Hoxa-5 in mouse developing lung: cell-specific expression and retinoic acid regulation

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Hoxa-5 in mouse developing lung: cell-specific expression and retinoic acid regulation. Am J Physiol Lung Cell Mol Physiol 279: L863–L871, 2000.—Hoxa-5 is a homeobox gene that is highly expressed in the developing mouse lung. However, little is known about the molecular mechanisms controlling expression. We characterized the ontogeny of Hoxa-5 gene and protein expressions during lung development and then studied the cell-specific effects of retinoic acid (RA) on Hoxa-5 mRNA in fetal lung fibroblasts and MLE-12 mouse lung epithelial cells. Strong but constant Hoxa-5 gene and protein expressions were detected from mouse lung on embryonic day 13.5 to postnatal day 2. At baseline, the gene was strongly expressed in the fibroblasts of day 17.5 fetal mouse lungs. A very weak but reproducible expression was present in the MLE-12 cells. RA stimulated gene expression in both cell types and that RA participates in the regulation of Hoxa-5 in these cells. The molecular mechanisms underlying the cell-specific response to RA were explored and found the action and function of Hoxa-5 can be overcome by RA. homeobox genes; fetal lung

A GROUP OF HIGHLY CONSERVED regulatory genes that control spatial and/or temporal patterning, structural identity, and cell fate in vertebrate development include the HOM-C/Hox homeobox genes. In the mouse, 39 Hox genes, arranged into four clusters (A, B, C, and D on chromosomes 2, 6, 11, and 15, respectively) have been identified (20, 27). Each of the Hox genes shares a common homeobox, a DNA sequence of 183 bp that codes for the 61-amino acid polypeptide region, which specifically binds DNA. The presence of the homeobox region in each Hox protein allows it to function as a transcription factor of downstream target genes, the identities of which are mostly unknown (20). The specific organization of the Hox genes within the clusters is critical to the induction, expression, control and function of these genes (6, 7, 14, 20). An important positive and negative controller of Hox genes is retinoic acid (RA), a signaling molecule that mediates differentiation and pattern formation throughout embryogenesis (4, 11, 15, 18, 19).

Of the numerous Hox genes identified in fetal mouse lung, Hoxa-5 (previously called Hox 1.3) in particular is highly expressed (3). This suggested a potentially significant role for Hoxa-5 in fetal lung development. The Hoxa-5 mRNA was localized to mesoderm-derived structures of the thoracic region as early as embryonic day 8 (E8). It was expressed in the area of early lung region beginning on E9 and definitively in lung tissue by E12. Within the lung on E12, Hoxa-5 mRNA signal was observed only in the mesoderm-derived mesenchyme and not in the endoderm-derived epithelium (5). Hoxa-5 is expressed in other specific embryonic tissues such as the hindbrain, trachea, thyroid, ribs, spinal cord, kidneys, stomach, and midgut during embryogenesis (E8, E9, E11, E12, E13, and E18) as well as in adult mouse tissues (5, 6, 9, 24). The molecular mechanisms governing Hoxa-5 expression in any of these tissues are not known.

Studies in Hoxa-5 knockout mice show that homozygous mutant newborns die soon after birth secondary to pulmonary insufficiency and that the expression of epithelial-specific genes is altered by the absence of Hoxa-5 gene expression (1). From this study, it is clear that Hoxa-5 plays a critical role in fetal lung development. By learning more about the Hoxa-5 expression pattern in the developing lung and the factors that influence it, the action and function of Hoxa-5 can be better delineated. We hypothesized that the Hoxa-5 gene is differentially expressed among different lung cell types and that RA participates in the regulation of Hoxa-5 gene expression in the developing mouse lung. We tested this by studying the ontogeny of Hoxa-5 gene and protein expression in fetal and postnatal mice, the cell-specific gene expression in cultured lung cells, and the ability of RA to differentially stimulate Hoxa-5 in these cells. The molecular mechanisms underlying the cell-specific response to RA were explored using cycloheximide (CHX) and actinomycin D treatments.
MATERIALS AND METHODS

Subjects. Swiss Webster mice were time mated, and the day after mating was designated day 0.5. Maternal mice were killed by CO₂ inhalation. The fetuses and newborn mice were collected and immediately decapitated. The animal protocol was approved by the institutional laboratory animal review committee. Cell culture studies used primary unpasaged fetal lung fibroblasts prepared and cultured as described below. MLE-12 cells are a cell line derived from lung tumors of transgenic mice carrying the simian virus 40 large tumor antigen (viral oncogene) under the transcriptional control of the human surfactant protein C promoter. These transformed mouse lung tumor cells have morphological and functional characteristics of alveolar type II cells (33). They were purchased (American Type Culture Collection, Manassas, VA) and repeatedly passaged for use in the experiments.

