ARTICLE

Perinatal lethality and multiple craniofacial malformations in MSX2 transgenic mice

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MSX2 is a homeodomain transcription factor that has been implicated in craniofacial morphogenesis on the basis of its expression pattern during mouse development and the finding of a missense mutation (P148H) in humans affected with Boston-type craniosynostosis. We have generated transgenic mice carrying a 34 kb DNA fragment encompassing a human MSX2 gene encoding either wild-type or mutant (P148H) MSX2. Inheritance of either transgene resulted in perinatal lethality and multiple craniofacial malformations of varying severity, including mandibular hypoplasia, cleft secondary palate, exencephaly, and median facial cleft, which are among the severe craniofacial malformations observed in humans. Transgenic mice also manifested aplasia of the interparietal bone and decreased ossification of the hyoid. Transgene-induced malformations involved cranial neural-crest derivatives, were characterized by a deficiency of tissue, and were similar to malformations associated with embryonic exposure to ethanol or retinoic acid, teratogens that cause increased cell death. Together with previous observations implicating MSX2 expression in developmentally-programmed cell death, these results suggest that wild-type levels of MSX2 activity may establish a balance between survival and apoptosis of neural crest-derived cells required for proper craniofacial morphogenesis.

INTRODUCTION

In contrast to the obvious segmentation of the axial skeleton, craniofacial development is a poorly understood process which must be executed properly for survival and which determines our physiognomy, the most uniquely human of all our physical attributes. Hox genes are expressed throughout the developing trunk and limbs, but the rostral border of expression is the third rhombomere (r3) of the embryonic hindbrain (1). The craniofacial skeleton forms primarily from neural crest cells (NCC) that migrate from hindbrain rhombomeres into the branchial arches. NCC from r1 and r2 migrate into the first arch, whereas cells from r4 and r6 migrate into the second and third arches, respectively (2). In response to a signal from the surrounding rhombomeres, Msn2 expression is induced within NCC of r3 and r5, which subsequently undergo apoptosis (3,4). Hox gene products, including Hoxa1, Hoxa2, and Hoxa3, play a role in the development of craniofacial structures derived from the second and third branchial arches, but they are not involved in the patterning of first arch derivatives (5–8). Other homeodomain proteins are expressed in cranial NCC that migrate into the first branchial arch, including Gsc, MHox, and members of the Dlx and Msx families (9–19). Msx1 and Msx2 are particularly remarkable for their patterns of expression within many of the developing craniofacial structures in which epithelial-mesenchymal interactions occur, including the developing eyes, ears, tooth buds, nasal, maxillary, and mandibular processes, and skull bones (17,20–22). NCC migrating into the craniofacial processes form mesenchymal condensations that differentiate into cartilage and bone of endochondral and membranous skull, respectively (23).

Both human mutations and knockout mice have demonstrated a role for Msx1 and Msx2 in craniofacial development. The first mutation identified caused a Pro-to-His substitution at residue 148 (P148H) of the human MSX2 protein (20). The amino acid
substitution involved the seventh residue of the homeodomain which is proline in all vertebrate Msx proteins. The P148H mutation segregated with autosomal-dominant Boston-type craniosynostosis in a large kindred (20). Affected individuals manifested varying degrees of craniofacial malformation ranging from mild fronto-orbital recession to severe cloverleaf skull malformation; cleft soft palate was also identified in one family member (24). Both wild-type and mutant Msx2 specifically recognized a high-affinity Msx1 binding site and repressed gene transcription, suggesting that the P148H mutation may exert its pathophysiologic effects on craniofacial development by a gain-of-function, rather than a loss-of-function or dominant-negative, mechanism (25).

Recently, a mutation resulting in an Arg-to-Pro substitution at residue 31 of the MSX1 homeodomain was shown to segregate with an autosomal dominant form of familial tooth agenesis (26). Finally, loss of Msx1 expression resulted in mice with cleft secondary palate, agenesis of alveolar tissue of mandible and maxilla, and abnormalities of the nasal, frontal, and parietal bones which all contain neural crest-derived tissues of the first branchial arch (27).

In order to determine the effect of increased MSX2 gene dosage on craniofacial development, we have generated mice transgenic for either the wild-type or mutant human MSX2 gene, which encodes a 267 amino acid protein that is 92% identical to mouse Mxs2, including 100% identity in the 60 amino acid homeodomain (20,28).

