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Early Postnatal Lethality in *Hoxa-5* Mutant Mice Is Attributable to Respiratory Tract Defects

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To uncover roles for the *Hoxa-5* gene during embryogenesis, we have focused on identifying structural and functional defects in organ systems underlying the perinatal lethality in *Hoxa-5* homozygous mutants. Analysis of the mutant phenotype shows that *Hoxa-5* is essential for normal organogenesis and function of the respiratory tract. In homozygous newborn mutants, improper tracheal and lung morphogenesis can lead to tracheal occlusion, and to respiratory distress associated with a marked decrease in the production of surfactant proteins. Collectively, these defects likely underlie the pronounced mortality of homozygous mutant pups. Furthermore, the loss of *Hoxa-5* function results in altered *TTF-1, HNF-3β,* and N-*myc* gene expression in the pulmonary epithelium. Since expression of *Hoxa-5* is confined to the mesenchymal component of the developing trachea and lung, the effects observed in epithelial cells may result from a disruption of normal epithelial–mesenchymal interactions. © 1997 Academic Press

Key Words: Hox **genes; trachea; lung development;** ***TTF-1; HNF-3.***

INTRODUCTION

The murine *Hoxa-5* gene is a member of the *Hox* gene family of transcription factors that act during development to specify regional identity along the anteroposterior body axis by regulating specific downstream sets of effectors, which in turn direct morphogenetic events (Krumlauf, 1994). The transcriptional activity of HOX proteins is mediated via a conserved DNA-binding homeodomain present in both invertebrate and vertebrate genomes. Mammalian *Hox* genes constitute a large family of 39 genes clustered in four independant linkage groups. The organization of the constituent genes in each complex is fundamental for the precise spatiotemporal regulation and the function of each gene, and hence for the correct patterning of the embryo (van der Hoeven *et al.,* 1996). Thus, the relative order of the *Hox* genes within a given cluster governs their time of activation and their precise domain of expression along the anteroposterior embryonic axis: the 3′-most genes are expressed earlier and in more anterior domains than the genes localized in more 5′ positions. Consequently, different members of the *Hox* clusters are expressed in discrete but overlapping domains along the developing axis, suggesting that the specific combination of HOX proteins within a particular region provides a unique address determining its identity.

To decipher the developmental role played by the mammalian *Hox* genes, targeted mutational analyses of multiple *Hox* genes have been performed in mice (reviewed in Stein *et al.,* 1996). The panoply of phenotypic consequences of these mutations fall into two general categories, namely homeotic transformations affecting the skeleton and defects in organogenesis. The abnormalities generally affect structures restricted to a rostrocaudal window corresponding to the most anterior portion of the expression domain (e.g., *Hoxa-1*), or to subregions showing the highest levels of gene expression (e.g., *Hoxc-4*). The homeotic transformations result in either a partial or complete change of identity of the vertebra into the likeliness of the neighboring vertebrae, with both anterior and posterior transformations having

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been described. Various defects in organogenesis have also been reported, some resulting in lethality in *Hox* mutant mice. In fact, perturbations of the 3′-most *Hox* genes involved in early patterning events have more dramatic consequences on the viability of the animals.

Lethal phenotypes are associated with mutations of *Hoxa-1, Hoxb-1, Hoxa-2, Hoxb-2, Hoxa-3, Hoxd-3, Hoxb-4, Hoxc-4,* and *Hoxa-5* (Chisaka and Capecchi, 1991; Lufkin *et al.,* 1991; Chisaka *et al.,* 1992; Condie and Capecchi, 1993; Gendron-Maguire *et al.,* 1993; Jeannotte *et al.,* 1993; Ramirez-Solis *et al.,* 1993; Rijli *et al.,* 1993; Barrow and Capecchi, 1996; Boulet and Capecchi, 1996; Goddard *et al.,* 1996). Resolving how defects in organogenesis result in lethality in different *Hox* mutant mice is critical for determining the exact developmental function of these genes. However, for the most part this has proven difficult. Nevertheless, as for the axial skeleton, the phenotypic consequences of *Hox* gene mutations on developing organs further endorse the important relationship between the position of individual genes within the *Hox* clusters and the structural defects observed along the anteroposterior axis.

To date, the most 5′ *Hox* gene mutation associated with a high rate of perinatal lethality is *Hoxa-5.* Preliminary characterization of *Hoxa-5* mutant mouse showed that specification of axial identity is perturbed and viability is markedly reduced (Jeannotte *et al.,* 1993). Homeotic transformations affect vertebrae confined to a region of the axis located between the third cervical and the second thoracic vertebrae (Jeannotte *et al.,* 1993; M. Lemieux and L. Jeannotte, unpublished). This region corresponds to the anterior part of the *Hoxa-5* expression domain along the prevertebral axis. *Hoxa-5* is also expressed in the developing spinal cord, where its anterior limit of expression corresponds to the posterior floor of the myencephalon and in the mesenchymal component of the lungs, trachea, stomach, intestine, and kidneys (Dony and Gruss, 1987; Gaunt *et al.,* 1988).

We report here that *Hoxa-5*-deficient pups display respiratory distress at birth. They suffer from severe obstruction of the laryngotracheal airways and presented with abnormal pulmonary histology. The lack of *Hoxa-5* function during respiratory tract ontogeny results in a profound dysmorphogenesis of the developing lung and in a decrease of expression of the surfactant-associated proteins. Collectively, these defects underlie the pronounced perinatal lethality phenotype associated with this mutation. Furthermore, the expression of some of the genes regulating production of the surfactant-associated proteins in the endoderm, such as *TTF-1* and *HNF-3β,* is affected by the *Hoxa-5* mutation. Moreover, enhanced N-*myc* expression in the pulmonary epithelium is observed in *Hoxa-5* mutants. Since *Hoxa-5* expression is confined to the mesenchymal component of the respiratory tract, a cell population that interacts with overlying endoderm during lung morphogenesis, these findings suggest that target genes regulated by *Hoxa-5* are likely to mediate these important tissue interactions in development.

MATERIALS AND METHODS

Generation of Mice and Genotyping

The establishment of the *Hoxa-5* mutant mouse line has been reported previously (Jeannotte *et al.,* 1993). *Hoxa-5* mutant mice were initially generated in a hybrid MF1-129/SvEv-C57B1/6 genetic background. To assess the effect of the mutation in an inbred genetic background, heterozygous *Hoxa-5* mice were backcrossed with either 129/SvEv or C57B1/6 animals for at least 10 generations. Heterozygous *Hoxa-5* mice were then intercrossed to generate embryos or newborn pups of all three possible genotypes. Embryonic age was estimated by considering the morning of the day of the vaginal plug as embryonic (E) day 0.5. Animals were genotyped by Southern blot analysis of yolk sac or tail DNA as previously described (Jeannotte *et al.,* 1993).

Histology and Skeletal Preparations

Newborn mice were asphyxiated with CO_2, skinned, and fixed in Bouin's fixative for 7 days. They were further processed as described in Mark *et al.* (1993), with the exception that toluene was used instead of Histolemon. For histological analyses of isolated organs and embryos, the samples were fixed in 4% paraformaldehyde for 16 h, before being processed and embedded in paraffin wax. Serial sections (5 μm) were collected, deparaffinized, rehydrated, and stained with hematoxylin and eosin according to standard histological procedures. For each embryonic age analyzed, at least two wild-type controls and four *Hoxa-5* homozygous mutants were used. Morphometric measurements made on E15.5 lungs consisted of the scoring of tubules and terminal acini per area of 0.25 μm^2. Measurements were made on at least six independent sections at different levels of the lungs of two wild-type controls and five *Hoxa-5* homozygous mutants. Statistical analyses were performed according to Student's t test.

For immunostaining, lung tissue from five wild-type specimens and four *Hoxa-5* homozygous mutant mice sacrificed at birth was fixed in Bouin's fluid for 16 h, dehydrated, and embedded in paraffin wax. Serial sections (5 μm) were used to detect surfactant proteins (SP) by immunofluorescence using polyclonal antibodies directed against SP-A, -B, and -C (kindly provided by Dr. J. Whitsett). For each specimen, alternate sections were stained with hematoxylin and eosin to correlate histological observations with the results obtained from the immunofluorescence experiment.