RNA isolation and Northern hybridization. Whole lungs and livers of Swiss Webster mice, E13.5 to postnatal day 2 (P2), were removed from the animals and separated according to gender. Total RNA was isolated with Trizol reagent (GIBCO BRL, Grand Island, NY) as outlined by the manufacturer. Total RNA was denatured at 65°C for 15 min in loading buffer containing 0.5x MOPS, 52% formamide, and 7% formaldehyde (Sigma, St. Louis, MO). The RNA was loaded on 1% agarose-6% formaldehyde-0.5x MOPS gels and then separated by electrophoresis under denaturing conditions. Ethidium bromide-stained RNA bands were visualized under ultraviolet light and quantified for use as an internal standard using a computerized digital imaging system (Alpha Innotech, San Leandro, CA). RNA was transferred onto GeneScreen Plus nylon membranes (NEN-DuPont, Boston, MA) in 10x saline-sodium citrate (SSC; Sigma) transfer buffer. The transfer occurred overnight, and the membranes were baked for 1.5 h at 80°C. The Hoxa-5-specific cDNA probe (gift of Dr. Peter Gruss, Goettingen, Germany) for MA) in 10x SSC plus 0.2x SDS at room temperature for 30 min each, once in 2x SSC plus 1x SDS at 60°C for 1 h, and twice in 0.1x SSC plus 0.2x SDS at room temperature for 30 min each. The resulting hybridization bands were visualized and evaluated by phosphorimaging analysis. The blots were then stripped and reprobed with rat α-tubulin as an internal standard for the amount of RNA loaded and for integrity of the mRNA message.

Protein analysis and Western hybridization. Whole lungs of fetal mice E12 to P2 were extracted and homogenized in phosphate-buffered saline (PBS) solution containing protease inhibitors (1 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml antipain, and 0.5 mM phenylmethylsulfonyl fluoride; Sigma). Protein assay was performed for protein quantification (17). Equal amounts of protein (16 µg) and molecular weight standards were loaded and subjected to 12% polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). After the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), Western blot analysis was performed. Blots were blocked by preincubation in 1% gelatin overnight and then incubated in 1:10,000 dilution of Hoxa-5 polyclonal rabbit antibody (Babco, Richmond, CA) for 1 h at room temperature. Blots were washed three times in 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (TBS-T), pH 7.4, for 10 min each with gentle agitation and then incubated in secondary antibody 1:6,000 (affinity-purified goat anti-rabbit IgG conjugated to alkaline phosphatase, Boehringer Mannheim, Indianapolis, IN) with agitation for 1 h at room temperature. The blots were then washed three times in TBS-T, pH 7.4, for 10 min and exposed to the alkaline phosphatase color developer.

Fibroblast cell culture and RA treatment. E17.5 fetal mouse lungs were minced and trypsinized. Fibroblasts were isolated using differential adhesion method (26). The cells were grown to confluence at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT), 0.05 mg/ml gentamicin (GIBCO BRL), 0.00025 mg/ml amphotericin B (Sigma), 3.7 mg/ml sodium bicarbonate (Sigma), and 0.11 mg/ml sodium pyruvate (GIBCO BRL) and brought to pH 7.28. All-trans-RA (Sigma) was initially prepared at 10⁻¹ M concentration in 100% ethanol (EtOH) and then diluted to the desired final concentration in fresh medium. The confluent fibroblasts were treated with RA at concentrations of 10⁻⁸ to 10⁻⁴ M for each treatment period of 3, 6, 14, 24, or 48 h. The control fibroblasts were treated with an equal volume of EtOH not containing RA. The final concentration of EtOH in all cultures was 0.1%. Total RNA was isolated as described previously, and 10 µg of total RNA were used for the Northern hybridization.

