**INTRODUCTION**

The neural crest, a migratory cell population originating in the dorsal neural tube, is required for the development of the mammalian heart. The cardiac subpopulation of cranial neural crest migrate from the hindbrain into the outflow tract of the heart where they contribute to the septum that divides the pulmonary and aortic channels. In *Splotch* mutant mice, which lack a functional *Pax3* gene, migration of cardiac neural crest is deficient and aorticopulmonary septation does not occur. Downstream genes through which *Pax3* regulates cardiac neural crest development are unknown. Here, using a combination of genetic and molecular approaches, we show that the deficiency of cardiac neural crest development in the *Splotch* mutant is caused by upregulation of *Msx2*, a homeobox gene with a well-documented role as a regulator of BMP signaling. We provide evidence, moreover, that *Pax3* represses *Msx2* expression via a direct effect on a conserved *Pax3* binding site in the *Msx2* promoter. These results establish *Msx2* as an effector of *Pax3* in cardiac neural crest development.

**SUMMARY**

Msx2 is an immediate downstream effector of Pax3 in the development of the murine cardiac neural crest

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**INTRODUCTION**

The neural crest, a migratory cell population originating in the dorsal neural tube, is required for the development of the mammalian heart. The cardiac subpopulation of cranial neural crest emerges from rhombomeres 6, 7 and 8 of the postotic neural tube and migrates into branchial arches 3, 4 and 6 (Miyagawa-Tomita et al., 1991). Some cells continue migrating into the cephalic region of the heart tube, or outflow tract. There they coalesce into ridges of connective tissue that grow together and ultimately fuse, forming the septum between the aortic and pulmonary channels. Deficiencies of cardiac neural crest development result in a failure of outflow tract septation and defective remodeling of the aortic arch arteries (Creazzo et al., 1998).

The mouse mutant *Splotch* provides an entrée into the molecular circuitry regulating neural crest development. In the homozygous state, the *Splotch* mutation results in several anomalies of neural crest that affect development of melanocytes, dorsal root ganglia, sympathetic ganglia, thymus, thyroid and the outflow septum of the heart (Auerbach, 1954; Conway et al., 1997b; Conway et al., 1997c; Epstein et al., 2000; Franz, 1989). Defects of neural tube closure and limb musculature development also occur. Several alleles of *Splotch* are known; these cause a spectrum of defects generally similar to those seen in the original *Splotch* mutant, but of varying severity (Dicke, 1964). The outflow tract anomaly of *Splotch* results from a deficiency in the migration of cardiac neural crest cells: *Splotch* cardiac neural crest cells initiate migration but do not reach the branchial arches or the outflow tract in sufficient numbers for aortic arch remodeling or outflow tract septation to occur (Conway et al., 1997b; Epstein et al., 2000; Serbedzija and McMahon, 1997). Deficiencies in myocardial function further compromise cardiac function (Conway et al., 1997a; Li et al., 1999), such that homozygous *Splotch* mutants die in utero at approximately E13.5 and exhibit signs of cardiac insufficiency (Conway et al., 1997c). This myocardial defect is probably an indirect consequence of reduced numbers of migrating crest cells, because neural crest does not contribute to the myocardium (Jiang et al., 2000).

The *Splotch* phenotype is caused by a loss-of-function mutation in *Pax3* (Epstein et al., 1993; Goulding et al., 1993),
one of nine mammalian Pax genes (Stuart et al., 1994; Walther and Gruss, 1991). In humans, loss-of-function mutations in Pax3 cause Waardenburg syndrome type I and III (Stuart et al., 1994; Machado et al., 2001) in which inactivation of a single Pax3 allele produces defects stemming from abnormal neural crest development, including sensorineural deafness, pigmentation anomalies, and abnormal skeletal and neurological features (Asher et al., 1996). Cardiac defects have also been reported in children bearing mutations in Pax3 (Banerjee, 1986; Mathieu et al., 1990). Within the tissues that form the heart, Pax3 expression is detectable only in cardiac neural crest cells and only during the early phases of their migration, consistent with findings that the Splotch mutation acts early in cardiac neural crest development (Conway et al., 2000; Epstein et al., 2000). The Splotch cardiac phenotype can be rescued by transgenic expression of Pax3 under the control of a region of the Pax3 promoter that expresses in the neural tube and neural crest, suggesting that the defect in neural crest development is cell autonomous (Li et al., 1999).

Little is known about the downstream targets of Pax3 in cardiac neural crest. Transcripts encoding the proteoglycan versican are upregulated in mesenchyme adjacent to the neural tube of Splotch mutant embryos, suggesting a role for this protein in crest migration (Henderson et al., 1997). In other tissues, genes that are potentially downstream of Pax3 include the gene for receptor tyrosine kinase, Met, which is required for the migration of limb muscle precursor cells (Epstein et al., 1996), Mif1, a transcription factor associated with Waardenburg syndrome type II (Tassabehjii et al., 1994; Watanabe et al., 1998), Ret, a tyrosine kinase receptor which is the most often mutated in Hirschsprung disease (Edery et al., 1994; Lang et al., 2000; Romeo et al., 1994), and Tyrd1, a tyrosine-phosphatase-related protein involved in the genesis of pigment (Galibert et al., 1999).