Skeletons were prepared according to the procedure described in Jeannotte *et al.* (1993).

RNA in Situ Hybridization and Semiquantitative RT–PCR Analyses

The *in situ* hybridization protocol was based on that of Jaffe *et al.* (1990). The following murine fragments were used as templates for synthesizing [^{35}S]UTP-labeled riboprobes: a 850-bp *Bgl*II–*Hin*dIII genomic fragment containing the 3′-untranslated region of the *Hoxa-5* gene; a 528-bp *TTF-1* cDNA clone (kindly provided by Dr. R. Di Lauro); a 1.7-kb *HNF-3β* cDNA and a 1.2-kb *HNF-3α* cDNA clones (kindly provided by Dr. E. Lai); and a 974-bp cDNA clone containing the 3′-untranslated region of the N-*myc* gene (kindly provided by Dr. J. Charron). A minimum of two wild-type controls and three *Hoxa-5* homozygous mutants were analyzed for

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TABLE 1
Comparison of Overall Grain Density for *TTF-1, HNF-3α, HNF-3β,* and N-*myc* in Wild-Type and Homozygous Lung Epithelium

	Nuclear grain density			
	WT	HM	Change (%)	Mean (%)
TTF-1				
Experiment 1	10.14 ± 0.51	3.67 ± 0.70	−64	
Experiment 2	6.29 ± 0.91	3.63 ± 0.58		−54 ± 14
	5.19 ± 1.84	3.22 ± 0.32		($P < 0.001$)
		2.80 ± 0.25		
Mean Expt 2	5.74 ± 1.50	3.22 ± 0.50	−44	
HNF-3α				
Experiment 1	3.84 ± 1.18	3.04 ± 0.58		
		3.64 ± 0.43		
Mean Expt 1	3.84 ± 1.18	3.34 ± 0.58	−13	−20 ± 10
				($P > 0.07$)
Experiment 2	2.68 ± 0.12	1.96 ± 0.68	−27	
HNF-3β				
Experiment 1	6.51 ± 1.05	3.62 ± 0.14	−44	
Experiment 2	6.32 ± 0.93	4.75 ± 0.39		−39 ± 6
		3.54 ± 0.37		($P < 0.005$)
		3.98 ± 0.24		
Mean Expt 2	6.32 ± 0.93	4.25 ± 0.56	−33	
N-*myc*				
Experiment 1	3.08 ± 0.26	6.26 ± 1.16		
	5.40 ± 1.90	6.41 ± 1.53		+43 ± 11
		5.56 ± 1.30		($P < 0.002$)
Mean Expt 1	4.24 ± 1.77	6.08 ± 0.45	+43	

each embryonic age studied. Overall grain density was estimated by counting nuclear silver grains staining on six independant areas per embryo analyzed at 1000× magnification. For each embryo, a minimum of 240 nuclei were counted and the mean value obtained is indicated in Table 1. Changes in the nuclear grain density was calculated for samples from the same experiment. Statistical analyses were performed according to Student's *t* test.

Total RNA from wild-type and homozygous lungs of E18.5 fetus was prepared as described in Jeannotte *et al.* (1993). The RT–PCR semiquantitative analysis for assessment of surfactant protein mRNA levels was performed as described in Bérard *et al.* (1994).

RESULTS

The Hoxa-5 Mutation Is Associated with Markedly Reduced Postnatal Viability

We previously observed that homozygous *Hoxa-5* mutant animals were not obtained in the expected ratio in the progeny of intercrosses of heterozygous animals of a mixed genetic background (Jeannotte *et al.,* 1993). Thus, only 13% of the weaned animals were homozygous mutants. To examine the effect of the genetic background on the expressivity of the phenotype, the *Hoxa-5* mutation was backcrossed into the 129/SvEv and C57B1/6 inbred backgrounds. In the 129/SvEv congenic background, a comparable proportion of homozygous mutant mice was observed (16%) at weaning age, while it fell as low as 3% in the C57B1/6 congenic genetic background (M. Lemieux and L. Jeannotte, unpublished).

To identify the underlying cause of the lethal phenotype, we undertook a thorough characterization of the *Hoxa-5* mutant phenotype in the 129/SvEv inbred background because a substantial number of mutants survive until adulthood, which facilitates the analyses. Daily monitoring of litters generated from heterozygous and homozygous intercrosses revealed that the majority of the dead pups (73/115 homozygous mutants) had a cyanotic complexion and presented with respiratory distress immediately following birth. Autopsies of the dead newborns revealed that a high proportion had collapsed lungs (37/44 homozygous animals analyzed) and had air in their stomach and intestines, indicating these animals failed to breathe properly. Eighty-five percent of the deaths in *Hoxa-5* homozygous mutant population (98/115) occurred within less then 24 h. In some cases, mutants survived for a few days after birth but showed a rapid wasting syndrome probably related to their incapacity to feed properly, as assessed by the absence of milk in their digestive tract. *Hoxa-5* homozygous animals that survived until adulthood were fertile but could be distinguished from heterozygous and wild-type littermates because the loss of *Hoxa-5* function hindered their growth and

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caused delayed eyelid opening (J. Aubin and L. Jeannotte, unpublished).

Laryngotracheal Malformations Are Completely Penetrant in Hoxa-5 Mutant Mice

Skeletal preparations and macroscopic observations revealed that the larynx and the trachea were greatly disorganized in all homozygous specimens. The cricoid and thyroid cartilages are the two major components of the larynx, with the thyroid cartilage being the larger of the two. In all homozygous individuals analyzed (37 newborns and 27 adults), the cricoid was always larger than the thyroid cartilage due to fusion with the first tracheal rings (Fig. 1). Furthermore, the tracheal rings did not display a normal banding pattern and the number of rings formed tended to be reduced. The cartilage did not extend as dorsally as in wild-type samples and the trachea was narrower.

Histological examination of sections of dead homozygous newborn pups revealed a striking reduction of the diameter of the tracheal lumen compared to wild-type littermates, confirming the macroscopic observations. However, although laryngotracheal malformations were fully penetrant, their expressivity was variable (Fig. 2). Another consistent observation was a profound disorganization of the tracheal epithelium and a thickening of the lamina propria derived from the mesenchymal layer during trachea ontogeny. In severely affected mutants, the lamina propria formed small folds sometimes accompanied with protusions in the tracheal aperture (data not shown). Normally, the respiratory epithelium undergoes a progressive transition from a tall pseudostratified columnar, ciliated form in the larynx and the trachea to a simple cuboidal, nonciliated form in the smallest airways. In *Hoxa-5* homozygous mutants, the tracheal epithelium appeared to be stratified rather than pseudostratified. The lamina propria was also wider along the entire length of trachea compared to wild-type. This cellular disorganization was also detected in the bronchial and bronchiolar epithelial lining and underlying lamina propria (data not shown). Thus, in the most severely affected homozygous newborn mutants, tracheal occlusion was likely the cause of the rapid perinatal death. However, the variation in the expressivity of the phenotype indicated that these defects were not the sole cause of death of the *Hoxa-5* homozygous mutant mice.

The Hoxa-5 Mutation Hinders Normal Lung Function

We next investigated whether normal lung function was also perturbed. The pulmonary surfactants and their associated proteins are essential for proper lung expansion and their absence or reduced production by type II pneumocytes results in respiratory distress. This condition is characterized by labored breathing, underinflated lungs, and alveoli partly filled with a proteinaceaous fluid (Carlson, 1994). The phenotype of *Hoxa-5* mutants showed similarities with the phenotypic consequences of the respiratory distress syndrome described in premature human infants. *Hoxa-5* is expressed in the lungs (Dony and Gruss, 1987; Gaunt *et al.,* 1988), which prompted us to address the state of lung function by assessing the presence of the pulmonary surfactant proteins SP-A, SP-B, and SP-C in lungs of wild-type and mutant newborn mice. A significant reduction in SP proteins was observed in mutant newborn lungs (shown for SP-B, Fig. 3). A semiquantitative RT–PCR analysis was performed on total RNA extracted from E18.5 wild-type and homozygous fetal lungs. The decrease at the protein level correlated with a diminution in mRNA levels of 56% for SP-B, and 31% for both SP-A and -C (data not shown). Thus, the loss of *Hoxa-5* function affected the production of surfactant-associated proteins.

