Craniofacial Abnormalities Induced by Ectopic Expression of the Homeobox Gene Hox-1.1 In Transgenic Mice

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Summary

Hox-1.1 is a murine homeobox-containing gene expressed in a time- and cell-specific manner during embryogenesis. We have generated transgenic mice that ectopically express Hox-1.1 from the chicken β-actin promoter. In these mice Hox-1.1 expression was changed to an almost ubiquitous pattern. Ectopic expression of Hox-1.1 leads to death of the transgenic animals shortly after birth and is associated with multiple craniofacial anomalies, such as cleft palate, open eyes at birth, and fused pinnae. This phenotype is similar to the effects seen after systemic administration of retinoic acid during gestation. This suggests that retinoic acid embryopathy and the specific developmental defects caused by ectopic expression of a potential developmental control gene share a common pathogenic mechanism.

Introduction

Antennapedia (Antp), a mutation in the fruit fly Drosophila melanogaster, is characterized by the presence of mesothoracic legs in the place of antennae. The molecular cause for this phenotype is a chromosomal rearrangement placing the Antp gene under the control of a heterologous promoter that transcriptionally activates the gene ectopically in the imaginal discs of the antennae (Frischer et al., 1986). The same phenotype can also be induced experimentally in transgenic flies carrying an Antp gene under the control of a heat shock promoter (Gehring and Gehring, 1988). Through the analysis of "gain-of-function mutations" like Antp, and complementing "loss-of-function mutations," molecular mechanisms of Drosophila embryonic development have been elucidated. In particular, genes affecting metamORIZATION and segment specification have been identified and cloned (Gehring and Hiromi, 1986; Scott and Carroll, 1987; Akam, 1987; Ingham, 1988). Many of these Drosophila developmental control genes share conserved DNA sequences, such as homeoboxes, paired boxes, or zinc finger encoding motifs. These motifs are also present in genes of other organisms such as frogs, mice, and humans, and many of the respective genes have been isolated (Holland and Hogan, 1986; Dressler and Gruss, 1988). Evidence that genes with sequence homology to Drosophila developmental control genes play a regulatory role during mammalian embryogenesis was obtained from the molecular analysis of the mouse mutant "undulated" (un) (Balling et al., 1988), characterized by malformations of the axial skeleton along the entire axis. A point mutation in the murine paired box containing gene Pax 1 (Deutsch et al., 1988) may be responsible for the un phenotype.

One of the main problems in mammalian embryology is the limited number of appropriate mutants, making a functional analysis particularly difficult. However, loss-of-function as well as gain-of-function mutants can be generated using recently developed methods. Specific genes can be inactivated in the genome of embryonic stem cells by homologous recombination. Such mutated alleles can be introduced into mice, and homozygous transgenic animals can be obtained by breeding (Mansour et al., 1988; Thompson et al., 1989). A similar approach has recently been applied to eliminate a functional Hox-1.1 gene from the mouse genome (Zimmer and Gruss, 1989).

In addition, designed genes can be introduced into the genome of transgenic mice to achieve ectopic expression during development, similar to the approach described above for the Drosophila Antp gene under the control of a heat shock promoter. This strategy has been applied in the functional analysis of oncogenes, genes that are important in the establishment of the immune repertoire, or genes that code for multimeric proteins (for review see Jaenisch, 1988).

In the present study we have expressed Hox-1.1 ectopically in transgenic mice using the ubiquitous transcriptional activity of the chicken β-actin promoter. Transgenic mice with deregulated expression of Hox-1.1 die within a few days after birth. These mice show a striking combination of craniofacial abnormalities, such as open eyes at birth, fused pinnae, and cleft secondary palate. This phenotype is very similar to the effects seen after application of retinoic acid during pregnancy (retinoic acid embryopathy, or RAE) and to the previously described mouse mutant "first arch malformation" (far). Based on the similarity between the phenotype of our transgenic mice and both RAE and the first arch malformation phenotype, the possibility that Hox-1.1 ectopic expression affects cranial neural crest cells is discussed.