We have focused on the potential role of Msx2 as a Pax3 effector. Msx genes encode homeodomain-containing transcription factors related to the msh gene of Drosophila (Holland, 1991; Bell et al., 1992). Vertebrates possess three Msx-related genes, Msx1, Msx2 and Msx3 (Davidson, 1995). Msx3 is expressed exclusively in the neural tube (Shimeld et al., 1996; Wang et al., 1996). Msx1 and Msx2 are expressed in complex and dynamic patterns in a variety of tissues and organs, including the dorsal neural tube and neural crest, where Pax3 also is expressed (Mansouri et al., 2001; Wang et al., 1996). Targeted inactivation of Msx1 reveals a role in the development of the molar tooth and palate (Satokata and Maas, 1994). Inactivation of Msx2 causes defects in cardiac, skin and mammary glands. Though not characterized in detail, Msx1/Msx2 double homozygous mutants die in utero at approximately E16, with profound defects in cranial neural crest development (Satokata et al., 2000).

The overlap in the expression domains of Pax3, Msx1 and Msx2, together with the involvement of each in neural crest development, prompted us to ask whether these genes interact functionally. Consistent with such a possibility, we found that loss of Msx2 function rescues the cardiac defect of Splotch mutant embryos, as well as defects in the dorsal root ganglia, thymus and thyroid. Both the endogenous Msx2 gene and transgenes comprising fragments of the Msx2 promoter fused to lacZ were upregulated in the dorsal postotic hindbrain of Splotch homozygous mutants, suggesting that Msx2 is negatively regulated by Pax3. Mutation of a Pax3-binding site within the Msx2 promoter increased Msx2 transgene expression in the hindbrain, which reproduced Msx2 expression in the Splotch mutant and provided evidence that Pax3 is a direct transcriptional repressor of Msx2.

## MATERIALS AND METHODS

### Mouse strains, transgenes and the production of transgenic mice

Splotch, wild-type C57BL/6J and BALB/cJ mice were obtained from the Jackson Laboratory (stocks 000311, 000664 and 000651, respectively). The Msx1 and Msx2 knockouts (Satokata and Maas, 1994; Satokata et al., 2000) were produced from ES cells derived from strain 129Sv/J. Germine chimeras were crossed initially into BALB/cJ and then into C57BL/6J for five to six generations. Wild-type 129Sv/J mice were obtained from Dr Peter Laird (USC Medical School). ΔMsx2-lacZ and ΔMsx2-αlacZ transgenic mice have been described previously (Liu et al., 1994).

To generate the Pax3 site 1 mutant, we used the QuikChange site-directed mutagenesis kit (Stratagene). PCR primers were designed to introduce point mutations in Pax site 1, in the context of the 560 bp promoter fragment ΔMsx2-hsplacZ, as indicated in Fig. 5A, and verified by sequencing. The ΔMsx2-hsplacZ Pax site 1M vector was generated in the same way as described above for the wild-type congener. Transgenic mouse embryos were produced as described previously (Liu et al., 1994).

### Histology, in situ hybridization and β-galactosidase detection

Embryos were fixed in 4% paraformaldehyde for 0.5-24 hours, depending on the size of the embryo. They were embedded either in paraffin (for standard histology) or in Historesin after β-gal staining (Lazik et al., 1996). Paraffin-embedded sections were cut at 6 μm, Historesin-embedded sections cut at 4 μm. Hematoxylin and Eosin staining was carried out for histology.

Whole-mount in situ hybridization was performed essentially as described elsewhere (Hogan, 1994). Msx2 sense and antisense probes were generated by transcription with T3 and T7 RNA polymerase, respectively, and labeled with digoxigenin-labeled UTP. The Pax3 probe, a kind gift from Martyn Goulding, was derived from a 530 bp HindIII-PstI fragment of the murine Pax3 cDNA (Goulding et al., 1991). After whole-mount in situ hybridization, frozen sections were prepared and photographed under Nomarski optics. Photographs were digitized and the images processed in Adobe PhotoShop.

### Genotyping and statistical analysis of genetic crosses

DNA was prepared from mouse tails as described (Hogan, 1994). Splotch and Msx2 knockout mutants, and Msx2-αlacZ transgenes (Liu et al., 1994) were determined by PCR. Distinguishing the Splotch and wild-type Pax3 alleles required two reactions, one with the UM and CD primers and the other with the UW and CD primers. PCR was performed for 35 cycles with an annealing temperature of 55°C and an expected product of 158 bp. Oligonucleotide primer
sequences for Pax3 were as follows: wild-type forward (UW), 5'-GTGTCGCCCTCCCCCCGGAG-3'; Splotch forward (UM), 5'-CGTGGCGCCTCTTCTCTC-3'; and reverse (CD), 5'-CTGCCTACTAGGATGACACT-3'. Genotyping of Mxs2 knockout mice was performed as previously described (Satokata et al., 2000).

Confidence intervals for Mxs1-Pax3 and Mxs2-Pax3 interactions were computed using the method of Louis (Louis, 1981), which allows calculation of a one-sided confidence interval for a binomial parameter after observing no successes.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides containing Pax site1 are as follows: Pax site1 upper (5'-GCCGAAGTCACACAGCGAA TG-3'), Pax site1 lower (5'-CTAGCAATGAATGACAGGAAA TAAGCGTGACA-3').

Oligonucleotides containing the high affinity Pax site Nf3 (Epstein et al., 1995): Nf3 upper (5'-CTAGTGTTGTCAGCCTTATTITCCGTAGTTATTGG-3'), Nf3 lower (5'-CTAGCAATGAGTACAGGAAA TAAGCGTGACA-3').

Oligonucleotides containing a mutant Pax site1 are as follows: 1M upper (5'-GCCGAAGGATAGCTGAAATG-3'), 1M lower (5'-TGACATTTCAGGATCCCT-3').