Defects in Lung Ontogeny in Hoxa-5 Mutants

Since *Hoxa-5* is strongly expressed during ontogeny of the respiratory tract, we wanted to determine if defective lung morphogenesis was related to impaired lung function in *Hoxa-5* mutants. Initially, a comparative histological analysis of control and *Hoxa-5* mutant lung development was performed. In embryonic development, the larynx and the trachea begin as a groove in the floor of the primitive pharynx. At around E9.5, the trachea develops from the midportion of the laryngotracheal tube and then elongates and the diverticulum thus formed descends into the thoracic cavity. Concomitantly, the diverticulum becomes invested with splanchnic mesenchyme and by day 10 of gestation, its distal end enlarges to form the primary lung buds. This process occurred normally in *Hoxa-5* homozygous mutants (data not shown). The lung buds become the mainstem bronchi and their formation is followed by appearance of secondary bronchi (E11.0–E11.5) that will undergo further ramification into

FIG. 1. Laryngotracheal malformations in *Hoxa-5* mutant newborn mice. Frontal (A–D) and lateral (E–H) views of a control wild-type (A, E) and three homozygous mutant newborns (B–D, F–H). Alcian blue staining revealed cricoid malformation, disorganized banding pattern, and narrowing of the trachea in all mutant specimens. The number of tracheal rings formed was reduced and the cartilage did not extend as dorsally in the mutants (F–H) when compared to the control (E).

FIG. 2. Effect of the *Hoxa-5* mutation on lumen diameter and tissue organization of the trachea. Transverse sections of the upper portion of the trachea of a sacrificed wild-type (A) and three homozygous stillborn mutants (B–D) showed the severe reduction in the lumen diameter in mutants. Concomitantly, disorganization of the epithelial layer and the adjacent submucosa was observed in all mutants. Expressivity of the mutant phenotype varied considerably. Original magnification, 100×.

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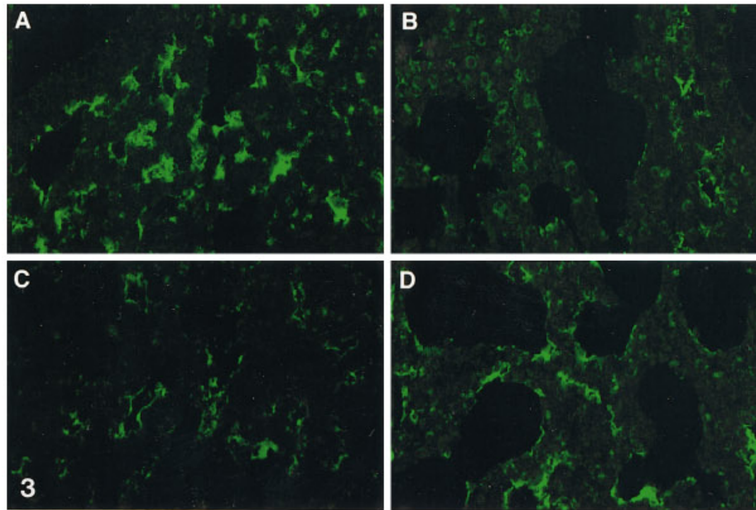
A B C D E F G H 1

A B C D 2


A
B
C
D
3

the surrounding mesenchyme to generate the bronchial tree ending in multiple alveoli. In *Hoxa-5* homozygous mutants, altered pulmonary morphogenesis was seen as early as E12.5. At this stage, a slight disorganization of the mesenchymal layer was apparent, especially in those mesenchymal cells surrounding the secondary bronchioli (Figs. 4A and 4B). At E15.5, the developing lung reaches the pseudoglandular stage and the ramifying bronchial tree gives rise to tubular branches ending in terminal acini. At this stage, the branching was less extensive in *Hoxa-5* mutants showing a mean reduction of 33% ($P < 0.001$) in the number of bronchioli and terminal acini per area scored (Figs. 4C and 4D). In addition, the diameter of the structures that formed was generally reduced in size, and accompanied by a disorganization of the surrounding mesenchyme. At the saccular stage (E17.5–18.5), the *Hoxa-5* mutant lungs displayed an abnormal compact appearance probably resulting from a thickening of the alveolar walls (Figs. 4E and 4F). At this stage of fetal development, the mesenchymal component of the lung is fully intermingled with the pulmonary epithelium, making it extremely difficult to distinguish if only one or both layers were affected in the mutants. By birth, the predominant defect was disorganization of both the proximal (trachea and mainstem bronchi) and distal respiratory pathways (Figs. 4G and 4H). The bronchial epithelium, which normally displays nonciliated columnar cells, was tangled in all mutant animals examined (total of 17). In addition, areas of poorly inflated alveoli were observed as well as the presence of liquid in bronchioli correlating histological findings with the fact that the mutants never breathe properly. In summary, no observable delay in lung ontogeny occurred in *Hoxa-5* homozygous mutants compared to wild-type littermates, but normal morphogenesis was altered by the absence of *Hoxa-5* function.

Expression of TTF-1 Is Affected during Lung Ontogeny in Hoxa-5 Mutants

As a transcription factor, HOXA-5 protein is likely to exert effects on lung morphogenesis via regulation of subset of lung genes. A good candidate target of HOXA-5 is *TTF-1* because its promoter region contains a binding site responsive to the HOXB-3 protein in cotransfection assay (Guazzi *et al.,* 1994). TTF-1 is a transcription factor involved both in lung ontogeny and in the regulation of the expression of surfactant proteins (Bohinski *et al.,* 1994). An essential role for TTF-1 in lung morphogenesis has been established because homozygous *TTF-1* mutants show dilated sac-like structures in the pleural cavity instead of normal lungs (Kimura *et al.,* 1996). Considering that both abnormal lung morphogenesis and lowered production of surfactant proteins were observed in *Hoxa-5* homozygous-deficient animals at birth, an interesting possibility is that expression of *TTF-1* may be affected by the loss of *Hoxa-5* function. We addressed this question by comparative *in situ* hybridization analyses at various stages of lung development on wild-type and mutant specimens.

Throughout development of the rat respiratory tract, *TTF-1* transcripts have been reported to be restricted exclusively to the more distal part of the developing lung epithelium, no signal being detectable in more proximal airways (Lazzaro *et al.,* 1991). A similar hybridization pattern was described for the mouse (Kimura *et al.,* 1996). However, we observed *TTF-1* expression in the epithelium of the laryngotracheal groove at the beginning of lung morphogenesis in wild-type embryos (E10.5; Figs. 5A and 5B). Furthermore, at E12.5, *TTF-1* expression was not restricted to the developing epithelium but was present in the mesenchymal layer of the trachea, being detected as two narrow bands restricted to the edge of the tracheal primordia (Figs. 5C and 5D). Finally, we also observed *TTF-1* transcripts in the proximal and distal airways at later stages tested (E18.5 and newborn; Figs. 5E, 5F, 5G, and 5H). By contrast, examining the hybridization signal obtained for *TTF-1* in *Hoxa-5* mutant embryos at E12.5, we observed a decrease in the intensity of the signal in the epithelial lining of mutant respiratory tract (Figs. 6D and 6I). A comparison of overall grain density confirmed a reduction ranging from 44 to 64% (mean of 54%) in the mutant pulmonary epithelia (Table 1). The results were reported on a per nuclei basis to take into account any alteration in the number of cells. Another difference noted comparing E12.5 wild-type and mutant trachea and primary bronchi was an expansion of the mesenchymal *TTF-1* signal along the primary bronchi of the *Hoxa-5* mutants (Figs. 6L and 6N).

Effect of the Lack of Hoxa-5 on the Expression of Members of the forkhead Gene Family

HNF-3α and HNF-3β are transcription factors belonging to the *forkhead (fkd)* gene family, and are both expressed

FIG. 3. Effect of the *Hoxa-5* mutation on the production of surfactant protein B. Immunofluorescence detection of SP-B was performed on a control wild-type (A) and three mutant lung samples of animals sacrificed at birth (B–D). The immunofluorescence staining pattern showed a marked reduction in SP-B production in mutant lungs.