RA treatment of MLE-12 cells. MLE-12 mouse lung epithelial cells were used at passages 5–12. Cells were plated and grown to confluence in DMEM with added 10% FCS, 1 U/ml penicillin (Sigma), 0.001 mg/ml streptomycin (Sigma), 3.7 mg/ml sodium bicarbonate, and 0.11 mg/ml sodium pyruvate at pH 7.28. The cells were treated with RA (10⁻⁷ to 10⁻⁴ M) for 3, 6, 14, 24, and 48 h. RNA isolation and Northern blot hybridization using 20 µg of total RNA were performed as previously described.

CHX and actinomycin D treatment of cells. Primary cultures of E17.5 fetal mouse lung fibroblasts and MLE-12 cells were grown to confluence in separate tissue culture plates. Optimal CHX and actinomycin D (Sigma) doses for each cell type were determined by trichloroacetic acid (TCA; Sigma) precipitation of [³H]uridine (>45 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) and [³⁵S]methionine (>1,000 Ci/mmol; ICN). CHX doses of 0.1–10 µg/ml were used. Fibroblasts were incubated in CHX with cell type-appropriate DMEM medium for 6 h, and MLE-12 cells were incubated for 14 h. Two hours before the incubation time was completed, 50 µCi/ml [³H]uridine or 100 µCi/ml [³⁵S]methionine were added for incorporation and the incubation was completed. Before the addition of [³⁵S]methionine, the cells were preincubated in methionine-free medium (GIBCO BRL) containing CHX for 15 min to deplete the cells of unlabeled methionine. Medium was changed to fresh warm methionine-free medium containing [³⁵S]methionine and appropriate doses of CHX, and incubation was completed. Radioactive medium was rinsed away three times with cold PBS. Cells were scraped into 2 ml of cold PBS, sonicated, and centrifuged at 300 g at 4°C for 5 min. The cell pellet was resuspended in 1 ml of cold PBS, and 50 µl of this sample were TCA precipitated in bovine serum albumin onto glass microfiber filters. Incorporation was measured by scintillation counting. [³H]Ur dine incorporation was measured by first rinsing the cells three times in cold PBS and then scraping cells into 2 ml of PBS and sonicating. Fifty microliters of suspended cell volume were TCA precipitated in salmon sperm DNA. Incorporation was measured by scintillation counting. Next, actinomycin D doses of 0.05–1 µg/ml were tested. After the cells
completed treatment in EtOH (control) or RA for 6 h in fibroblasts and 14 h for MLE-12 cells, the medium was changed. The cells were further incubated in actinomycin D for 4 h. $[^3]$H]uridine (50 µCi/ml) or $[^35]$S]methionine (100 µCi/ml) was added, and incorporation was measured as described previously for CHX.

After the optimal doses of CHX and actinomycin D were defined, the effects of protein synthesis inhibition and of RNA synthesis inhibition on RA stimulation of Hoxa-5 mRNA expression were studied in each cell type. Cells were pretreated with 1 µg/ml CHX for 30 min. Then $10^{-5}$ M RA was added and incubated with CHX for the time required for maximal Hoxa-5 mRNA stimulation (6 h for fibroblasts and 14 h for MLE-12 cells) to occur. Cells were then harvested for total RNA isolation, and Northern hybridization was performed as described above. Next, cells were treated with actinomycin D as follows: Hoxa-5 mRNA was maximally stimulated with RA or EtOH diluent for 6 h in fibroblasts and 14 h in MLE-12 cells. RA was removed and actinomycin D was added (0.25 µg/ml for fibroblasts and 1 µg/ml for MLE-12 cells). Total RNA was harvested at subsequent time intervals of 0, 0.5, 1, 2, 4, and 6 h. Northern hybridization was done as described previously using 10 µg of total RNA for fibroblasts and 20 µg for MLE-12 cells. Results are expressed as percent of untreated controls.

**Statistical analysis.** The Hoxa-5-to-α-tubulin ratios in developing mouse lung experiments were analyzed by two-way analysis of variance (ANOVA). The effect of RA dose, the time of treatment, and the relationship between time and treatment dose in fibroblasts and MLE-12 cells treated with varying doses of RA at increasing treatment times were analyzed by multiple ANOVA. The CHX results were analyzed by Tukey-Kramer multiple comparisons test. Data obtained from the actinomycin D study were evaluated by analysis of covariance (ANCOVA). For all the statistical methods used, $P < 0.05$ was accepted as statistically significant (35).