Upper and lower strands were annealed, 3' filled in with 32P-dCTP and Klenow fragment, and purified on Sephadex G-25 columns. Pax3 protein was generated by coupled transcription/translation using the Promega T7 TrxRT kit. Binding reactions contained 10,000 cpm of oligonucleotide probe, reaction buffer (10 mM Tris-HCl pH7.5, 50 mM NaCl, 7.5 mM MgCl2, 1 mM EDTA, 0.1% NP-40, 5 mM DTT, 5% sucrose, 5% glycerol), 0.5 µg poly d(I-C), and 0.5, 1, 2 or 3 µl lysate in a 15 µl volume. Bovine serum albumin (BSA) was used to equalize the protein concentrations in different reactions. Reactions were carried out at 4°C for 30 minutes, then run on 6% acrylamide gels in Tris-glycine buffer. Gels were dried and autoradiographed.

RESULTS

Suppression of embryonic lethality of the homozygous Splotch mutation by a targeted mutation in Mxs2 but not in Mxs1

To investigate the relationship between Pax3, Msx1 and Msx2, we intercrossed Splotch mutant mice with Msx1 and Msx2 knockout mice. Extensive studies conducted on the Splotch mutant have shown that virtually 100% of Pax3Sp/Sp animals die in utero at approximately E13.5 of heart failure (Auerbach, 1954; Li et al., 1999). Nearly all such Pax3Sp/Sp animals exhibit spina bifida, which can occur with or without exencephaly (Auerbach, 1954). Msx1−/− animals exhibit cleft palate and tooth defects but survive to term (Satokata and Maas, 1994). Msx2−/− animals have defects in craniofacial structures, skin and mammary gland, but are viable and able to reproduce (Satokata et al., 2000). Neither Msx1 nor Msx2 mutant animals have detectable anomalies in the cardiac outflow tract or neural tube (S. K. and R. M., unpublished observations).

Double heterozygous mutant combinations (Msx1+/−; Pax3Sp/+ and Msx2+/−; Pax3Sp/+ did not exhibit changes in any Splotch mutant phenotypes (not shown). However, intercrosses of Msx2+/−; Pax3Sp/+ mice revealed a strong and unexpected genetic interaction. Whereas control intercrosses of Pax3Sp/+ mice yielded no newborn mice with the Pax3Sp/Sp genotype (0/78), Msx2+/−; Pax3Sp/+ intercrosses produced a significant fraction of newborns (17/77; 22%) that were homozygous for the Splotch mutation (Fig. 1A). All of these animals had spina bifida with or without exencephaly, and all died in the neonatal period (Fig. 1B-D). As Pax3Sp/Sp homozygotes have not been shown to live beyond midgestation, the expected incidence of neural tube defects in term pups generated by crossing Splotch heterozygotes is zero. These results suggest that the genetic combination that includes the Splotch mutation and the targeted Mxs2 allele enables Pax3Sp/Sp embryos to survive to term.

In contrast to these results, the cross Msx1+/−; Pax3Sp/+ × Msx1+/−; Pax3Sp/+ produced no Pax3+/− animals at the newborn stage (0/45) (Fig. 1A). This outcome was significantly different (P<0.05) from the expected result if the Msx1+/− or Msx1+/− genotypes could rescue Pax3Sp/Sp embryos to viability. The rescue of Pax3Sp/Sp embryos was therefore correlated specifically with the Mxs2 knockout allele.

We next sought to exclude the possibility that a strain background effect was responsible for the apparent rescue of Splotch mutant embryos by the Mxs2 knockout. In the crosses described above, the Splotch mutant was in a C57BL/6J background; the Msx1 and Mxs2 knockouts were in a largely C57BL/6J background (5-6 generations), but were initially in a mixed BALB/cJ and 129/SvJ background (Satokata et al., 2000). Arguing against a strain effect was, first, the fact that Msx1 mutant mice did not rescue, yet had the same background composition as the Mxs2 mutant mice. Second, there was a tight correlation between the rescue phenotype and the Mxs2 genotype: we did not see any rescued animals that did not carry

![Fig. 1. Targeted inactivation of Mxs2 but not of Mxs1 suppresses the embryonic lethality of the homozygous Splotch genotype. Mice with targeted mutations in Msx1 or Msx2 were intercrossed with Splotch mutant mice as indicated in A. (B) A gross view of a newborn Msx2+/−; Pax3Sp/+ pup. These mice typically had exencephaly (C), spina bifida (D) and craniofacial abnormalities including a foreshortened snout.](image-url)
the targeted **Msx2** allele. A third argument came from the results of test crosses in which we examined the effect of the BALB/cJ and 129/SvJ backgrounds on the viability of **Pax3^{Sp/+}** embryos. We produced **Pax3^{Sp/+}** in mixed C57BL/6J/BALB/cJ and C57BL/6J/129/SvJ backgrounds. We then carried out inter se matings between **Pax3^{Sp/+}** C57BL/6J/BALB/cJ and between **Pax3^{Sp/+}** C57BL/6J/129/SvJ individuals, and examined the progeny for term **Pax3^{Sp/Sp}** animals. Of 64 newborns in the 129SvJ background, and 85 in the BALB/cJ background, none had neural tube defects and none was **Pax3^{Sp/Sp}**. These data show clearly that neither the 129SvJ nor the BALB/cJ background per se had a detectable effect on the frequency of viable **Pax3^{Sp/Sp}** at the newborn stage (**P**<0.001). We conclude that reduced **Msx2** gene function