FIG. 4. Comparative histology of lung development. Left panels represent wild-type lung specimens whereas right panels correspond to *Hoxa-5* homozygous mutant counterparts. At E12.5, mutant lung showed a slight disorganization of the mesenchymal layer (A, B). At E15.5, mesenchymal disorganization was obvious in the mutant specimen, and an overall decrease in both the number and diameter of the bronchiolar tubules and terminal acini was observed (C, D). At E18.5, mutant lung displayed an abnormal compact appearance compared to the control sample (E, F). At birth, mutant lung was collapsed and a fluid filled the bronchioli and alveoli, while proper lung expansion was observed in the wild-type sample (G, H). Original magnification, 200×.

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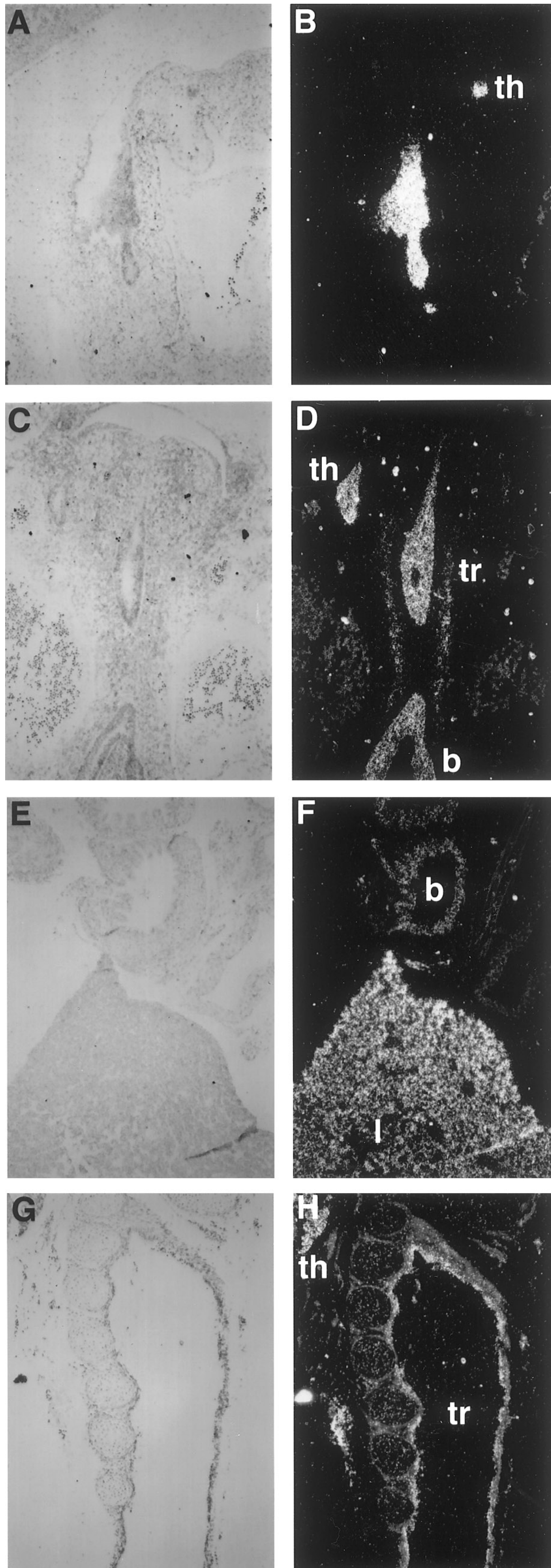

in endoderm derivatives during embryogenesis (Sasaki and Hogan, 1993; Monaghan *et al.,* 1993; Ang *et al.,* 1993). The expression of *HNF-3α* and *HNF-3β* is maintained throughout lung development and in adult pulmonary tissue. Moreover, it has been shown that HNF-3α and HNF-3β are required for the proper expression of pulmonary-specific genes such as the surfactant proteins and the Clara cell secretory protein (Cardoso, 1995). *HNF-3β* mutants fail to form foregut structures, including lung primordia (Weinstein *et al.,* 1994; Ang and Rossant, 1994). Additionally, in *Drosophila, fkd* expression is regulated by the product of the *Sex combs reduced* gene, the *Hoxa-5* ortholog (Zhao *et al.,* 1993). To explore if expression of *HNF-3α* and *HNF-3β* is perturbed in *Hoxa-5* homozygous mutants, *in situ* hybridization experiments were performed on control and mutant embryonic lungs at E12.5. As reported, expression in the lung was restricted to the epithelium (Figs. 6B, 6C, 6G, and 6H). In the *Hoxa-5* mutant lungs, a decrease in *HNF-3β* signal intensity was observed, while no statistically significant change was seen for *HNF-3α* (Figs. 6B, 6C, 6G, and 6H). Quantitation by comparison of overall grain density showed that *HNF-3β* mRNA levels was reduced by 39% in homozygous mutant lung epithelia (Table 1).

N-myc Expression Is Affected by the Loss of *Hoxa-5* Function

The proto-oncogene N-*myc* is involved in growth and differentiation during mammalian embryogenesis (Charron *et al.,* 1992; Moens *et al.,* 1992; Stanton *et al.,* 1992; Sawai *et al.,* 1993). During organogenesis, N-*myc* transcripts are detected in the epithelium of several organs including the lung (Mugrauer *et al.,* 1988; Hirning *et al.,* 1991). The essential role of N-*myc* in lung morphogenesis was established by the analysis of N-*myc* homozygous mutant mice carrying either a null or a hypomorphic allele (Moens *et al.,* 1992; Stanton *et al.,* 1992; Sawai *et al.,* 1993). In null mutants, branching does not proceed normally, while in the case of the hypomorphic mutation, the mutant mice die at birth of respiratory distress caused by the improper proliferation and branching of the pulmonary epithelium. The phenotype of the N-*myc* mutant combined with the localization of N-MYC protein in the lung epithelium suggests that the level

FIG. 5. Expression of *TTF-1* during proximal respiratory tract development. At E10.5, *TTF-1* transcripts were detected throughout the laryngotracheal groove and in the thyroid diverticulum (A, B). At E12.5, *TTF-1* expression was observed as two narrow hybridization bands at the edge of the tracheal mesenchyme, in the entire respiratory tract epithelium, and in the thyroglossal duct (C, D). At E18.5, primary bronchi and lung epithelia were both expressing *TTF-1* (E, F). Signal persisted in newborn trachea (G, H). All specimens were wild-type samples. b, bronchi; 1, lung; th, thyroid; tr, trachea. Original magnification, 100×.

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of N-*myc* expression in this tissue is critical to allow a normal response to inducing signals from the mesenchyme. Considering the reduced lung branching and the profound disorganization observed in *Hoxa-5* mutant lungs, we tested whether N-*myc* expression was affected by the *Hoxa-5* mutation. At E12.5, N-*myc* expression was detected in the lung epithelium. In *Hoxa-5* mutants, a 43% increase in N-*myc* signal intensity was observed in the lung epithelium (Table 1, Figs. 6E and 6J). Consequently, the loss of *Hoxa-5* gene function resulted in the altered expression of essential regulators involved in specialized pulmonary epithelial cell function and proliferation.

Hoxa-5 Gene Expression in the Developing Lung

It was important to confirm that the structural defects observed in the *Hoxa-5* mutant mice were restricted to regions contained within the normal *Hoxa-5* gene expression domain. *Hoxa-5* is strongly expressed in the cervicothoracic region of the prevertebral column with an anterior boundary corresponding to prevertebra 3 and in the mesodermal component of the trachea and the lung during their organogenesis (Gaunt *et al.,* 1988). *In situ* hybridization experiments on E12.5 embryos confirmed the *Hoxa-5* expression in the mesenchyme of the larynx, trachea, and lungs in the developing embryo (Fig. 7). Restriction to the mesenchymal component of the lung was also confirmed for E10.5, E11.5, and E15.5 (data not shown). Thus, disruption of the *Hoxa-5* gene in the lung mesenchyme resulted in deregulated expression of epithelial-specific genes. The role of *Hoxa-5* in correct respiratory tract morphogenesis and function may therefore proceed via epithelial–mesenchymal interaction pathways.