## RESULTS

### Generation of MSX2 F0 transgenic mice

In order to obtain a large DNA fragment that included the MSX2 gene as well as essential cis-acting transcriptional regulatory sequences, clone 38C8, containing a 34 kb insert of human genomic DNA encompassing the MSX2 gene, was isolated from a chromosome 5-specific cosmid library. Restriction endonuclease analysis revealed that the two exons and one intron of the MSX2 gene extended over ~9 kb, and that the clone also contained 11 kb and 14 kb of 5′- and 3′-flanking DNA, respectively (Fig. 1A). A unique 3.1 kb ScaI fragment containing the MSX2 homeobox was subcloned, the nucleotide substitution responsible for the P148H missense mutation associated with Boston-type craniosynostosis was introduced by site-directed mutagenesis, and the ScaI fragment was returned to the cosmid. The 34 kb wild-type and mutant transgenes (tgMSX2wt and tgMSX2mut, respectively) were isolated by NotI digestion and microinjected into fertilized mouse eggs. These two transgenes were thus identical except that they encoded wild-type and mutant (P148H) forms of the MSX2 protein.

### Phenotype analysis of F1 transgenic mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>F1 tgMSX2mut4</th>
<th>F1tgMSX2wt19</th>
<th>F1tgMSX2wt24</th>
</tr>
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<tbody>
<tr>
<td>Perinatal lethality (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Mandibular hypoplasia (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Cleft secondary palate (%)</td>
<td>65.9</td>
<td>66.7</td>
<td>100</td>
</tr>
<tr>
<td>Exencephaly (%)</td>
<td>24.3</td>
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<td>0</td>
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<td>Midline facial cleft (%)</td>
<td>9.8</td>
<td>8.3</td>
<td>0</td>
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<tr>
<td>Eyelid aplasia (%)</td>
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<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>Mandibular hypoplasia only (%)</td>
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<td>8.3</td>
<td>0</td>
</tr>
<tr>
<td>Cleft palate + exencephaly (%)</td>
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Table 1: Genotype analysis of F1 mice

<table>
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<th>tg(+/-)</th>
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<tr>
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<tr>
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<td>tgMSX2mut15</td>
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<td>96.2%</td>
<td>3.8%</td>
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<td>tgMSX2wt19</td>
<td>60</td>
<td>80.0%</td>
<td>20.0%</td>
</tr>
<tr>
<td>tgMSX2wt24</td>
<td>41</td>
<td>61.0%</td>
<td>39.0%</td>
</tr>
<tr>
<td>tgMSX2wt25</td>
<td>69</td>
<td>100%</td>
<td>0%</td>
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</table>

Table 2: Phenotype analysis of F1 transgenic mice
Figure 2. Craniofacial malformations in neonatal MSX2 transgenic mice. (a) Non-transgenic mouse [littermate of mouse in (b)]. Normal mandibulo-maxillary profile. (b) tgMSX2mut4.1.72. Mandibular hypoplasia. A single central incisor can be seen protruding from the lower jaw which is too small to fully appose the upper jaw (note also the cleft lip). (c) tgMSX2mut4.1.79. Complete nasofacial cleft and mandibular hypoplasia. (d) tgMSX2wt19.1.42. Cleft lip. (e) tgMSX2mut4.1.75 (at left with non-transgenic littermate at right). Exencephaly and eyelid aplasia. (f) tgMSX2wt19.1.42 [see also panel (d)]. Exencephaly with normal eyelids. Note that the malformations are less severe than in the mouse shown in (e). (g) tgMSX2mut4.1.36. Skull malformation and eyelid hypoplasia in absence of exencephaly. (h) tgMSX2mut4.1.78. Eyelid aplasia and ocular malformation (see Fig. 3c for histology).
Figure 3. Histological analysis of craniofacial malformations. Frontal sections through the heads of non-transgenic (a, e) and transgenic (b–d and f–h) neonatal mice. Sections are arranged in a posterior to anterior sequence. (a) Non-transgenic mouse. The two well-developed mandibular rami (closed arrows) are separated by the symphysis menti. The intact palate (open arrow) separates the nasopharynx above from the oropharynx below. (b) tgMSX2wt19.1.14. Note the large cleft palate with tongue apposed to nasal septum and the hypoplastic, fused mandible (closed arrow) with closely-spaced central incisors. (c) tgMSX2mut4.1.35. In (g), note the single central lower incisor and severely hypoplastic fused mandible (closed arrow) which barely extends to the region included within the section shown in (h) (horizontal arrow). The more posterior section shown in (g) demonstrates complete separation of the nasal processes and cleft palate with disorganized neural tissue (which may have been disrupted during delivery) extending to just above the tongue (open arrow). In the more anterior section shown in (h), the nasal processes are partially fused (vertical arrow). E, eye; N, nasal septum; n, non-fused nasal process; O, olfactory lobe; P, palate; T, tongue.