Results

Production of Transgenic Mice

A genomic expression vector was constructed (Figure 1) to express Hox-1.1 in transgenic mice. The vector contains a 340 bp promoter fragment of the chicken β-actin gene fused to genomic Hox-1.1 sequences at a XbaI site located in the 5' end of the cDNA clone C1 (Kessel et al., 1987). The Hox-1.1 sequences extend from 500 bp upstream of the translational initiation codon to 300 bp downstream of the poly(A) addition site.

Eleven transgenic mice were identified that carry the
Between one and five copies of the transgene had been inserted into a single integration site (data not shown). Seven out of the 11 transgenic mice died shortly after birth (FO animals) or were sacrificed when they developed very poorly and survival seemed unlikely (Table 1). Four transgenic mice carrying gp survived without any obvious pathological phenotype and were used as founders for establishing transgenic lines. One of the four lines (Tg-gp-471) had a rearrangement in the Hox-7.7 transgene, and aberrantly sized RNAs were detected in the brain. Therefore this line was not analyzed further. Tg-gp-938 was healthy and gave birth to ten normally sized litters (Table 2). Although Tg-gp-938 itself showed no abnormalities, all of her transgenic offspring were nonviable.

In all litters from animal Tg-gp-938 we found a strict correlation between transgenicity and postnatal death in the first few days after birth (Table 2). This phenotype was identical to that of the seven transgenic FO animals that died shortly after birth. The observation that a phenotype caused by a transgene is observed in the F1, but not in the F0, generation of a particular line has also been described for mice carrying human immunodeficiency virus transgene.

The data for the transgenic animals (Table 1) are complemented by Figure 1, which shows the structure of the Hox-1.1 transcription unit and the derived expression vector gβ. Three types of cDNAs for Hox-1.1 have been isolated and are indicated: B21 from differentiated F9 cells (Kessel et al., 1987), B1 from 3T3 cells, and O2 from M866 transformed fibroblasts (M. Kessel, unpublished data). The structure of the Hox-1.1 gene as deduced from the depicted cDNA clones is shown. The main open reading frame is hatched, and the homebox is a filled box. Two small upstream frames are shown stippled. The β-actin promoter is fused to genomic sequences at the indicated XhoI site. Details for the construction of the expression vector gβ are given in Experimental Procedures. RNA probes for the mapping studies were generated from sequence between the XhoI (actin) and the XhoI (Hox-1.1) sites, and between the MatII (intron) and PstI sites. The protected RNAs are indicated as stippled boxes. The homebox-containing fragment between the EcoRI and PvuII sites was labeled for the Northern analysis.
Table 2. F1 Generations from the Transgenic Animal Tg-gl-938

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ND, not determined.

DNA (Leonard et al., 1988). Several F1 animals were sacrificed for histological and RNA analysis as soon as it became apparent that they would not survive.

Expression of the Hox-1.1 Transgene

RNA from newborn transgenic animals was analyzed for expression of the transgene by Northern blotting and RNAase mapping (Figure 2). Transcripts of the predicted length (Figure 2A) and with the correctly protected chimeric 5' sequences (Figure 2B) were detected in transgenic F0 animals as well as in the transgenic offspring of Tg-gl-938. We were unable to detect gβ transcripts by in situ hybridization.