DISCUSSION

The present study demonstrates the importance of *Hoxa-5* to the process of tracheal and lung development. The lung is derived from an outpocketing of the foregut endoderm into the mesenchyme of the fetal thorax. Proliferation of the primitive pulmonary epithelial cells results in a continuous epithelium from the trachea to the alveoli. Concomitant with this cellular proliferation, regional differentiation along the length of the developing epithelium gives rise to the formation of highly specialized cell types that determine lung function (Cardoso, 1995). The mechanisms of tracheal and lung morphogenesis are known to be governed by epithelial–mesenchymal interactions. Classical experiments involving *in vitro* embryonic lung explants have shown that during early lung morphogenesis, the bronchial mesenchyme induces budding of the epithelium and controls its specific pattern of branching (Wessells, 1970). Multiple transcription factors are expressed in patterns consistent with roles in lung morphogenesis, and the involvement of some of them has been provided by the analysis of mutant mouse lines.

In *Hoxa-5* homozygous mutants, the defective respiratory tract morphogenesis underlies the high rate of perinatal lethality. Among the causes involved is the diminished tracheal lumen. Laryngotracheal malformations are fully penetrant in *Hoxa-5* mutant mice, leading in some cases to a pronounced reduction of the lumen at the level of the cricoid cartilage and extending along the length of the trachea that is clearly incompatible with life. Even in milder cases of occlusion, a reduction of the proximal airways diameter may jeopardize fetal breathing, known to contribute to fetal lung growth (Harding and Hooper, 1996). Impaired breathing may also be due to the reduced production of the surfactant proteins. Targeted disruptions of *SP-A* and *SP-B* genes have been reported (Clark *et al.,* 1995; Korfhagen *et al.,* 1996). The lack of *SP-A* function does not alter postnatal survival, whereas the disruption of *SP-B* causes respiratory failure in newborn pups. *SP-B* heterozygous mice survive normally; however, it may be possible that in the context of a reduced production of SP-A and SP-C as for *Hoxa-5* mutants, a marked decrease in SP-B level may impair respiratory function at birth. Compound heterozygotes between the different *SP* mutants might reveal if this holds true. In *Hoxa-5* mutants, it is not yet clear if the decrease in SP production represents reduced expression of *SP* genes in individual cells, changes in cellularity, or both. Histology on adjacent sections of the ones used for immunofluorescence analysis showed that the cellular density was higher in homozygous mutant lungs (data not shown). However, due to the important lung collapse in *Hoxa-5* mutants, it was difficult to determine if the proportion of type II pneumocytes was changed in the mutant specimens. Therefore, we cannot exclude either the reduced SP production by type II pneumocytes or a change in the relative proportion of this cell population to explain the lower SP abundance in the *Hoxa-5* mutant lungs. Nevertheless, the low levels of SP may reflect the state of lung dysmorphogenesis because all observed defects could result from altered development of the respiratory tract. Alternatively, a delay in lung maturation may also lead to a SP decreased production (Carlson, 1994). However, no lag is observed in the occurrence of each developmental stage of the lung between *Hoxa-5* homozygous mutant and wild-type embryos. Moreover, a high proportion of sacrificed *Hoxa-5* homozygous mutant adults showed a partial collapse of the lungs, which cannot be attributed to lung immaturity (data not shown). Thus, dysmorphology rather than delayed development of the respiratory tract is most likely to underlie the fatal outcome in *Hoxa-5* homozygous mutant newborns.

Hox Genes and Lung Morphogenesis

Hoxa-5 is essential for correct laryngeal and tracheal ontogenesis. In human infants, malformations of the cricoid cartilage can cause subglottic stenosis, a narrowing of the subglottic larynx, which can results in death (Holinger and Oppenheimer, 1989). However, to date the genetic basis of

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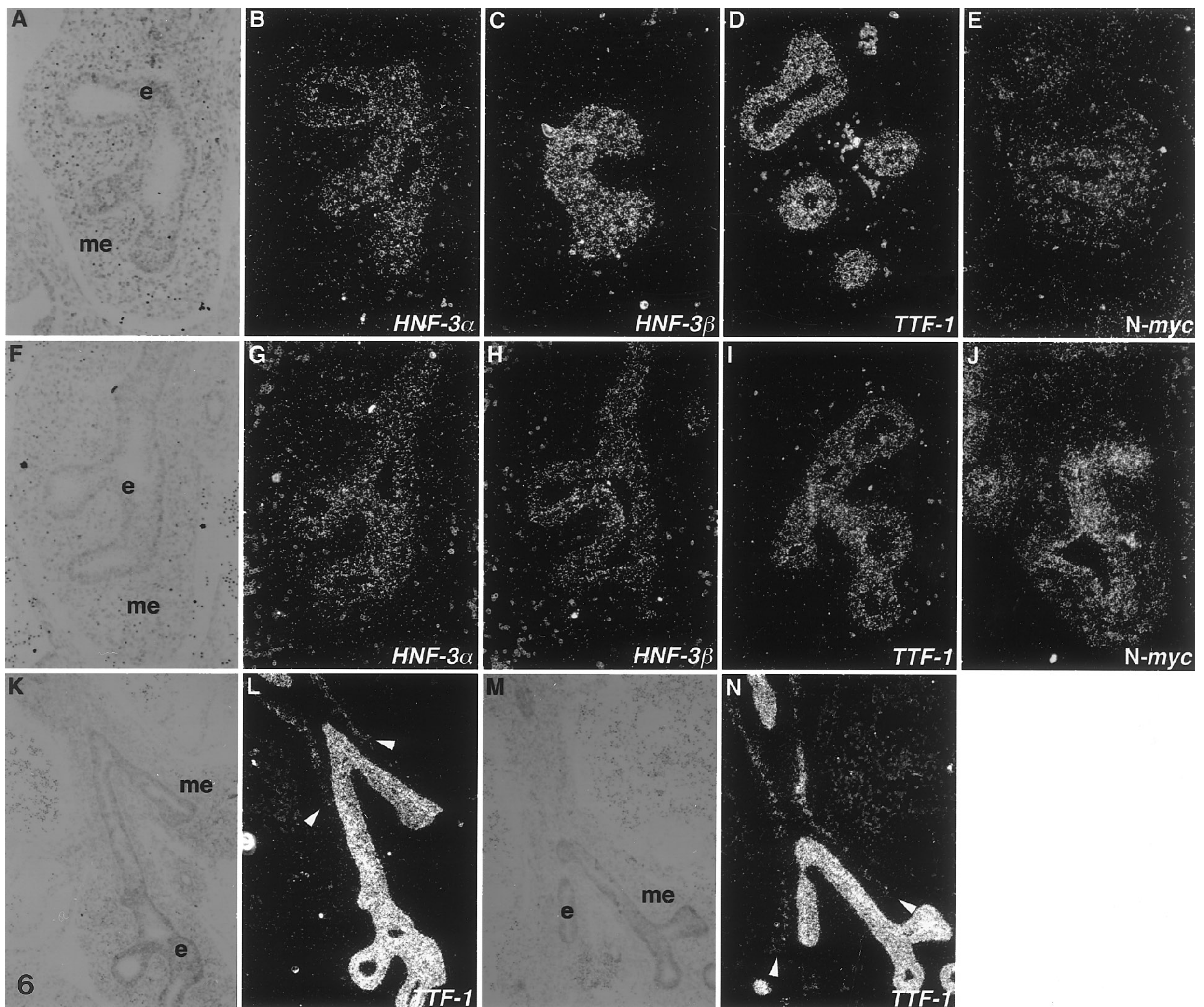