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**Fig. 2.** Targeted inactivation of **Msx2** selectively rescues derivatives of the cardiac neural crest in **Splotch** mutant mice. We show a histological analysis of the influence of a targeted mutation in **Msx2** on neural crest derivatives affected by the **Splotch** mutation. These included the cardiac outflow septum (A-I), the glossopharyngeal (IXth) ganglion (N-Q), dorsal root ganglia and sympathetic ganglia (R-U). The myocardium (J-L), though not derived from neural crest, is known to be affected by the **Splotch** mutation (Li et al., 1999). Note the lack of septation of the aortic and pulmonary channels in the **Pax3^{Sp/+}** embryo (C) but normal septation in the **Msx2^{−/−}**; **Pax3^{Sp/Sp}** embryo (E). Note normal morphology of a **Msx2^{−/−}**; **Pax3^{Sp/Sp}** at the newborn stage, both at level of outflow vessels (I) and myocardium (L) compared with wild-type (G,J) and **Msx2^{−/−}** (H,K) animals. Both the glossopharyngeal ganglion and the thoracic sympathetic ganglion are absent in **Pax3^{Sp/Sp}** embryos (O,S). In contrast to the cardiac outflow septum, neither is rescued in **Msx2^{−/−}**; **Pax3^{Sp/Sp}** embryos (Q,U). The dorsal root ganglia, also reduced or absent in **Pax3^{Sp/Sp}** (S), are partially rescued in **Msx2^{−/−}**; **Pax3^{Sp/Sp}** (U) embryos. IX, glossopharyngeal ganglion; a, aorta; aa, arch of aorta at site of entrance of ductus arteriosus; ap, aorticopulmonary trunk; drg, dorsal root ganglion; hm, hypaxial muscle; lv, left ventricle; p, pulmonary trunk; rv, right ventricle; sg, sympathetic ganglion. Scale bar: 500 μm.
specifically rescued Pax3<sup>Sp/Sp</sup> embryos to viability at the newborn stage.

**Loss of Msx2 function rescues a subset of neural crest defects in Pax3<sup>Sp/Sp</sup> embryos**

To determine whether the Msx2<sup>−/−</sup> genotype rescued the cardiac neural crest-related phenotypes of Splotch homozygous mutants, we compared the morphologies of wild-type, Msx2<sup>−/−</sup>, Pax3<sup>Sp/Sp</sup> and Msx2<sup>−/−</sup>; Pax3<sup>Sp/Sp</sup> hearts at E13.5 (Fig. 2B-E) and at newborn (Fig. 2G-L) stages. Analysis of serial transverse sections at E13.5 showed normal outflow tract and aortic arch anatomy in wild-type and Msx2 knockout embryos (Fig. 2B,D). As expected, Pax3<sup>Sp/Sp</sup> embryos (5/7) exhibited either persistent truncus arteriosus, defective remodeling of aortic arch arteries, or both, consistent with an effect on cardiac neural crest development (Fig. 2C). By contrast, all Msx2<sup>−/−</sup>; Pax3<sup>Sp/Sp</sup> hearts examined (3/3) had normal outflow tract and aortic arch development (Fig. 2E). At the newborn stage, hearts of Msx2<sup>−/−</sup>; Pax3<sup>Sp/Sp</sup> newborns were grossly normal, with both the ascending aorta and pulmonary artery clearly visible (data not shown). Transverse sections confirmed that the aorta and pulmonary artery were distinct and that there were no aortic arch anomalies (5/5) (Fig. 2I). Sections at the level of the ventricles revealed that the myocardia of the double mutant embryos were indistinguishable from those of wild-type and Msx2<sup>−/−</sup> embryos (5/5) (Fig. 2J-L). Thus the Msx2<sup>−/−</sup> genotype rescued not only the embryonic lethality of the homozygous Splotch mutation, but also the developmental anomalies of the neural crest-related and neural crest-dependent structures of the Splotch mutant heart.

We next asked whether other neural crest-derived structures affected by the Splotch mutation were rescued. In Pax3<sup>Sp/Sp</sup> embryos, as previously described (Auerbach, 1954; Li et al., 1999), the glossopharyngeal ganglion of the IXth cranial nerve and the sympathetic ganglia were completely absent (Fig. 2O,S; Table 1). The dorsal root ganglia were absent or substantially reduced. The thyroid was reduced in size (data not shown), and the thymus, though visible, failed to undergo migration (data not shown). Analysis of serial transverse sections showed that in Msx2<sup>−/−</sup>; Pax3<sup>Sp/Sp</sup> double mutant embryos, the sympathetic ganglia were absent at all axial levels (Fig. 2U). Similarly, the glossopharyngeal ganglion, which is derived from r6 (Manzanares et al., 2000), was not rescued by the Msx2<sup>−/−</sup> genotype (Fig. 2P-Q). The dorsal root ganglia were visible in double mutant embryos but were reduced in size compared with wild-type or Msx2<sup>−/−</sup> embryos (Fig. 2R-U). This partial rescue of the dorsal root ganglia occurred to an equal extent along the rostrocaudal axis (data not shown). Both the thymus and thyroid were normal (data not shown). The Msx2<sup>−/−</sup> genotype thus had a differential effect on different neural crest-derived structures, ranging from a complete rescue of the cardiac outflow septum to no effect on the glossopharyngeal ganglion. We conclude that the functional relationship between Pax3 and Msx2 differs in individual subpopulations of neural crest.

We did not observe any modification of non-neural crest phenotypes in Pax3<sup>-/Sp</sup> double mutants compared with Splotch mutants. Two classes of neural tube defects were found in the double homozygous (Msx2<sup>−/−</sup>; Pax3<sup>Sp/Sp</sup>) pups that escaped midgestation death and survived to term: spina bifida alone (64%) and exencephaly and spina bifida together (36%). Analyses of Pax3<sup>Sp/Sp</sup> animals in utero yielded similar percentages (data not shown). The diaphragm muscle of Msx2<sup>−/−</sup>; Pax3<sup>Sp/Sp</sup> double mutant pups was absent, and these mice did not breathe at birth (data not shown), demonstrating that the Msx2<sup>−/−</sup> genotype did not rescue muscle development in Splotch. The effect of the Msx2<sup>−/−</sup> genotype on Splotch mutants was therefore specific to a limited number of neural crest-derived structures.