**RESULTS**

*Hoxa-5 gene and protein expression in developing mouse lung.* The developmental expression of Hoxa-5 in the lung was determined by Northern analysis using RNA isolated from whole lungs of E13.5 to P2 mice. In both female and male lungs, specific Hoxa-5 mRNA appeared as a single band at 1.9 kb, with constant expression across gestation (Fig. 1A). Hoxa-5 was not expressed in the liver (data not shown), consistent with previous studies (5). This and the absence of the common homeobox sequence in the Hoxa-5-specific cDNA probe used in this study indicated that hybridization was specific to Hoxa-5 and not to a paralogous homeobox gene.

The Hoxa-5 signal intensity was quantified by phosphorimage analysis and expressed as a Hoxa-5-to-α-tubulin ratio and analyzed by two-way ANOVA. The statistical analysis showed no significant differences in Hoxa-5 gene expression either by gestational age ($P = 0.3015$) or by gender ($P = 0.8732$) from E13.5 to P2 (Fig. 1B). The validity of α-tubulin as an internal standard was evaluated by studying the ratio of α-tubulin expression to the intensity of the 18S ribosomal bands seen on ethidium bromide stain of the gels. There was a small difference in the ratio between the sexes, with slightly increased expression in females compared with males ($P = 0.009$), but there was no difference in this ratio between the sexes with advancing gestational age ($P = 0.3780$, Fig. 1C).

The evaluation of Hoxa-5 protein during lung development was accomplished using Western blots of pro-
tein obtained from mouse lungs of E13.5 to P2. The Western blots showed a strong protein band migrating to ∼32 kDa and a slightly weaker band migrating to ∼43 kDa (Fig. 2). Consistent with the developmental pattern of Hoxa-5 mRNA, protein expression was also constant throughout gestation with no apparent male-female differences. The specificity of the antibody for Hoxa-5 protein was confirmed by the absence of these bands in control blots treated with normal rabbit serum as well as lack of cross-reaction with its closest paralogous counterpart Hoxb-5 antigen (32).

Lung fibroblasts and MLE-12 cells treated with RA. After the temporal expression of Hoxa-5 gene and protein was identified in mouse lung tissue, cell-specific regulation of its expression within the lung was explored by studying cultured E17.5 mouse lung fibroblasts and MLE-12 cells.

Northern blots of RNA from fibroblasts treated with varying doses of RA for up to 48 h showed hybridization of the Hoxa-5 cDNA to a major band at 1.9 kb and a minor band at 3.9 kb. Figure 3A illustrates results after 24-h exposure to RA. The control fibroblasts showed a fourfold increase in Hoxa-5 gene expression by 48 h in culture (data not shown). To control for this effect, the data for RA-exposed cells were normalized to their respective controls and represented as an increase in stimulation by RA (Fig. 3B). An overall stimulation of Hoxa-5 by RA was observed, with a trend toward greater stimulation of Hoxa-5 expression with increasing dose at any given time. However, this was not statistically significant (P = 0.6783, ANOVA). Maximal stimulation occurred within 3–6 h for every RA concentration used. The stimulation of Hoxa-5 by RA was time dependent (P = 0.0003, ANOVA) and the response over 48 h was different at different doses of RA. In fibroblasts treated with lower concentrations of RA (10⁻⁸ to 10⁻⁶ M), the expression peaked and then decreased with further time of exposure to RA. Treatment with high doses of RA (10⁻⁵ to 10⁻⁴ M) resulted in no decrease from peak stimulation with time. This interaction of RA dose and response over time of treatment also was statistically significant (P < 0.045, ANOVA).

MLE-12 mouse lung epithelial cells were used in our experiments as a model of type II cells to determine whether epithelial cells were involved in the expression and control of Hoxa-5. On Northern analysis, Hoxa-5 mRNA was weakly but reproducibly detectable in untreated MLE-12 cells (Fig. 4A). Contrary to untreated lung fibroblasts, Hoxa-5 expression in untreated MLE-12 cells did not change with time. When treated with RA, however, these cells expressed Hoxa-5 mRNA in a dose- (P < 0.00005) and time-dependent (P < 0.00005) manner (Fig. 4B). All doses of RA stimulated Hoxa-5 expression, with peak stimulation occurring between 6 and 14 h. Subsequently, expression tended to decrease to control levels by 48 h.