FIG. 6. *HNF-3α, HNF-3β, TTF-1,* and N-*myc* expression in E12.5 *Hoxa-5* wild-type and mutant respiratory tract. A, F, K, and M correspond to the bright-fields of B, G, L, and N, respectively. In the lung epithelium (A–J), comparative *in situ* hybridization patterns are shown for *HNF-3α* (B, G), *HNF-3β* (C, H), *TTF-1* (D, I), and N-*myc* (E, J) in control (B–E) and mutant embryonic lungs (G–J). For *HNF-3α, HNF-3β,* the hybridization pattern was restricted to the lung epithelium and a decrease in signal was observed for *HNF-3β*. *TTF-1* also showed a decrease in its hybridization signal in the mutant lung epithelium (D, I; see Table 1 for comparison of overall grain density). An increase in N-*myc* expression was observed in mutant lung epithelium (E, J). Moreover, in the bronchial mesenchyme, *TTF-1* expression (arrowheads), which was normally restricted to the edge of the trachea in wild-type sample (L), expanded into the mainstem bronchial mesenchyme at this stage (N). Overexposure (compared to D and I) was used for L and N to clearly show the mesenchymal *TTF-1* expression. This, along with the fact that the sections are not equivalent on the basis of the epithelial cellular density (K and M), contributes to mask the difference in signal intensity in the epithelium. e, epithelium; me, mesenchyme. Original magnification, A–J, 200×; K–N, 100×.

this malformation has not been established. In addition to *Hoxa-5,* the *Hoxa-3* gene has also proven to be important for correct formation of the laryngotracheal cartilage (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). Loss of *Hoxa-3* function results in anomalies of the laryngotracheal cartilage that share some similarities with the phenotype of the *Hoxa-5* mutants. However, in contrast to *Hoxa-5* mutants, tracheal occlusion does not occur in *Hoxa-3* mutants. The lethal *Hoxa-3* phenotype is presumed to result from cardiovascular dysfunction compounded by throat problems. Thus, both *Hoxa-3* and *Hoxa-5* genes fulfill distinctive but complementary functions for the specification of the larynx. In *Hoxa-3* mutants, the thyroid and cricoid cartilages were both shorter and thicker, whereas *Hoxa-5* mutants exhibited a larger cricoid and an unaltered thyroid cartilages. Therefore, these two

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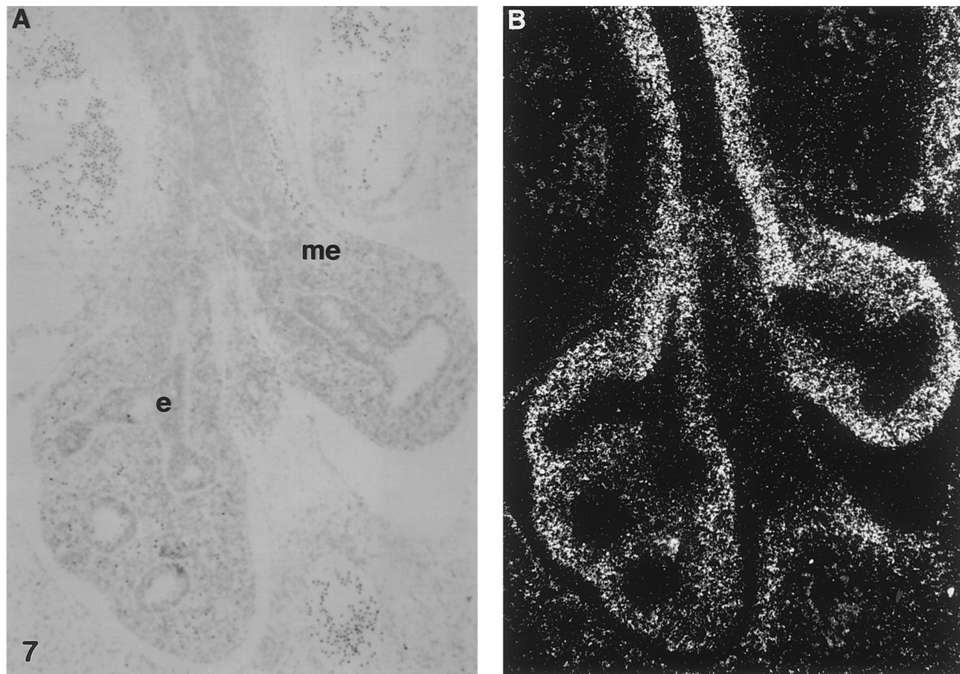

FIG. 7. Expression of *Hoxa-5* in the developing lung at E12.5. *Hoxa-5* expression was strictly restricted to the mesenchymal layer of the trachea and lung. e, epithelium; me, mesenchyme. Original magnification, 100×.

genes must participate in the proper patterning of the laryngotracheal cartilages.

Despite the fact that loss-of-function mutations for several *Hox* genes known to be expressed in the lungs have been generated (*Hoxa-2* to *Hoxa-6, Hoxb-4, Hoxb-5, Hoxc-4, Hoxd-3,* and *Hoxd-4;* Boulet and Capecchi, 1996; Stein *et al.,* 1996), only the *Hoxa-5* mutation has been shown to cause a distinctive lung phenotype. Except for *Hoxa-5,* the fact that lung defects have not been reported in these mutant strains suggests that compensation by other *Hox* genes may mask the anomalies expected on the basis of the expression pattern. On the other hand, it is also possible that these genes may not have any function in lung patterning, since the loss of *Hox* gene expression in the more caudal regions has never been associated with phenotypic consequences in the posterior parts of the embryo. Consequently, the *Hoxa-5* mutation appears to be less subject to compensation by other *Hox* members because of the severity of the phenotype observed. Interestingly, mutants for the other members of paralogous group V, *Hoxb-5* and *Hoxc-5,* are fully viable (Rancourt *et al.,* 1995; Boulet and Capecchi, 1996). However, only *Hoxa-5* is strongly expressed in the trachea, the bronchi, and the distal lungs, while *Hoxb-5* expression is confined to the lung (Krumlauf *et al.,* 1987), and *Hoxc-5* is only weakly expressed in the lung (Gaunt *et al.,* 1990). Therefore, the marked phenotype associated with the *Hoxa-5* mutation correlates with the strong *Hoxa-5* expression in the respiratory tract.

Expression of TTF-1 during Trachea Formation

Lung mesenchyme can be subdivided into two types: bronchial, which promotes formation of the bronchial bud from tracheal epithelium, and tracheal, which inhibits bronchial formation (Wessells, 1970). Lazzaro *et al.* (1991) have proposed that the *TTF-1* gene expression in the bronchiolar epithelium and its absence in more proximal structures could be the result of specific interactions of endodermal cells with regionally specialized mesoderm. Interestingly, in contrast to the rat, we found that in mouse, *TTF-1* gene expression is not restricted to the distal respiratory epithelium, since the proximal epithelium expresses *TTF-1* transcripts at high level (Fig. 5). Our results are further supported by the presence of TTF-1 protein in epithelial cells of the trachea during mouse embryonic development as shown by Zhou *et al.* (1996). Moreover, the macroscopic and histologic organization of the respiratory tract of *TTF-1* homozygous mutants suggests that the formation of the trachea is affected by the loss of *TTF-1* function (Kimura *et al.,* 1996).

Hoxa-5 Loss-of-Function Affects Expression of Transcription Factors Involved in Lung Morphogenesis

The abnormal respiratory tract morphogenesis resulting in tracheal occlusion and impaired lung function most

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likely contributes to the perinatal lethality of the majority of *Hoxa-5* homozygous mutants. In addition to HOXA-5, other transcription factors have been shown to be involved in lung morphogenesis. These include RAR nuclear receptors, N-MYC, members of the HNF3/forkhead family, and TTF-1 (Cardoso, 1995). The analysis of the *RAR* mutants indicates an involvement of RA-dependent processes in initial lung budding and subsequent branching morphogenesis via epithelial–mesenchymal interactions (Mendelsohn *et al.,* 1994; Luo *et al.,* 1996). Moreover, the severity of the phenotype observed in the *RARα/β* double mutants suggests that RA-dependant patterning of the larynx and trachea could be partially mediated by *Hox* genes.

The importance of N-MYC in lung morphogenesis was uncovered by the analysis of homozygous mutant mice carrying a hypomorphic allele of the N-*myc* gene. The mutant mice display improper proliferation and branching of the pulmonary epithelium leading to respiratory distress at birth (Moens *et al.,* 1992). Homozygous mutants for a null N-*myc* mutation die around E10.5 and fail to exhibit lung branching morphogenesis (Charron *et al.,* 1992; Stanton *et al.,* 1992; Sawai *et al.,* 1993). The phenotype of the N-*myc* mutant combined with the localization of N-MYC protein in the lung epithelium suggests that the level of N-*myc* expression in this tissue is critical to allow a normal response to inducing signals from the mesenchyme. The loss of *Hoxa-5* function leads to an increased N-*myc* expression, reinforcing the hypothesis that correct level of N-*myc* expression is important for the proper lung epithelium specification.