**Upregulation of Msx2 in the postotic hindbrain of Splotch mutant embryos**

The simplest model to explain the genetic interaction between Msx2 and Pax3 in cardiac development is that Pax3 negatively regulates Msx2 gene activity in the cardiac neural crest or in a tissue that influences the development of the cardiac neural crest. The Splotch cardiac phenotype can be rescued by expression of Pax3 driven by a segment of the Pax3 promoter that directs expression to the neural tube and neural crest (Li et al., 1999); thus, we expected the interaction between Msx2 and Pax3 would occur in one or both of these tissues. As Pax3 is downregulated soon after cardiac neural crest cells emigrate from the postotic hindbrain at E9-10 (Conway et al., 1997c; Epstein et al., 2000), we also predicted that any functionally important change in Msx2 expression would occur in the dorsal neural tube or in the neural crest during the early phases of its migration. Accordingly, we tested the effect of the Splotch mutation on Msx2 expression in the hindbrain and neural crest at E9.5.

Whole-mount in situ hybridization experiments on wild-type embryos showed that Msx2 was expressed strongly in r5, and lower at levels caudally (Fig. 3B,E). Pax3, by contrast, was expressed at relatively lower levels in the otic region (r4 and r5) but at higher levels caudally (Fig. 3A,D). In Splotch mutant embryos, there was an increase in the intensity of the Msx2 hybridization signal in the postotic hindbrain relative to the otic region (Fig. 3C,F).

Transverse sections of whole-mount preparations showed that at the level of the otic vesicle (r5), Msx2 was expressed in the dorsal neural tube while Pax3 was expressed more ventrally and laterally (Fig. 3G,H). In the postotic neural tube (r6-8), Msx2 was expressed in a medial to lateral gradient, with the highest levels medially (Fig. 3K). Msx2 was expressed at a relatively low level in the mid-dorsal region of the neural tube (Fig. 3K), while Pax3 transcripts were present throughout the dorsal neural tube (Fig. 3I). In Splotch mutant embryos, Msx2 expression expanded, occupying a greater proportion of the Pax3 domain than in wild-type embryos. This was particularly

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**Table 1. Influence of Msx2<sup>−/−</sup> genotype on Pax3<sup>Sp/Sp</sup> neural crest phenotypes**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Wild-type</th>
<th>Msx2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Pax3&lt;sup&gt;Sp/Sp&lt;/sup&gt;; Msx2&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac outflow tract</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thoracic sympathetic ganglia</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thyroid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thymus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glossopharyngeal nerve (IX)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The indicated structures were examined histologically (see Fig. 2). –, a substantial morphological deficiency; +, a wild-type appearance. See text for details.
evident in the postotic hindbrain (r6-8), where Msx2 expression expanded into the dorsal and lateral regions of the neural tube (Fig. 3L). These data are consistent with the prediction of our genetic experiments that Pax3 negatively regulates Msx2 in the postotic hindbrain and neural crest.

A 560 bp segment of the Msx2 promoter is sufficient for upregulation of Msx2 transgenes in the postotic hindbrain of Splotch mutant embryos

To determine whether the increase in the Msx2 expression in Splotch mutant embryos was caused by an effect on Msx2 promoter activity, we asked whether transgenes containing regions of the Msx2 promoter fused to a lacZ reporter were upregulated in Splotch mutant embryos. We first used two previously characterized transgenic lines (Liu et al., 1994; Liu et al., 1999). One included a 13 kb Msx2 genomic fragment with a lacZ gene inserted near the 5’ end of the first exon (Δ1Msx2-lacZ; Fig. 4A), the other a 6.2 kb genomic fragment fused to a lacZ reporter (Δ2Msx2-lacZ; Fig. 4A). The embryonic expression patterns of several independent Δ1Msx2-lacZ and Δ2Msx2-lacZ lines have been documented previously (Liu et al., 1994; Liu et al., 1999). Both were found to recapitulate endogenous Msx2 expression accurately in a variety of sites in the embryo, including the hindbrain and migratory cardiac neural crest (Liu et al., 1994; Liu et al., 1999; Lazik, 1999).