Genotype analysis of F1 transgenic mice

Three F0 mice carried the mutant MSX2 (P148H) transgene. Founder mouse tgMSX2mut13 died on the second day of life due to unknown causes. Female founder tgMSX2mut15 died at 4 months of age after delivering three litters and a total of 26 offspring (Table 1). The only F1 transgenic mouse died on the first day of life due to respiratory insufficiency and postmortem examination revealed mandibular hypoplasia and cleft palate. Male founder tgMSX2mut4 had a total of 110 offspring of which 61% were non-transgenic and 39% were transgenic. All of the female founder tgMSX2mut15 died at 4 months of age after delivering three litters and a total of 26 offspring (Table 1). The only F1 transgenic mouse died on the first day of life due to respiratory insufficiency and postmortem examination revealed mandibular hypoplasia and cleft palate. Male founder tgMSX2mut4 had a total of 110 offspring of which 61% were non-transgenic and 39% were transgenic. All of the transgenic mice died on the first day of life due to respiratory insufficiency and all manifested craniofacial malformations (Table 2).

Three female founders carrying the wild-type MSX2 transgene were mated to non-transgenic males. tgMSX2wt19, tgMSX2wt24, and tgMSX2wt25 each gave rise to greater than 40 F1 mice, of which 20, 39, and 0% were transgenic, respectively (Table 1). All of the transgenic F1 mice died on the first day of life with multiple craniofacial malformations (Table 2). These data indicate that there was 100% penetrance for the general phenotype of perinatal lethality and craniofacial malformations, regardless of whether the transgene encoded wild-type or mutant MSX2. The viability of F1 mice, the lack of viability of F1 mice, and the lower-than-expected transgene transmission frequencies suggest that founders 13, 15, 19, and 25 were mosaic for transgene integration. In the case of founders 4 and 24, the frequency of transgenic F1 mice was higher (39%), although still less than expected, and the founders may have been either mosaic or non-mosaic but non-expressing. There was no evidence for prenatal lethality of F1 transgenic mice, although this possibility was not definitively excluded.

Transgene copy number in genomic DNA was determined by blot hybridization using a standard curve based upon known amounts of cosmid DNA (Fig. 1B). This analysis indicated that tgMSX2mut4, tgMSX2wt19, and tgMSX2wt24 F1 mice carried 14, 22, and 13 copies of the transgene, respectively.

Craniofacial malformations in F1 transgenic mice

The types of malformations identified in F1 mice carrying the wild-type and mutant transgenes were similar as determined by an analysis of 41 F1tgMSX2mut4, 12 F1tgMSX2wt19, and 16 F1tgMSX2wt24 transgenic mice (Table 2). In addition to perinatal lethality, the other aspect of the transgenic phenotype with 100% penetrance was a variable degree of mandibular hypoplasia. This defect was often easily diagnosed by the failure of the upper and lower jaws to fully appose (compare Figs. 2a and b). Histologic analysis demonstrated a markedly hypoplastic lower jaw containing either two closely-spaced (Fig. 2b) or a single (Fig. 3f, g) central incisor. Cleared skeletal preparations of transgenic mice with a single central incisor (data not shown)
confirmed the midline fusion of the mandibular rami and absence of the symphysis menti that was detected histologically. Maxillary malformations of variable severity were also seen in most transgenic mice. In the mildest cases, the snout lacked the normally rounded appearance of non-transgenic littermates (Figs 2a, b, 4a and 6a). More severe malformations of the maxillary region are described below. In general, however, the mandible appeared to be disproportionately reduced in size relative to the maxilla.