Line Tg-gl-938 provided transgenic animals for a more detailed analysis of expression and phenotype (Table 2). The newborn Tg-gl-1546, an F1 animal of the Tg-gl-938 line, was identified initially by lower weight as compared with its littermates; it was sacriﬁed directly after birth, and RNA was prepared from various organs. Owing to the limited amount of material, RNA was not separately prepared from tissues such as spinal cord and bone marrow. RNA expressed from the transgene was readily detectable in brain, head, stomach, heart, lung, bladder, muscle, rib cage, and cervical and thoracic vertebral columns (Figure 3). The RNA from head tissue was also mapped using simultaneously the transgene-specific and the homeobox probes (see Experimental Procedures; Figure 5B). A comparison of the intensities of the two protected bands indicates that the chimeric RNA is the only RNA species found in the head of newborn Tg-gl-1516. The levels of this RNA and of chimeric Hox-1.1 RNAs in the other
Figure 3. Expression of the Transgene in Organs and Tissues from the Newborn Animal Tg-g6-1516

RNA preparation and RNAase mapping were performed as outlined in Experimental Procedures. The "vertebral column" was dissected out by cutting as near as possible laterally of the vertebrae, and then subdividing the column after the eighth and thirteenth ribs into a cervical, a thoracic, and a lumbar segment. "Rib" tissue was prepared by taking out the entire rib cage. "Head" tissue comprised the head after removal of the brain. As a positive control, RNA from Tg-g6-1163 was mapped in the same experiment; brain and lung RNAs of a nontransgenic littermate were used as negative controls. In all lanes 40 µg of total RNA was analyzed, with the following exceptions: bladder (14 µg), spleen (5 µg), thymus (10 µg), and heart (30 µg).

Phenotype of Hex-1.7 Transgenic Mice

Offspring expressing the transgene were live-born, but died from 1 hr to 14 days after birth. The only exception was animal Tg-g6-938, which exhibited no pathological phenotype. Those individuals with briefest survival were unable to nurse, as shown by the absence of milk in the stomach. With increasing survival, transgenic newborns experienced poor weight gain and failed to thrive. The animal that survived the longest (Tg-g6-492) died on day 14 after birth (Figure 6).

In different animals multiple craniofacial abnormalities were noted, such as cleft secondary palate, incomplete eyelid fusion, and detached pinnae (Figures 7-9). In all of these sites there was a deficiency of underlying connective tissues resulting in small palatal shelves, hypoplastic palpebrae, and nonfused pinnae. Exophthalmos, which may secondarily delay eyelid fusion, was not present. A pointed snout, consistent with a poorly formed maxilla, was noted in one of the animals with cleft palate (Tg-g6-794, Figure 7).

An F1 transgenic animal (Tg-g6-1530) from line Tg-g6-938 was serially sectioned along with a control nontransgenic littermate for comparison, and stained for routine histological analysis. Particular attention was paid to morphological parameters of visceral maturity and congenital defects. Careful evaluation of the oral and nasopharyngeal region showed cleft secondary palate (Figure 6) without ectopic chondrification of the palatal shelf. The primary hard palate, located rostra1 to the incisive foramen, was closed, and associated cleft lip was absent. Lung and renal development were age appropriate, indicating that the observed delayed eyelid (Figure 6) and ear fusions are not part of a generalized immaturity. The ocular orbit and retro-orbital structures were normal. The middle and inner ear were unremarkable. Pulmonary infection or aspirated foreign material was not observed. Gastrointestinal defects that might inhibit feeding such as tracheoesophagi...
Expression of Hex-1.1 in Transgenic Mice

Figure 5. Expression of Transgenic and Endogenous Hex-1.1 RNAs in Embryos and Newborn Head

(A) Expression of the transgene in embryos. The foster mothers were sacrificed at day 12 of gestation. Embryos, yolk sacs, and placentas were dissected out and used for DNA and RNA analysis. RNAase mapping data are shown for five transgenic embryos (Tg-gp-1620, -1621, -1630, -1631, and -1636) and two wild-type littermates (WT 1622 and 1634).

(B) Relative levels of transgenic versus total Hex-1.1 homeobox-containing RNAs. The RNAs analyzed in (A) from the day 12 embryos Tg-gp-1621 and WT 1622 and in Figure 3 from nontransgenic and transgenic head tissue, prepared from the newborn littermates WT 1519 and Tg-gp-1516, respectively) were simultaneously mapped with the g6 transgene probe and the Hox-1.1 homeobox-specific probe (see Experimental Procedures). Note that the 214 base protected band (arrow, Hox-1.1 RNA) in the lane from animal Tg-gp-1516 results from the presence of transgene RNA only, while the corresponding band in the lane from animal Tg-gp-1621 is the sum of chimeric plus endogenous RNAs.