Fig. 2. Representative Western blot for female mouse lungs from E13.5 to P2 immunoreacted with Hoxa-5 antibody. Male data were similar to female data. Total proteins (16 µg) were separated on 12% SDS-PAGE gel. The antibody detected a strong protein band migrating to ∼32 kDa and a weaker band migrating to 43 kDa. The protein expression is constant across gestation with no gender-specific differences. NRS, normal rabbit serum.

Fig. 3. A: representative Northern blot demonstrating the effect of increasing doses of retinoic acid (RA) on Hoxa-5 mRNA expression in the E17.5 fetal mouse lung fibroblasts after 24 h of RA treatment. Reprobing for α-tubulin showed equal RNA loading. Signals of both the major 1.9-kb transcript and the weaker band at 3.9 kb increase in intensity with increasing RA concentration. B: RA time-course and dose-response curves of Northern blot phosphorimage data normalized to their respective controls and expressed as the treated-to-control RNA ratio (increase stimulation). Each data point is the mean of 3 sets of experiments. Maximal stimulation by RA of Hoxa-5 gene expression occurred within 3 to 6 h of treatment for each RA concentration studied. With increasing exposure time to RA, Hoxa-5 gene expression decreased relative to controls at lower doses of RA (10⁻⁶ to 10⁻⁴ M) but remained constant relative to controls at higher doses (10⁻⁵ to 10⁻⁴ M).
The interaction between RA dose and length of treatment also was significant ($P = 0.042$, ANOVA). The Northern blots of the fibroblasts and MLE-12 cells in Figs. 3A and 4A also illustrate that the RNA loading is equal by internal standard $\alpha$-tubulin and that there is no stimulation of $\alpha$-tubulin by RA.

Lung fibroblasts and MLE-12 cells treated with CHX and actinomycin D. CHX and actinomycin D were used to determine whether cell-specific regulation of Hoxa-5 in lung cells involved changes in protein synthesis and/or mRNA stability. We first identified optimal doses of each substance in each cell type. The dose-response effects of CHX and actinomycin D doses are shown in Table 1. The optimal dose of CHX was determined to be 1 $\mu$g/ml, with 80% protein synthesis inhibition and 19% RNA synthesis inhibition in fibroblasts and 81% protein synthesis inhibition and 45% RNA synthesis inhibition in MLE-12 cells. The optimal dose of actinomycin D was determined to be 0.25 $\mu$g/ml for fibroblasts, with 14% protein synthesis inhibition and 77% RNA synthesis inhibition, and 1 $\mu$g/ml for MLE-12 cells, with 25% protein synthesis inhibition and 95% RNA synthesis inhibition.

CHX was used to test whether the difference in peak stimulation of Hoxa-5 by RA between fibroblasts and MLE-12 cells reflected a need for new protein synthesis in the MLE-12 cells. In fibroblasts (Fig. 5A) CHX treatment alone had a minimal effect on baseline expression. When the fibroblasts were treated with RA alone, mRNA expression was stimulated more than twofold compared with controls ($P < 0.001$). In the RA plus CHX group, CHX completely inhibited the induction of gene expression ($P < 0.001$).

In MLE-12 cells treated with CHX alone, Hoxa-5 mRNA expression was stimulated greater than twofold compared with the control (Fig. 5B). RA alone significantly stimulated Hoxa-5 gene expression greater than sixfold compared with the control ($P < 0.001$). RA plus CHX treatment eliminated RA-induced stimulation of mRNA compared with the RA-only group ($P < 0.001$), with stimulation at the level of CHX treatment alone.

Actinomycin D was used to determine whether the different responses to RA between fibroblasts and MLE-12 cells reflected a change in mRNA stability. In the fibroblasts treated with actinomycin D (Fig. 6A), the mRNA degradation rates of the control and RA-treated groups were not different ($P = 0.17$, ANCOVA).