All F1 mice transgenic for tgMSX2mut or tgMSXwt thus demonstrated maxillary defects, mandibular hypoplasia, and perinatal lethality. However, many tgMSX2mut4 and tgMSX2wt19 mice also presented with either cleft palate or exencephaly, but never both, suggesting that these two phenotypic manifestations were mutually exclusive. In contrast, all of the tgMSX2wt24 F1 mice had cleft secondary palate and none had exencephaly (Table 2). Cleft secondary palate (Fig. 4a) was observed in two-thirds of the tgMSX2mut4 and tgMSX2wt19 F1 mice (Table 2). In these mice, the tongue was malpositioned within the cleft (Fig. 3b–d) and the combination of mandibular hypoplasia, glossoptosis, and cleft palate led to both airway obstruction and insufflation of the gastrointestinal tract.

Figure 4. Cleft secondary palate and airway obstruction. (a) Rostral projection of Bouin’s-fixed heads (with mandibles removed) from non-transgenic littermate (left) and tgMSX2mut4.1.1 (right). Cleft palate and maxillary hypoplasia are evident in the transgenic skull. (b) Transgenic (top row) and non-transgenic (bottom row) tgMSX2wt24 F1 littermates. Note the gaseous abdominal distension of the transgenic mice. The mandible of the transgenic mouse at left has been removed to demonstrate the cleft secondary palate, whereas the transgenic mice to its right demonstrate mandibular hypoplasia.
Figure 5. Histologic demonstration of exencephaly. Section from tgMSX2mut4.1.75 (see Fig. 2e) demonstrating continuity between choroid plexus (open arrow) and squamous epithelium (closed arrow). N, neural tissue; P, petrous bone with ossicles.

The other mutually-exclusive phenotype was exencephaly, which was observed in one-quarter of the tgMSX2mut4 and tgMSX2wt19 F1 transgenic mice, with failure of cranial neural tube closure and a defect of the overlying skull (Fig. 2e, f). These animals also died of respiratory insufficiency, possibly on a neurogenic basis. Exencephaly was confirmed by histological demonstration that the ependymal layer covering the brain was in direct continuity with skin covering the cranial base (Fig. 5). The degree of cerebral development in the exencephalic mice varied in severity, ranging from more mild malformations to apparent anencephaly. These cranial defects were identified in mice delivered by caesarean section at E19 and thus were not traumatic in their primary etiology, although mice delivered spontaneously did show evidence of hemorrhage and tissue disruption due to the absence of boney protection for the brain. In several mice with cleft palate, less severe skull malformations were identified rather than exencephaly (Fig. 2g).

Whereas tgMSX2wt24 mice had a fairly uniform phenotype consisting of cleft palate and mandibular hypoplasia (without midline fusion), several other craniofacial malformations were variably present in tgMSX2mut4 and tgMSX2wt19 mice (Table 2). A subset of mice with cleft secondary palate or exencephaly also manifested a midline facial cleft that included incomplete midline fusion of the maxillary and nasal processes that was variable in its severity, ranging from cleft lip due to incomplete midline fusion of the premaxillary soft tissues (Fig. 2b, d) to complete failure of nasal process fusion (Fig. 2c) with neural tissue virtually apposed to the tongue (Fig. 3g, h). The more severe facial clefts were seen in tgMSX2mut4 mice and the more mild clefts were seen in tgMSX2wt19 mice. Hypoplasia or aplasia of the eyelids was observed in 25% of tgMSX2mut4 F1 mice (Figs 2e, g, h and 3c, d) and 12.5% of tgMSX2wt19 F1 mice (Table 2). More severe ocular defects were observed in several mice, including in coloboma of the retina and optic nerve (Fig. 3d). Transgenic mice also manifested a variable reduction in the size of the external ear which was not analyzed quantitatively.

The results described above indicate that most of the malformations were seen in mice carrying either tgMSX2wt or tgMSX2mut. However, analysis of cleared skeletal specimens revealed two defects which were present in tgMSX2mut4 mice and absent in the non-transgenic and tgMSX2wt24 mice that were studied. First, examination of the skull bones revealed that compared to non-transgenic mice (n = 21), tgMSX2wt24 mice (n = 3) demonstrated a complete midline cleft of the interparietal bone (data not shown), whereas in tgMSX2mut4 mice without exencephaly (n = 10) the interparietal bone was completely absent (Fig. 6a). Second, ossification of the hyoid bone was markedly decreased in tgMSX2mut4, relative to non-transgenic and tgMSX2wt24 mice (Fig. 6b). Whereas many of the observed malformations involved structures derived from the first and second branchial arches, the hyoid defect indicates involvement of the third arch as well. Cleared skeletal preparations from tgMSX2wt19 mice were not examined. Since the phenotypes of tgMSX2mut4 and tgMSX2wt19 mice were more similar than the phenotypes of tgMSX2wt24 and tgMSX2wt24 mice (Table 2), it is unclear whether the observed differences between tgMSX2mut4 and tgMSX2wt24 mice were due to inheritance of mutant vs wild-type transgenes or another cause, such as different levels of transgene expression, that might account for the overall differences in phenotype between tgMSX2mut4 and tgMSX2wt24 mice.