Figure 4. Transgene Expression in Animals with No Obvious Pathological Phenotype

(A) RNAase mapping of total RNA isolated from organs of animal Tg-gp-1526 and of a nontransgenic (wt) littermate. Both mice were sacrificed with no pathological symptoms 2 days after birth. RNA from animal Tg-gp-1183 was used as a positive control.

(B) RNAase mapping of total RNA isolated from organs of the adult animal Tg-gp-938, the only expressing transgenic mouse surviving to adulthood and fertility. RNA from animal Tg-gp-1183 was used as a positive control.

Discussion

Endogenous versus Ectopic Hox-1.1 Expression

To create a gain-of-function mutant by ectopic expression of the murine homeobox gene Hox-1.1, we generated transgenic mice with a chicken β-actin promoter–Hox-1.1 expression vector. Endogenous Hox-1.1 expression is maximal at day 12 of gestation, when RNA is found in the neural tube, spinal ganglia, and sclerotomes (Colberg-Poley et al., 1985a; Mahon et al., 1988). The well-defined anterior borders of expression differ between endodermally and mesodermally derived tissues. In the neural tube and the spinal ganglia, expression starts at the level of the fourth ganglion, whereas mesodermal expression extends caudally from the second thoracic prevertebra (Mahon et al., 1988). Posterior boundaries are less well defined, and levels of Hox-1.1 RNA decrease toward the
The relatively long survival of Tg-β-492 demonstrates the developmental retardation after birth as compared with a nontransgenic littermate. (a) Five days after birth. (b) Fourteen days after birth.

caudal end of the embryo. At no time in development can Hox-1.1 RNA be detected in the head region (M. Kessel, unpublished data).

The specifically restricted pattern of Hox-1.1 expression in normal mice was changed to an almost ubiquitous expression pattern in the transgenic mice. The observed expression of the transgene results from the ubiquitous activity of the β-actin promoter. The promoter sequences used for the β construct are active in a variety of tissue culture systems, including teratocarcinoma stem cells (B. Paterson, personal communication; M. Kessel, unpublished data; Guild et al. 1988). The relatively low levels of expression initiated by the chicken β-actin promoter may have been a parameter that allowed the animals to survive until birth.

It is likely that additional control regions from the Hox-1.1 sequences influence the expression of the transgene. The presence of two untranslated open reading frames upstream of the initiation codon for the sequence encoding the homeodomain protein may modulate Hox-1.1 translation (Kessel and Gruss, 1988), and a posttranscriptional destabilization signal may be present in the second exon (Colberg-Poley et al., 1987). The postnatal lethality was strictly correlated with the presence and transcription of the transgene. Therefore we conclude that it is the ectopic or ubiquitous expression of Hox-1.1 that causes the observed phenotype.

Craniofacial Abnormalities in Transgenic Animals
Craniofacial abnormalities were detected among the non-viable newborn transgenic animals expressing Hox-1.1 ectopically. Morphological alterations were restricted to the head, which does not normally contain Hox-1.1 RNA at any time of development. At birth, visceral and somatic structures caudal to the head were normally configured, developmentally age appropriate, and without pathological findings. It seems that the majority of the embryonic tissues are well buffered against effects of ectopic Hox-1.1 expression at the level obtained with the β construct. While a cleft palate would be sufficient to explain postnatal lethality, we cannot yet explain the dominant lethal phenotype in animals without cleft palate. Possibilities include functional or metabolic defects not reflected in the morphology of the tissues examined, or structural changes too subtle to be resolved by routine histology.