As for *Hoxa-5* (Jeannotte *et al.,* 1993; Bogue *et al.,* 1994), expression of *HNF-3β* is maintained throughout lung development and in adult pulmonary tissue (Sasaki and Hogan, 1993; Monaghan *et al.,* 1993; Ang *et al.,* 1993). Since homozygous *HNF-3β* mutant embryos show very early defects of the foregut derivatives preventing the formation of the lung primordia (Weinstein *et al.,* 1994; Ang and Rossant, 1994), the generation of conditional mutants for *HNF-3β* will be required to establish the specific role of this gene during lung development. An essential role for TTF-1 in lung formation has been demonstrated since homozygous *TTF-1* mutants show only distal sac-like structures (Kimura *et al.,* 1996). Interestingly, coexpression of *HNF-3* and *TTF-1* in some cells of the developing respiratory epithelium has suggested that interactions between these regulatory factors may contribute to cell differentiation and gene expression critical to lung development (Bohinski *et al.,* 1994; Ikeda *et al.,* 1996). Of all the transcription factors described above, *TTF-1, HNF-3,* and N-*myc* genes are strictly expressed in the epithelium, *RAR*s are expressed in both the mesenchyme and the epithelium, while *Hoxa-5* is present only in the mesenchyme of the lung.

We have shown that loss of *Hoxa-5* leads to a reduction in the expression level of *TTF-1* and *HNF-3β* and to up-regulation of N-*myc* levels at early stages of lung morphogenesis. The fact that *HNF-3α* expression is not significantly affected suggests that *Hoxa-5* exerted its action via specific pathways. The quantitative changes in *TTF-1, HNF-3β,* and N-*myc* expression levels indicate that additional genes regulate the transcriptional activity of these loci. Altered expression of these genes in isolation is unlikely to explain the lethality per se. For instance, animals heterozygous for the *TTF-1* mutation do not display any decreased viability linked to respiratory problems (Kimura *et al.,* 1996). In contrast, many *HNF-3β* heterozygous mutants die prematurely from unknown causes (Ang and Rossant, 1994; Weinstein *et al.,* 1994). In *Hoxa-5* homozygous mutants, small changes in the expression level of multiple genes may underscore global alterations in cell function resulting from the lack of *Hoxa-5* function. Interestingly, analogous observations were recently reported for the *Fkh6* mutant phenotype (Kaestner *et al.,* 1997). Moreover, modest differences in gene expression are to be expected since multiple *Hox* genes are expressed in the same developing structures. Analysis of compound heterozygotes for both *TTF-1* and *HNF-3β* mutations might help to clarify the impact of cumulative molecular repercussions detected in *Hoxa-5* mutants during lung morphogenesis.

Given that *Hoxa-5* expression is restricted to the mesenchyme implies that its action on *TTF-1, HNF-3β,* and N-*myc* gene expression is a secondary consequence to defects in epithelial–mesenchymal interactions. Interestingly, down-regulation of *TTF-1* expression may be a direct result of reduced *HNF-3* expression, as it has been recently shown that *TTF-1* gene transcription is activated by *HNF-3β* and, to a lesser extent, by *HNF-3α* in respiratory cells (Ikeda *et al.,* 1996). Therefore, it is possible that the lack of *Hoxa-5* function in the mesenchyme results in diminished expression of *HNF-3β* gene in the epithelial layer, which in turn affects *TTF-1* expression levels. In keeping with this possibility, *Drosophila Sex combs reduced* gene, the *Hoxa-5* ortholog, is a genetic regulator of *fkd* gene expression (Zhao *et al.,* 1993). However, our results do not exclude the possibility that *Hoxa-5* may directly repress the *TTF-1* expression in the respiratory tract mesenchyme, since *TTF-1* expression extended into the primary bronchial mesenchyme in the absence of *Hoxa-5* function (Figs. 6L and 6N).

Hoxa-5 Gene Function and Lung Epithelial–Mesenchymal Interactions

Our results suggest that HOXA-5 regulates expression of signals provided by mesenchyme for correct epithelial lung morphogenesis. It is known that tissue morphogenesis involves the coordination of several cellular processes, including paracrine activation, deposition of extracellular matrix, proliferation, and differentiation into specialized lineages (Gumbiner, 1992). Several of these processes are altered in the respiratory tract of *Hoxa-5* homozygous mutants. Indeed, in *Hoxa-5* mutant lungs, the disorganization of the mesenchymal layer concurs with the profound perturbation of the epithelial layer suggesting that the extracellular ma-

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trix may be affected (Fig. 4). Targeted disruptions of the TGF-β3 growth factor gene and the EGF receptor gene lead to a neonatal respiratory distress similar to that observed in *Hoxa-5* homozygous newborn mutants (Kaartinen *et al.,* 1995; Miettinen *et al.,* 1995; Sibilia and Wagner, 1995). Both genes show a developmental expression pattern compatible with their involvement in epithelial–mesenchymal interactions during lung ontogenesis. Thus, both *TGFβ*s and *EGFR* genes represent candidate effectors of *Hoxa-5* function in lung development.

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REFERENCES

Ang, S. L., and Rossant, J. (1994). *HNF-3β* is essential for node and notochord formation in mouse development. *Cell* **78,** 561–574.

Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J., and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: Involvement of HNF3/forkhead proteins. *Development* **119,** 1301–1315.

Barrow, J. R., and Capecchi, M. R. (1996). Targeted disruption of the *Hoxb-2* locus in mice interferes with expression of *Hoxb-1* and *Hoxb-4. Development* **122,** 3817–3828.

Bérard, J., Gaboury, L., Landers, M., De Repentigny, Y., Houle, B., Kothary, R., and Bradley, W. E. C. (1994). Hyperplasia and tumours in lung, breast and other tissues in mice carrying a RARβ4-like transgene. *EMBO J.* **13,** 5570–5580.

Bogue, C. W., Gross, I., Vasavada, H., Dynia, D. W., Wilson, C. M., and Jacobs, H. C. (1994). Identification of *Hox* genes in newborn lung and effects of gestational age and retinoic acid on their expression. *Am. J. Physiol.* **266,** 448–454.

Bohinski, R. J., Di Lauro, R., and Whitsett, J. A. (1994). The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.* **14,** 5671–5681.

Boulet, A. M., and Capecchi, M. R. (1996). Targeted disruption of *hoxc-4* causes esophageal defects and vertebral transformations. *Dev. Biol.* **177,** 232–249.

Cardoso, W. V. (1995). Transcription factors and pattern formation in the developing lung. *Am. J. Physiol.* **13,** 429–442.

Carlson, B. M. (1994). ''Human Embryology and Developmental Biology,'' (R. Farrell, Ed.), Mosby, St. Louis, MO.

Charron, J., Malynn, B. A., Fisher, P., Stewart, V., Jeannotte, L., Goff, S. P., Robertson, E. J., and Alt, F. W. (1992). Embryonic lethality in mice homozygous for a targeted disruption of the N-*myc* gene. *Genes Dev.* **6,** 2248–2257.

Chisaka, O., and Capecchi, M. R. (1991). Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene *hox-1.5. Nature* **350,** 473–479.

Chisaka, O., Musci, T. S., and Capecchi, M. R. (1992). Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene *Hox-1.6. Nature* **355,** 516–520.

Clark, J. C., Wert, S. E., Bachurski, C. J., Stahlman, M. T., Stripp, B. R., Weaver, T. E., and Whitsett, J. A. (1995). Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc. Natl. Acad. Sci. USA* **92,** 7794–7798.

Condie, B. G., and Capecchi, M. R. (1993). Mice homozygous for a targeted disruption of *Hoxd-3 (Hox-4.1)* exhibit anterior transformations of the first and second cervical vertebrae, the atlas and the axis. *Development* **119,** 579–595.

Dony, C., and Gruss, P. (1987). Specific expression of the *Hox1.3* homeobox gene in murine embryonic structures originating from or induced by the mesoderm. *EMBO J.* **6,** 2965–2975.