DISCUSSION

In this paper we demonstrate the presence of multiple severe craniofacial malformations, involving structures that arise from the first, second, and third branchial arches, in mice that inherited human MSX2 transgenes. There are several possible pathogenetic mechanisms that might underlie the observed phenotypes. First, transgene integration may have disrupted a mouse gene that is essential for craniofacial development. However, the observation that the transgenic phenotype was dominated in multiple, independently- derived lines rules out insertional mutagenesis as a possible etiology. Second, we introduced a large human genomic DNA fragment into the mouse germline in order
to increase the probability of including all cis-acting transcriptional control elements required for proper developmental regulation of MSX2. We cannot exclude the presence of a second gene within the 34 kb fragment, although this possibility is unlikely as the gene would need to be small and in close proximity to MSX2 in order to be included intact within the transgene. It should be noted that MSX1 and MSX2 map to human chromosomes 4p16.1 and 5q34-35, respectively (20,29), and are therefore not contiguous. A third and favored explanation for the observed phenotypes is that expression of human MSX2 in cells already expressing mouse Msx2 adversely affects craniofacial development. Additional experiments will be required to demonstrate transgene expression within Msx2-expressing NCC of the rhombencephalon and/or branchial arches. These studies require a transgenic male for mating to females that are sacrificed at various gestational intervals in order to correlate transgene expression with abnormal embryonic development. The only male founder, tgMSX2mut4, became infertile at the time those experiments were initiated. It will therefore be necessary to generate new transgenic founders in order to extend these studies. A transgene consisting of the intact mouse Msx2 gene (with LacZ inserted into exon 1) as well as 5.2 kb and 3 kb of 5′- and 3′-flanking DNA was expressed in a pattern that closely resembled the endogenous Msx2 gene, including expression in craniofacial mesenchyme at E12.5 (30). It is therefore likely that our 34 kb transgene, consisting of the intact human MSX2 gene with 11 kb and 14 kb of 5′- and 3′-flanking DNA, includes positive and negative transcriptional regulatory elements that are functionally analogous to those present within the less extensive mouse transgene.

**MSX2 (P148H) and craniofacial malformations in humans**

Our studies were performed in part to investigate the molecular basis by which inheritance of the MSX2 (P148H) allele results in craniosynostosis. We hypothesized that this missense mutation might result in increased biological activity of the MSX2 protein. If such were the case, then overexpression of the wild-type protein (as a consequence of increased gene dosage in transgenic mice) might also have phenotypic effects. The presence of craniofacial malformations in both tgMSX2mut and tgMSX2wt F1 mice was consistent with these hypotheses. However, the observed mouse malformations were more severe than those observed in humans with Boston-type craniosynostosis (20,24). Two likely explanations which are not mutually exclusive involve MSX2 gene dosage and genetic background. We have demonstrated that all transgenic lines contained multiple copies of the human MSX2 gene in addition to the endogenous mouse Msx2 genes. Although we have no data relating copy number to the level of transgene expression, it is likely that expression of Msx2/MSX2 protein is increased in the transgenic mice and that the effect of this overexpression on craniofacial development is considerably greater than the effect of simple heterozygosity for the MSX2 (P148H) allele. A second probable contributory factor is genetic background. Two mutually-exclusive phenotypic presentations, cleft secondary palate and encephaly, were identified in the offspring of founders that arose from the mating of (C57BL/6 × SJL)F1 mice. Genetic differences between the C57BL/6 and SJL strains segregating in the F1 mice may have influenced the phenotypic presentation. Analysis of offspring derived from matings of transgenic mice to isogenic C57BL/6 and SJL mice would be informative in this regard, but we are prevented from doing so by the lack of fertile transgenic mice. In MSX2 transgenic mice in which a complete skull developed, there was no evidence of craniosynostosis (data not shown). Our results stand in contrast to the human syndrome and also differ from the analysis of transgenic mice in which either wild-type or
transgenic mice and cleft secondary palate, respiratory insufficiency, gaseous therefore surprising that the phenotype of mandibular hypoplasia, Msx1 and Msx2 are functionally redundant (27,32). It was development, although not identical, overlap considerably. These Msx1 and Msx2 both function as repressors (25,32). In addition, sequences diverge outside of the residues immediately flanking the homeodomain, suggesting that they bind to similar DNA homeodomain, suggesting that they bind to similar DNA. This prediction was confirmed in the context of in vitro electrophoretic mobility shift assays (25). Msx1 and Msx2 sequences diverge outside of the residues immediately flanking the homeodomain, suggesting that these proteins may have distinct biological functions. However, in transcription assays, Msx1 and Msx2 both function as repressors (25,32). In addition, Msx1 and Msx2 expression patterns during craniofacial development, although not identical, overlap considerably. These observations have led several investigators to hypothesize that Msx1 and Msx2 are functionally redundant (27,32). It was therefore surprising that the phenotype of mandibular hypoplasia, cleft secondary palate, respiratory insufficiency, gaseous abdominal distension, and perinatal lethality were associated with the development of NCCs derived from the Hoxa1 promoter (44). However, aplasia of the retinoic acid (46). Midline nasomaxillofacial clefts, and exencephaly were also seen in mice deficient for both retinoic acid receptor (RAR) z and y (47). Other malformations shared by these mice and MSX2 transgenic mice include cleft palate, coloboma of the retina and optic nerve, and eyelid abnormalities. The similar phenotypes of these mice suggest that AP-2, MSX1, MSX2, RARz, and RARY may be required together for the proper execution of NCC of one or more specific genetic programs required for normal craniofacial development.

