2C), down-regulation of *Pax3* was still observed. However, in cases where the misexpression of *Dach2* was successfully targeted to the barrier regions (n = 4/24), *Pax3* expression was observed in the lateral dermomyotmal region that had been covered by a barrier (Fig. 2E,G). This expression corresponded to the domains of the lateral dermomyotome, which were infected with the *Dach2* retrovirus (compare purple *Pax3* expression with orange 3C2 stain, Fig. 2E,G). Note that, because the retrovirus is replication-competent and continues to spread in the injected embryo, tissue outside the dermomyotome is also infected. These other tissues do not up-regulate *Pax3*, as expected from the lack of response to virus injected into intact somites (see above). In rescue experiments involving *Pax3* misexpression, we observed *Dach2* expression in the lateral dermomyotomal regions that had been covered by the barrier (n = 2/11) (Fig. 2F,H). The patches of *Pax3* or *Dach2* expression observed in these experiments were found in dermomytomal regions but clearly had irregular borders and did not exhibit a normal, wild-type expression pattern (Fig. 2E–H). Such patches were never seen when the barrier was inserted alone. Moreover, because of the irregular expression domains of *Pax3* or *Dach2*, we could be certain that this expression was due to rescue and not simply due to a failure of barrier inhibition of ectodermal signals. The rescue of dermomyotomal *Pax3* or *Dach2*, down-regulated in the absence of ectodermal signals, by RCAS *Dach2* or *Pax3* demonstrates that in vivo *Pax3* and *Dach2* positively regulate each other's expression. This finding result strongly suggests that the regulatory gene network defined in the somite culture system is important for normal myogenic regulation.

Previous somite culture experiments demonstrated that the ability of *Dach2* to up-regulate *Pax3* expression was improved when *Dach2* was misexpressed in combination with *Eya2* (Heanue et al., 1999). We might expect such synergistic action of *Dach2* and *Eya2* to be similarly observed in vivo. By using the rescue protocol described here, it would be possible to directly test for synergy of *Dach2* and *Eya2* to regulate *Pax3* expression, by misexpressing *Dach2* together with *Eya2* (by means of RCAS(A) and RCAS(B) retroviral coinjections; Morgan and Fekete, 1996). If *Dach2* and *Eya2* synergistically regulate *Pax3* in vivo, a more pronounced rescue of *Pax3* expression would be expected. In the future, these and other aspects of the *Pax3*/*Six1*/*Eya2*/*Dach2* network identified in vitro can be confirmed in vivo by using the approach presented here. In addition, barriers isolating the somite from other in vivo influences (e.g., notochord or lateral plate) could allow other aspects of the regulation of myogenesis to be examined.