Gaunt, S. J., Coletta, P. L., Pravtcheva, D., and Sharpe, P. T. (1990). Mouse *Hox-3.4:* Homeobox sequence and embryonic expression patterns compared with other members of the *Hox* gene network. *Development* **109,** 329–339.

Gaunt, S. J., Sharpe, P. T., and Duboule, D. (1988). Spatially restricted domains of homeo-gene transcripts in mouse embryos: Relation to a segmented body plan. *Development* **104,** 169–179.

Gendron-Maguire, M., Mallo, M., Zhang, M., and Gridley, T. (1993). *Hoxa-2* mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. *Cell* **75,** 1317–1331.

Goddard, J. M., Rossel, M., Manley, N. R., and Capecchi, M. R. (1996). Mice with targeted disruption of *Hoxb-1* fail to form the motor nucleus of the VIIth nerve. *Development* **122,** 3217–3228.

Guazzi, S., Lonigro, R., Pintonello, L., Boncinelli, E., Di Lauro, R., and Mavilio, F. (1994). The thyroid transcription factor-1 gene is a candidate target for regulation by Hox proteins. *EMBO J.* **13,** 3339–3347.

Gumbiner, B. M. (1992). Epithelial morphogenesis. *Cell* **69,** 385–387.

Harding, R., and Hooper, S. B. (1996). Regulation of lung expansion and lung growth before birth. *J. Appl. Physiol.* **81,** 209–224.

Hirning, U., Schmid, P., Schulz, W. A., Rettenberger, G., and Hameister, H. (1991). A comparative analysis of N-*myc* and c-*myc* expression and cellular proliferation in mouse organogenesis. *Mech. Dev.* **33,** 119–126.

Holinger, L. D., and Oppenheimer, R. W. (1989). Congenital subglottic stenosis: the elliptical cricoid cartilage. *Ann. Otol. Rhinol. Laryngol.* **98,** 702–706.

Ikeda, K., Shaw-White, J. R., Wert, S. E., and Whitsett, J. A. (1996). Hepatocyte nuclear factor 3 activates thyroid transcription factor 1 in respiratory epithelial cells. *Mol. Cell. Biol.* **16,** 3626–2636.

Jaffe, L., Jeannotte, L., Bikoff, E. K., and Robertson, E. J. (1990). Analysis of β_2-microglobulin in the developing mouse embryo and placenta. *J. Immunology* **145,** 3474–3483.

Jeannotte, L., Lemieux, M., Charron, J., Poirier, F., and Robertson, E. J. (1993). Specification of axial identity in the mouse: Role of the *Hoxa-5 (Hox1.3)* gene. *Genes Dev.* **7,** 2085–2096.

Kaartinen, V., Voncken, J. W., Shuler, C., Warbuton, D., Bu, D.,

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

Heinsterkamp, N., and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-β3 indicates defects of epithelial–mesenchymal interaction. *Nature Genetics* **11,** 415–420.

Kaestner, K. H., Silberg, D. G., Traber, P. G., and Schütz, G. (1997). The mesenchymal winged helix transcription factor *Fkh6* is required for the control of gastrointestinal proliferation and differentiation. *Genes Dev.* **11,** 1583–1595.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M., and Gonzalez, F. J. (1996). The T/ebp null mouse thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10,** 60–69.

Korfhagen, T. R., Bruno, M. D., Ross, G. F., Huelsman, K. M., Ikegami, M., Jobe, A. H., Wert, S. E., Stripp, B. R., Morris, R. E., Glasser, S. W., Bachurski, C. J., Iwamoto, H. S., and Whitsett, J. A. (1996). Altered surfactant function and structure in SP-A gene targeted mice. *Proc. Natl. Acad. Sci. USA* **93,** 9594–9599.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78,** 191–201.

Krumlauf, R., Holland, P. W. H., McVey, J. H., and Hogan, B. L. M. (1987). Developmental and spatial patterns of expression of the mouse homeobox gene, Hox2.1. *Development* **99,** 603–617.

Lazzaro, D., Price, M., de Felice, M., and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113,** 1093–1104.

Lufkin, T., Dierich, A., LeMeur, M., Mark, M., and Chambon, P. (1991). Disruption of the *Hox-1.6* homeobox gene results in defects in a region corresponding to its rostral domain of expression. *Cell* **66,** 1105–1119.

Luo, J., Sucov, H. M., Bader, J.-A., Evans, R. M., and Giguère, V. (1996). Compound mutants for retinoic acid receptor (RAR) β and RARα1 for multiple RARβ isoforms. *Mech. Dev.* **55,** 33–44.

Manley, N. R., and Capecchi, M. R. (1995). The role of *Hoxa-3* in mouse thymus and thyroid development. *Development* **121,** 1989–2003.

Mark, M., Lufkin, T., Vonesch, J. L., Ruberte, E., Olivo, J. C., Dollé, P., Gorry, P., Lumsden, A., and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119,** 319–338.

Mendelsohn, C., Lohnes, D., Décimo, D., Lufkin, T., LeMeur, M., Chambon, P., and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development. II. Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120,** 2749–2771.

Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z., and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376,** 337–341.

Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L., and Rossant, J. (1992). A targeted mutation reveals a role for N-*myc* in branching morphogenesis in the embryonic mouse lung. *Genes Dev.* **6,** 691–704.

Monaghan, A. P., Kaestner, K. H., Grau, E., and Schutz, G. (1993). Postimplantation expression patterns indicate a role for the mouse *forkhead*/HNF-3 α, β and γ genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119,** 567–578.

Mugrauer, G., Alt, F. W., and Ekblom, P. (1988). N-*myc* proto-oncogene expression during organogenesis in the developing mouse as revealed by *in situ* hybridization. *J. Cell. Biol.* **107,** 1325–1335.

Ramirez-Solis, R., Zheng, H., Whiting, J., Krumlauf, R., and Bradley, A. (1993). *Hoxb-4 (Hox-2.6)* mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. *Cell* **73,** 279–294.

Rancourt, D. E., Tsuzuki, T., and Capecchi, M. R. (1995). Genetic interaction between *hoxb-5* and *hoxb-6* is revealed by nonallelic noncomplementation. *Genes Dev.* **9,** 108–122.

Rijli, F. M., Mark, M., Lakkaraju, S., Dierich, A., Dollé, P., and Chambon, P. (1993). A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2,* which acts as a selector gene. *Cell* **75,** 1333–1349.

Sasaki, H., and Hogan, B. L. (1993). Differential expression of multiple forkhead related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118,** 47–59.

Sawai, S., Shimono, A., Wakamatsu, Y., Palmes, C., Hanaoka, K., and Kondoh, H. (1993). Defects of embryonic organogenesis resulting from targeted disruption of the N-*myc* gene in the mouse. *Development* **117,** 1445–1455.

Sibilia, M., and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269,** 234–238.

Stanton, B. R., Perkins, A. S., Tessarollo, L., Sassoon, D. A., and Parada, L. F. (1992). Loss of N-*myc* function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev.* **6,** 2235–2247.

Stein, S., Fritsch, R., Lemaire, L., and Kessel, M. (1996). Checklist: Vertebrate homeobox genes. *Mech. Dev.* **55,** 91–108.

van der Hoeven, F., Zakany, J., and Duboule, D. (1996). Gene transpositions in the *HoxD* complex reveal a hierarchy of regulatory controls. *Cell* **85,** 1025–1035.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Piezioso, V. R., Jessel, T. M., and Darnell, J. E. (1994). The winged-helix transcription factor HNF-3β is required for notochord development in the mouse embryo. *Development* **78,** 575–588.

Wessells, N. K. (1970). Mammalian lung development: Interactions in formation and morphogenesis of tracheal buds. *J. Exp. Zool.* **175,** 455–466.

Zhao, J. J., Lazzarini, R. A., and Pick, L. (1993). The mouse *Hox-1.3* gene is functionally equivalent to the *Drosophila Sex combs reduced* gene. *Genes Dev.* **7,** 343–354.

Zhou, L., Lim, L., Costa, R. H., and Whitsett, J. A. (1996). Thyroid transcription factor-1, hepatocyte nuclear factor-3β, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung. *J. Histochem. Cytochem.* **44,** 1183–1193.

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