The observed head abnormalities—open eyes, non-
fused pinnae, and cleft secondary palate—share several features that may hint at an underlying pathogenesis. A deficiency in the quantity of mesenchyme is associated with aberrant coordinate morphogenesis with the overlying epithelium. Embryonic fusion of these structures may be delayed as a result of inadequate mesenchymal proliferation or modeling, a process that normally serves to juxtapose adjacent elements in preparation for subsequent merger. Palate processes are formed in the primitive oral cavity. These swellings, composed of mesenchyme derived from migratory neural crest cells and covered by craniopharyngeal ectoderm, grow vertically, then elevate to appose opposite sides, and eventually fuse in the midline around day 15 (for review see Ferguson, 1988). Growth of the eyelids and pinnae occurs later in embryogenesis, with fusion between day 15 and day 17 (Maconnachie, 1979). In more caudal regions of the body, similar processes such as fusion of the digits and formation of the septum of the heart occur at similar times. These processes were not found to be affected in the transgenic animals.

A phenotype resembling that of ectopic Hox-7.7 expression, including open eyes at birth, cleft palate, and postnatal lethality, has been described for far mouse mutants (McLeod et al., 1989; Juriloff and Harris, 1983). However, our transgenic animals lack several features of far mutant animals, including facial skin tags and vibrissae abnormalities. On the other hand, detached pinnae are not a described finding in the far mutants. Depending on the genetic background, far is an incomplete dominant mutation and thus may represent a gain-of-function mutation (Juriloff et al., 1987). Further molecular and morphological examinations and comparisons between gB transgenic and far mice are under investigation.

Ectopic overexpression of the Drosophila genes Antp and Deformed leads predominantly to the alteration of anterior structures in transgenic flies (Gibson and Gehring, 1988; Kuziora and McGinnis, 1988). We observe a similar predominance in the transgenic mice. In addition, homeotic transformations such as ectopic legs or ectopic maxillary structures are induced in flies. To generate the homeotic phenotype, the timing and degree of ectopic expression must be precisely controlled. Experimentally induced ectopic expression of the pair-rule gene fushi tarazu in segments not normally expressing this gene results in abnormal segmentation during development (Struhl, 1985). The experiments demonstrate that both expression and nonexpression are equally instructive in determining gene function, since the development of specific body regions requires selector genes to be induced or repressed in a combinatorial manner. In this study, homeotic transformations could not be detected with the methods available. Rather, the transgenic phenotype is abnormal development of appropriate regional structures. The induction of specific developmental defects through ectopic expression of a mammalian Hox gene strengthens the assumption that these genes play an important role in the regulation of vertebrate embryogenesis.

Neural Crest Cells as Possible Targets of Transgene Action
The mesenchyme in all sites affected in the gB mice is partly (in the ear) or predominantly (in the palpebrae and palate) derived from the first arch neural crest (for review
see Hall and Hörstadius, 1988; Ferguson, 1988; Noden and Van De Water, 1988). Neural crest cells originate from the dorsal ectoderm along each side of the neural plate during the final stage of neural tube closure. After migrating to their final position, they differentiate into a variety of different cell types (see Hall and Hörstadius, 1988). Cranial neural crest cells differ from trunk crest cells in having skeletogenic properties. The cells emigrating from the level of the rostral hindbrain normally contribute to structures of the first arch (Tan and Morriss-Kay, 1988). It is at present unclear whether neural crest cells show a predisposition toward a specific cell fate at the time of migration or whether they remain pluripotent until later stages of development. Several experiments indicate that at least some crest cells are pluripotent before migration from the neural tube (Hall and Hörstadius, 1988; Bronner-Fraser and Fraser, 1988), whereas transplantation experiments suggest that cranial neural crest cells are intrinsically predetermined at emigration (Noden, 1983). Our data indicate that cranial neural crest cells are possible targets of the ectopic Hox-1.1 gene expression. Determining the specific step affected by the transgene may reveal important aspects of craniofacial development.