We crossed the Δ1Msx2-lacZ and Δ2Msx2-lacZ transgenic mice with Splotch mutant mice. Embryos hemizygous for the Msx2-lacZ transgenes and homozygous for the Splotch mutation were examined at E9.5 for β-galactosidase (β-gal) activity. In whole-mount preparations of E9.5 Splotch mutant embryos, both the Δ1Msx2-lacZ and Δ2Msx2-lacZ transgenes were upregulated in the postotic hindbrain (Fig. 4B,C) similarly to the endogenous Msx2 gene (Fig. 3F). We prepared both transverse sections and longitudinal sections through the hindbrains of such embryos and examined them under dark field, which affords greater sensitivity than standard bright field imaging (Lazik et al., 1996). Stronger signals are blue, weaker signals pink. Transverse sections through the postotic hindbrain showed an increase in β-gal activity in the dorsal neural tubes of homozygous Splotch mutant embryos relative to wild type (Fig. 4D,E). These changes were similar to those of endogenous Msx2 transcripts (Fig. 3K,L).
Fig. 4. Transgenic analysis identifies a 560 bp Pax3-responsive region within the Msx2 promoter. (A) Schematic maps of Msx2-lacZ transgene constructs. Δ1 and Δ2 contain a lacZ gene inserted in the first exon. Δ3 and Δ4 comprise the indicated promoter fragments fused to an hsp68 minimal promoter and lacZ reporter. (B-N) Effect of the Splotch mutation on transgene expression in E9.95 embryos. (B,C,L) Dorsal views of whole-mount preparations. Brackets indicate the approximate location of the cardiac neural crest. Note increased staining in the postotic hindbrain of Δ1Msx2-lacZ, Δ2Msx2-lacZ and Δ4Msx2-lacZ transgenes in Pax3Sp/Sp embryos. The embryos in L are at a slightly earlier stage than those in B,C, which accounts for the lower overall level of staining in the hindbrain and neural tube. Embryos in B and L were sectioned in the transverse plane. Shown below the whole mounts are sections (D,E,M,N) at the levels indicated by arrows to the right of whole mounts. β-gal expression is imaged in dark field. Pink indicates a low to moderate signal, blue a more intense signal. Note the expansion of β-gal expression into the dorsal (arrowhead) and lateral (arrow) regions of the neural tube of Splotch mutant embryos, mirroring the change in endogenous Msx2 expression (Fig. 3). (F,G) Longitudinal sections through the hindbrains of E9.5 Δ1Msx2-lacZ embryos. (H-K) Higher magnification views of the boxed regions in F,G. Note increased staining in both preotic crest and cardiac crest in Splotch embryos compared with wild type. cc, cardiac crest; n, neural fold; o, otic vesicle; pc, preotic crest; r5, rhombomere 5. Scale bars: 100 μm.
In longitudinal sections of Splotch mutant embryos, increased β-gal staining was also evident in streams of migratory cranial neural crest (Fig. 4F-K). Serial sections revealed that in wild-type embryos, these streams extended from the preotic and postotic hindbrain into the branchial arches and cardiac outflow tract (data not shown). In Splotch mutant embryos, the postotic stream of β-gal-positive crest did not extend into the branchial arches and outflow tract, but rather remained in a small cluster adjacent to the dorsal neural tube. Preotic streams of β-gal-positive crest were unaffected in Splotch mutant embryos (data not shown). These results are consistent with previous findings that cardiac neural crest cells in Splotch mutant embryos exit the neural tube but do not reach the outflow tract in normal numbers (Epstein et al., 2000).

To further localize the Pax3-responsive region of the Msx2 promoter, we surveyed a series of fragments within the 6.2 kb 5′ flanking sequence for their ability to direct reporter gene expression to the hindbrain. A 1.8 kb fragment (ΔMsx2-hsplacZ, Fig. 4A) was found to be sufficient for hindbrain expression (not shown). Further deletion analysis within this fragment identified a 560 bp fragment, designated ΔMsx2-hsplacZ (Fig. 4A), that was expressed in neural tubes of E9.5 embryos in a manner consistent with endogenous expression. We crossed transgenic mice carrying the 560 bp hsplacZ transgene (two independent lines) with Splotch mutants and examined transgene expression in the hindbrain. As can be seen in whole-mounts (Fig. 4L), transgene expression was upregulated in the postotic hindbrain of Splotch mutants relative to wild-type or heterozygous mutant embryos. Transverse sections immediately caudal to the otic vesicle showed that transgene expression in the neural tube expanded laterally and ventrally (Fig. 4M,N). This upregulation was similar to that of the expression in the neural tube expanded laterally and ventrally or heterozygous mutant embryos. Transverse sections of E9.5 embryos revealed a lateral and ventral expansion of the β-gal positive crest. A genetic manipulation that prevents the upregulation of Msx2 in the postotic neural tube (data not shown), supporting the view that the Pax site 1 mutation inactivates a repressive cis-regulatory element. In summary, these data suggest that Pax site 1 is capable of binding Pax3 specifically.

To test whether Pax site 1 has a functional role in the control of Msx2 transgene expression in the hindbrain, we introduced the 6 bp Pax site 1 mutation (Pax site 1M into the 560 bp lacZ transgene (ΔMsx2-hsplacZ Pax site 1M). We injected this construct into mouse zygotes and analyzed transgenic embryos transiently at E9.5. As is apparent in Fig. 5D, the ΔMsx2-hsplacZ bearing the Pax site 1 mutation exhibited expanded expression in the neural tubes of E9.5 embryos relative to the ΔMsx2-hsplacZ control embryo (Fig. 5C) (Table 2). Transverse sections of E9.5 embryos revealed a lateral and ventral expansion of the β-gal signal (Fig. 5E,F) similar to that observed for the ΔMsx2-lacZ and ΔMsx2-hsplacZ transgenes in the Splotch background (Fig. 4E,N). Some β-gal activity was also observed in the ventromedial region of the neural tube, outside the domain of Pax3 expression (Fig. 5F), suggesting that trans-regulatory factors other than Pax3 may act through Pax site 1 to inhibit the expression of the ΔMsx2-hsplacZ transgene in the ventral neural tube. A 140 bp deletion of the Pax site 1 and surrounding DNA caused a similar upregulation of transgene expression in the postotic neural tube (data not shown), supporting the view that the Pax site 1 mutation inactivates a repressive cis-regulatory element. In summary, our results suggest that Pax site 1 functions in the control of Msx2 expression in the neural tube and neural crest, and that it does so through a direct interaction with Pax3.

**DISCUSSION**

We provide genetic and molecular evidence in support of a functional interaction between Pax3 and Msx2 in mammalian cardiac development. We show that loss of Pax3 function leads to upregulation of Msx2 in the neural tube and cardiac neural crest. A genetic manipulation that prevents the upregulation of Msx2 rescues Splotch mutants to viability and also rescues the cardiac morphological defects, providing evidence that increased expression of Msx2 is causally related to the deficiency in cardiac neural crest development in the Splotch mutant mouse. A mutation that interferes with Pax3 binding to a conserved consensus site within the Msx2 promoter causes an upregulation of Msx2 transgene expression in the postotic hindbrain similar to that observed for the endogenous Msx2 and Msx2 transgenes in Splotch. These results suggest that Pax3 normally acts to suppress Msx2 function, and furthermore suggest that Msx2 is a direct transcriptional target of Pax3.