**Msx2, teratogens, and apoptosis**

In addition to sharing phenotypic similarities with several knockout mice, the phenotype associated with inheritance of an MSX2 transgene is also remarkably similar to the effect of teratogens. Exposure of mouse embryos to ethanol on E8 or E9 resulted in the development of cleft palate, exencephaly, and midline facial clefts (48–50). Analysis of these mice revealed that at sites of developmentally programmed cell death there was an increased area and density of dying cells. Within the rhombencephalon, r3 and r5 were sites of markedly increased cell death (51). Exposure of mouse embryos to retinoic acid at E8 or E9 also resulted in increased death of rhombencephalic NCC, leading to cleft secondary palate, mandibular and maxillary deficiencies (50). Expression of Msx2 in the rhombencephalon was also temporally and spatially correlated with the selective apoptosis of NCC in r3 and r5 (3,4). Taken together, these results suggest that in Msx2 transgenic mice, the combined levels of Msx2/MSX2 may induce the death of an increased number of NCC, resulting in a deficiency of cells that populate the branchial arches and participate in craniofacial morphogenesis. Msx2 may thus regulate a balance between survival and apoptosis of rhombencephalic neural crest-derived cells that is required for proper craniofacial development. One intriguing hypothesis which follows from these conclusions is that the increased death of neural crest-derived cells associated with the development of craniofacial malformations, either in embryos with genetic defects such as AP-2 deficiency (45) or in embryos exposed to secondar
teratogens such as ethanol or retinoic acid (50), may involve an induction of Msx2 expression within apoptotic cells.

MATERIALS AND METHODS

Construction of transgenes

The Los Alamos human chromosome 5-specific cosmId library was screened by hybridization with a mixture of two probes representing PCR-amplified MSX2 exons 1 and 2. The 34 kb NotI insert fragment from clone 38C8 (in the vector SuperCos-1) was transferred to the NotI site of the cosmId vector pWE15sm, in which the Scal site of pWE15 (Strategene) was converted to an MnlI site. A 3.1 kb Scal fragment was isolated from pWE15sm/38C8 (Fig. 1) and subcloned into pUC19MScalR, in which the Scal site in the ampR gene was converted to an EcoRI site and the KpnI site in the polylinker was converted to a Scal site. Site-directed mutagenesis was performed with pUC19MSclalR/Sca3.1 using the mutagenesis primer 5′-CCGCAGCGAATCTTACACATCCC-3′ (mutation underlined) which converts MSX2

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Pathological analysis

For histological analysis, newborn mice were sacrificed by methoxyflurane inhalation overdose, fixed in 10% neutral-buffered formalin or Bouin’s solution, and paraffin-embedded sections were sectioned in a frontal plane and stained with hematoxylin and eosin. For skeletal analysis, neonates were sacrificed by passage through increasing concentrations of glycerol (33).

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