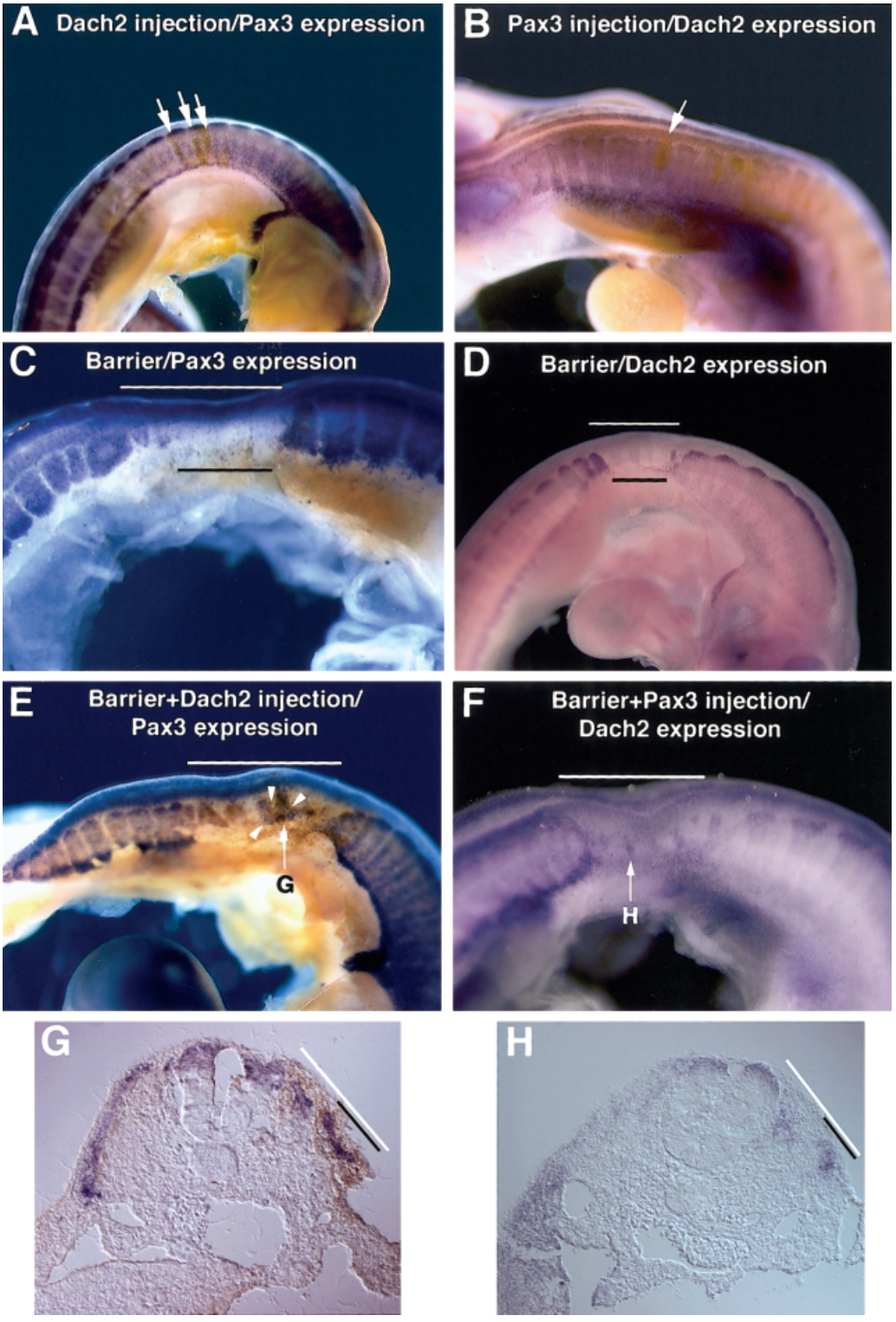

Fig. 2. In vivo manipulation of *Pax3* and *Dach2* expression. **A,B:** Replication-competent retroviral vector (RCAS) misexpression of *Dach2* or *Pax3* does not lead to ectopic expression of *Pax3* or *Dach2*. 3C2 antibody staining in brown shows location of virus, and *Pax3* or *Dach2* expression is shown in purple. Arrows show regions of RCAS misexpression of either *Dach2* (A) or *Pax3* (B) that do not result in ectopic *Pax3* (A) or *Dach2* (B) expression. **C,D:** Ectodermal signals are required for *Pax3* and *Dach2* somitic expression. White bars show position of barriers, which were removed before processing for in situ hybridization. No differences were observed in the morphology of the somites on the experimental and control sides of the embryo before in situ hybridization and immunohistochemistry. Black bars show regions of down-regulation of somitic *Pax3* (C) or *Dach2* (D). In these two examples, the barrier interfered with ectodermal signals, leading to loss of lateral *Pax3* or *Dach2* expression, and interfered with neural tube signals, leading to loss of medial *Pax3* or *Dach2* expression. The loss of endogenous *Pax3* or *Dach2* expression was seen either when the embryo was not infected (as in D) or when injected *Dach2* or *Pax3* virus failed to infect the domain blocked by the barrier (as in C, 3C2 staining in brown shows virus anterior to barrier). **E–H:** *Dach2* or *Pax3* can rescue down-regulation, in the absence of ectodermal signals, of *Pax3* (E,G) or *Dach2* (F,H). White bars show placement of barriers, which have been removed before processing. Again, no gross morphologic changes were observed in the somites as a result of the manipulations. Arrows show rescued *Pax3* (E) or *Dach2* (F) in purple. 3C2 staining in brown in E shows regions of *Dach2* viral infection, which coincide with locations of patchy *Pax3* expression. 3C2 staining was not carried out in the example shown in F so as not to obscure the lower level of *Dach2* expression. Sections through specimens in E and F (indicated with labeled arrows) are shown in G and H, respectively. The rescued *Pax3* and *Dach2* expression is somitic, but has irregular borders of expression not seen in wild-type embryos.

EXPERIMENTAL PROCEDURES

Embryo Surgery and Virus Injection

Fertilized chick eggs were obtained from SPAFAS (Norwich, CT), incubated to specified stages, and windowed before surgery. Three treatments of the psm and somites I-III (Christ and Ordahl, 1995) on stage 10–12 embryos were performed: (1) misexpression of *Dach2* or *Pax3* in somitic cells by means of injection of RCAS containing the genes of interest, (2) isolation of somitic tissue from ectoderm by placement of barriers, and (3) combination of misexpression of *Dach2* or *Pax3* and isolation from ectoderm of somitic tissue. For misexpression experiments, RCAS virus was injected into the somites with a micropipette and Hamilton syringe. For barrier experiments, ectoderm was separated from the underlying psm and somites by application of a small amount of pancreatin to loosen the tissue and teasing away the ectoderm with a sharpened tungsten needle. Fifteen-micron-thick cellophane barriers were then placed on top of the psm and somites, and the ectoderm was allowed to grow over the barrier. For combination experiments, the ectoderm was first removed, then virus was injected, and finally the barrier was placed. In all experiments, embryos were sealed and incubated for an additional 36 hr, fixed in 4% paraformaldehyde, and analyzed by whole-mount RNA in situ hybridization and antibody staining (see below). Before fixation, the position of the barrier and the morphology of the somites were recorded and then the barrier was removed.

RCAS Virus Construction

Viral constructs were generated and high titer virus was produced following the protocols of Logan and Tabin (1998). Both the *Pax3* and *Dach2* RCAS constructs have been described previously (Maroto et al., 1997; Heanue et al., 1999).

Whole-Mount In Situ RNA Hybridization and Antibody Staining

Whole-mount RNA in situ hybridization with nonradioactive riboprobes was performed as described previously (Riddle et al., 1993). On a subset of embryos, RCAS viral protein was visualized by staining embryos with the 3C2 antibody after whole-mount in situ hybridization (Logan and Tabin, 1998). After photographing the hybridization and staining results, a few embryos were embedded in 7.5% gelatin, cryosectioned (20 μm thick), and rephotographed.

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