Up to now, few candidates for head selector genes have been identified. Most homeobox genes are not expressed rostral to the myelencephalon, except for the engrailed-related genes En-1 and En-2, which are expressed in the complete central nervous system including the embryonic forebrain, or the mesencephalon, respectively (Davis and Joyner, 1988). Furthermore, the recently described Hox-7 gene is expressed in cranial neural crest cells and derived facial structures and thus is an interesting gene to study in the transgenic g6 mice (Robert et al., 1989; Hill et al., 1999).

In Xenopus embryos microinjection of antibodies against a homodomain protein (XII-box1) resulted in absence of anterior dorsal root ganglia and deletion of the dorsal fin (Cho et al., 1988). The dorsal fin mesenchyme and the cervical dorsal root ganglia are derived from the neural crest, and removal of a small region of neural crest cells in amphibians leads to a loss of the dorsal fin (Hadorn, 1970). By increasing the gene dosage, the Hox-
The 1.4 gene was specifically overexpressed in transgenic mice (Wolgemuth et al., 1989). The overexpression of Hox-1.4, although not ectopic, resulted in a functionally stenotic colon. It was postulated that Hox-1.4 overexpression affected neural crest-derived ganglia of the enteric nervous system. Thus these results, in addition to our own study, implicate neural crest cells as potential targets for Hox gene function.

RAE Resembles the Transgenic Phenotype

Vitamin A and its derivatives, the retinoids, have very important functions in vivo at physiological concentrations, whereas high doses of most retinoids are strongly teratogenic in vertebrates. RAE is characterized by severe limb and cranial defects, including cleft secondary palate, midfacial malformations, abnormal external and middle ear, and open eyes at birth (Shenefelt, 1972; Ferm and Ferm, 1979; Sulik et al., 1988). Women who took retinoid acid (Accutane) during pregnancy bore children with defects of the ear and craniofacial abnormalities (Lammers et al., 1979). In chicken, retinoid treatment in vivo specifically affected the upper but not the lower beak (Tarnarini et al., 1984; Wedden et al., 1988). When 13-cis-retinoid acid was administered to mice, defects in the visceral arches were particularly prominent (Goulding and Pratt, 1986; Sulik et al., 1988). The phenotypes of gß mice and the far mutants are similar to RAE. The human syndrome mandibulofacial dysostosis (Treacher Collins syndrome) also displays a similar phenotype (Sulik et al., 1988).

Since in vivo effects of retinoic acid suggested an effect on neural crest cells, in vitro experiments were performed that indeed demonstrated an inhibitory effect on migration of neural crest cells (Thorogood et al., 1982). Hassel et al. (1977) showed that retinol prevented the appearance of cranial neural crest cells in the first aortic arch of cultured chick embryos. It has been suggested that retinoids may interfere with positional cues that operate as axial primordia development (Wedden et al., 1988).

Retinoic acid is a potential morphogen during the embryonic development of vertebrates, as recently supported by experimental evidence (Thaller and Eichele, 1987). In teratocarcinoma cells retinoic acid induces the expression of many homeobox-containing genes, including Hox-1.1 (Culberg-Puley et al., 1985a, 1985b, Breier et al., 1986; Kessel et al., 1987; Mavilio et al., 1988; Fibi et al., 1989). Furthermore, Deechamp et al. (1987) were able to correlate this induction strictly with the application of retinoic acid and not with the onset of differentiation. Thus the Hox genes may be induced by retinoic acid during embryogenesis. Our experiments pointed out similar consequences of systemic retinoic acid administration and ectopic Hox-1.1 expression during embryogenesis, suggesting a common mode of action. It can be demonstrated that retinoic acid acts via an induction of Hox-1.1 in vivo, the similar phenotypes of RAE and our transgenic mice could be explained. The establishment of this correlation may help in understanding the teratogenic activity of retinoids and the physiological role of retinoic acid as a morphogen.
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References