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**Table 2. Effect of Pax site 1 mutation on ΔMsx2-hsplacZ transgene expression**

<table>
<thead>
<tr>
<th>Transgene expression</th>
<th>Transgene upregulation in neural tube</th>
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<tbody>
<tr>
<td>ΔMsx2-hsplacZ</td>
<td>0/11</td>
</tr>
<tr>
<td>ΔMsx2-hsplacZ (Pax site 1 mutant)</td>
<td>7/10</td>
</tr>
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Embryos were analyzed for β-gal expression transiently at stages from E9.5 to E11.5. Upregulation of mutant transgenes in the dorsal neural tube was assessed by comparison with the expression of control transgenes (ΔMsx2-hsplacZ and ΔMsx2-lacZ), which were expressed identically in the dorsal neural tube. All staining was performed under the same conditions.
Msx2 is a target of Pax3

Upregulation of Msx2 is necessary for the development of the Splotch cardiac phenotype

Despite a report suggesting that the ability of neural crest cells to migrate in vitro is impaired by the Splotch mutation (Moase and Trasler, 1990), more recent studies have shown that the Splotch mutation does not affect migration per se, but rather results in (1) a reduction in the number of cardiac neural crest cells emigrating from the neural tube (Conway et al., 2000); and (2) subtle impairment of the guidance of migrating crest cells such that they do not reach the outflow region in sufficient numbers to allow normal morphogenesis of the outflow septum or remodeling of the aortic arch arteries (Epstein et al., 2000). Furthermore, these defects are likely to be cell-autonomous, as they can be rescued by expression of wild-type Pax3 driven by a neural crest- and neural tube-specific region of the Pax3 promoter (Li et al., 1999).

Our genetic analysis shows that Msx2 is a part of the regulatory machinery through which Pax3 controls neural crest development. Whereas homozygous Splotch mutants die at E13.5, Msx2-Pax3 double homozygous mutants survive to the
newborn stage and exhibit normal cardiac outflow and myocardial anatomy. That Msx2 is upregulated in the hindbrains of Splotch mutant embryos, the site of origin of the cardiac neural crest, together with the finding that a genetic manipulation that reduces Msx2 gene function can rescue the cardiac neural crest defects of Splotch suggests that upregulation of Msx2 is required for the Splotch cardiac neural crest defects. The simplest explanation for how a deficiency in Msx2 can rescue the Splotch cardiac neural crest phenotype is that the Msx2 deficiency restores the ability of cardiac neural crest cells to migrate appropriately to their destinations in the outflow tract. However, other explanations, such as the re specification of other crest populations or a compensatory increase in the proliferative activity of cardiac crest cells, remain possible.

The interaction between Pax3 and Msx2 is remarkably cell-type specific. Reduced Msx2 activity does not rescue the Splotch muscle or neural tube defects, nor does it rescue all Splotch neural crest defects. While the cardiac neural crest, thymus and thyroid defects are suppressed completely in the Msx2-Pax3 double mutant, other Pax3-related neural crest defects are either partially rescued or unaffected. It is particularly striking that the glossopharyngeal ganglion, which is formed from neural crest originating from r6, within the rostrocaudal domain of the cardiac neural crest (Manzanares et al., 2000), is not rescued. This differential effect of the Msx2 genotype on different crest populations suggests that the regulatory interaction between Pax3 and Msx2 in the cardiac neural crest occurs after the specification of the crest population that gives rise to the outflow septum, thymus and thyroid, and the crest population that gives rise to the cranial and sympathetic ganglia. As Pax3 is downregulated shortly after crest cells emigrate from the neural tube (Epstein et al., 2000), it is likely that cardiac crest is specified before its migration into the outflow tract.

**Nonequivalence of Msx1 and Msx2 as modifiers of Pax3**

Our genetic data show that loss of Msx1 function does not rescue Splotch mutants to viability. Although we have not examined the hearts of Msx1-Pax3 double mutant embryos, the simplest explanation for this lack of rescue is that the Msx1 knockout fails to suppress the lethal cardiac defect of Splotch. This apparent non-equivalence of Msx1 and Msx2 is surprising as analysis of Msx1-Msx2 double mutant phenotypes suggests that these genes function redundantly in the development of at least some structures derived from the cranial neural crest, including the calvarial bones (Satokata et al., 2000). The inability of the Msx1 mutation to rescue the Splotch mutant is not due simply to non-responsiveness of Msx1 to regulation by Pax3: our in situ hybridization results show that Msx1 is upregulated in the postotic hindbrain of Splotch mutant embryos in a manner similar to Msx2 (data not shown). The failure of the Msx1 mutant to rescue is therefore more likely to be related to functional differences between the Msx1 and Msx2 proteins, which have virtually identical homeodomains but distinct N-terminal and C-terminal domains (Bell et al., 1993).

**Role of Pax3 in the regulation of Msx2 transcription**

Three lines of evidence support the hypothesis that Pax3 regulates Msx2 through a direct effect on its promoter. First, Msx2 lacZ transgenes are upregulated in the dorsal neural tube of Pax3<sup>385sp</sup> embryos in a manner similar to the endogenous Msx2 gene. Second, the 560 bp Pax3-responsive region of the Msx2 promoter includes a 520 bp stretch that is highly conserved (87%) in 5′ flanking DNA of the human MSX2 gene. Within this stretch is a single conserved Pax3 consensus site that Pax3 binds with high affinity. Third, mutation of this element, designated Pax site 1M, causes upregulation of Msx2 lacZ transgene expression in the dorsal neural tube. This upregulation is similar in spatial pattern to that of the Msx2 lacZ transgenes in the Splotch mutant background. These data strongly suggest that Pax3 regulates Msx2 lacZ transgenes through a direct interaction with Pax site 1. Whether Pax site 1 is functional in the context of the endogenous Msx2 promoter is unclear, though our in situ hybridization data show that in the Splotch mutant background, the changes in the pattern of endogenous Msx2 expression are strikingly similar to those of the A4Msx2-hsplacZ transgene bearing a mutation in Pax site 1. An analysis of approximately 13 kb of genomic sequence flanking the Msx2 gene has thus far failed to identify additional elements capable of driving hsp68-lacZ expression in the neural tube and neural crest; thus Pax site 1 may be of crucial importance in the context of the endogenous Msx2 promoter.

The molecular defect in Splotch is an A→T transversion in a splice acceptor site, which results in four aberrant transcripts (Epstein et al., 1993). Three of these are predicted to produce truncated proteins; one can direct the synthesis of a near full-length protein that lacks only 45 amino acids encoded by exon 4 (Pax3-Δ exon4). This deleted segment comprises a region of the paired domain and the highly conserved octapeptide motif. The paired domain is required for DNA binding (Machado et al., 2001). The octapeptide motif is involved in dimerization and other protein-protein interactions (Chalepakis et al., 1994; Machado et al., 2001). Although transcripts that lack exon 4 have been detected in Pax3<sup>385sp</sup> embryos, whether they are translated is not known. Our finding that a mutation in Pax site 1 in the Msx2 promoter results in upregulation of an Msx2 transgene similar to the upregulation of endogenous Msx2 in Pax3<sup>385sp</sup> mutant mice is consistent with the view that the molecular lesion in Splotch mutants is either a simple absence of Pax3 protein (owing, for example, to destabilization of the mutant protein), or is a result of a failure of the mutant Pax3 protein to form a functional complex on the target DNA. Despite lacking a portion of the paired domain, the Pax3-Δ exon 4 protein is capable of binding DNA (Chalepakis et al., 1994). That this protein lacks the octapeptide motif suggests that if the Pax3-Δ exon 4 transcript is in fact produced and translated in the cardiac neural crest, then its protein product may fail to recruit a partner required for transcriptional repression. Candidates for such a repressor include Groucho-related and Daxx proteins, both of which can interact with Pax3 through the octapeptide motif (Eberhard et al., 2000; Hohenbichl et al., 1999). Daxx requires, in addition to the octapeptide, sequences within the homeodomain for interaction with Pax3 (Hohenbichl et al., 1999). It is intriguing that the Splotch<sup>2H</sup> mutant, which also exhibits defective outflow tract development (Conway et al., 1997b), has a 32 bp deletion in the homeodomain (Epstein et al., 1993). Thus a common molecular mechanism, which involves an interaction between Pax3 and Daxx or another transcriptional repressor,
may lead to increased Msx2 expression and consequently to cardiac neural crest defects in both Splotch and Splotch2H mutant mice.

Finally, we note that the mutation in Pax site 1 not only causes Msx2 transgene expression to expand into the Pax3 expression domain, but also causes a low level of ectopic expression in the ventromedial neural tube, outside the Pax3 domain. This ectopic expression raises the possibility that proteins in addition to Pax3 may bind Pax site 1 and repress Msx2 expression in the ventral neural tube. Candidates for such proteins include Pax6, which, like Pax3, binds Pax site 1 avidly (M. R., unpublished), and is known to be expressed in the ventral neural tube (Grunn and Walther, 1992). Members of the brachury-related T-box protein family are also candidates. The Tyrp1 regulatory elements MSEu and MSEi contain the sequence TCACAC, which has been shown to bind not only Pax3, but also Tbx2 (Carreira et al., 1998; Galibert et al., 1999). The MSEu and MSEi sites are required for both Pax3 and Tbx2 mediated regulation of Tyrp1 in melanocytes (Galibert et al., 1999).

**Msx2, Pax3 and BMP signaling**

That Msx2 has a well-documented role as a bone morphogenetic protein (BMP) effector (Weinstein and Hemmati-Brivanlou, 1999) raises the question of whether the BMP pathway figures in the Splotch neural crest defects and the genetic interaction between Msx2 and Pax3. Misexpression experiments have shown that elevated levels of Bmp4 can induce apoptosis in neural crest that originates from the preotic and otic hindbrain (r3 and r5) (Graham et al., 1994). Overexpression of Msx2 alone can cause a similar effect (Takahashi et al., 1998). Although apoptosis has not been reported in neural crest cells in Splotch mutants, the possibility that the neural crest defects of Splotch are a result of ectopic or upregulated BMP signaling, and that loss of Msx2 function suppresses these effects by attenuating such signaling, is nevertheless an attractive hypothesis.

It is intriguing that Msx1, whose expression is positively regulated by BMP2/4, can inhibit the myogenic activity of Pax3 (Bendall et al., 1999). This effect, which occurs via a direct protein-protein interaction, antagonizes the stimulation of MyoD transcription by Pax3 and consequently antagonizes the terminal differentiation of muscle precursor cells. It is important to point out that this mechanism is fundamentally different from the interaction between Msx2 and Pax3 in cardiac crest development on both genetic and molecular levels. In the cardiac crest, Msx2 is genetically downstream of Pax3, not upstream as would be predicted by the Msx1-Pax3 model. In addition, Pax3 regulates Msx2 via an effect on its promoter. Nevertheless, findings that Msx1 and Msx2, both BMP effectors, can influence the activity of Pax3 in muscle precursors and neural crest respectively, raise the possibility of a general role for Mxs genes, Pax3 and the BMP pathway in the development of migratory mesenchymal cell populations.

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