Table 1. CHX and actinomycin D doses tested for optimal RNA and protein synthesis inhibition using $[^{3}H]$uridine and $[^{35}S]$methionine incorporation in lung fibroblasts and MLE-12 cells

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<th>CHX Doses, $\mu$g/ml</th>
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In both the fibroblasts and MLE-12 cells, CHX dose of 1 $\mu$g/ml was selected. Actinomycin D dose of 0.25 $\mu$g/ml was used in fibroblasts and 1 $\mu$g/ml was used in MLE-12 cells. CHX, cycloheximide; MLE, mouse lung epithelial.
The mRNA half-lives, extrapolated from the 50% mRNA level, were 3.45 h for the controls and 2.97 h for the RA-treated fibroblasts. The mRNA half-life of control MLE-12 cells ($t_{1/2} = 3.84$ h) was similar to that of the control fibroblasts. However, in MLE-12 cells (Fig. 6B), RA treatment significantly increased the rate of Hoxa-5 mRNA disappearance ($P < 0.0004$), resulting in mRNA half-life of 1.72 h in RA-treated MLE-12 cells.

DISCUSSION

To improve our understanding of Hoxa-5 expression in fetal mouse lung development, we described the pattern of its gene and protein expression in mouse lungs of E13.5 to P2 embryos through the major lung developmental stages from pseudoglandular to alveolar periods. We then investigated the effect of RA on Hoxa-5 gene expression in specific lung cell types in culture to learn what molecular factors were involved in the activation and regulation of Hoxa-5. RA stimulated Hoxa-5 expression in a variety of embryonal carcinoma cell lines (8, 21, 22), human bronchial fibroblasts (2), tracheobronchial fibroblasts (1), and whole mouse and rat fetal lungs (3). However, a cell-specific effect of RA on Hoxa-5 in the fetal lung has not been studied.

In the developing whole mouse lung, a single Hoxa-5 transcript size of 1.9 kb was seen that exhibited no gender-specific or gestation-specific differences from E13.5 to P2. The absence of a change with gestation is in contrast to the study of Bogue et al. (3). These authors reported decreasing Hoxa-5 expression with gestation as well as a second band of 2.9 kb. The differing results in terms of developmental regulation may have been procedural. Our study used an internal standard for RNA loading, which was further validated by the Northern hybridization method.

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In the developing whole mouse lung, a single Hoxa-5 transcript size of 1.9 kb was seen that exhibited no gender-specific or gestation-specific differences from E13.5 to P2. The absence of a change with gestation is in contrast to the study of Bogue et al. (3). These authors reported decreasing Hoxa-5 expression with gestation as well as a second band of 2.9 kb. The differing results in terms of developmental regulation may have been procedural. Our study used an internal standard for RNA loading, which was further validated by the Northern hybridization method.

The mRNA half-lives, extrapolated from the 50% mRNA level, were 3.45 h for the controls and 2.97 h for the RA-treated fibroblasts. The mRNA half-life of control MLE-12 cells ($t_{1/2} = 3.84$ h) was similar to that of the control fibroblasts. However, in MLE-12 cells (Fig. 6B), RA treatment significantly increased the rate of Hoxa-5 mRNA disappearance ($P = 0.0004$), resulting in mRNA half-life of 1.72 h in RA-treated MLE-12 cells.

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against a second independent standard. We also used a smaller amount of total RNA in our Northern blots; this may have increased the accuracy of the internal standard and could also cause us to not detect a less abundant second band. Although others also have reported multiple transcripts for Hoxa-5 depending on the probe and tissue or cell type that was studied (8, 16, 24), the 1.9-kb transcript is the most consistently and abundantly expressed transcript in the lung. The Northern blots of the whole mouse lung did not show a minor 3.9-kb band observed in the fetal lung fibroblasts. Expression of the 3.9-kb transcript was very low in the control fibroblasts not treated with RA. Only on stimulation with RA, the expression became more significant. The difference in expression of this minor band may be due to the fact that the whole lungs were not treated with RA and that the total RNA of the whole lungs loaded on the gel also included RNA of cells other than fibroblasts.

Concordant with its gene expression, Hoxa-5 protein was also expressed in the developing lung, at constant levels from as early as E13.5 to at least as late as P2. The Hoxa-5 protein sizes (32 and 43 kDa) found in our study closely approximated the molecular mass range of ~35–43 kDa previously determined in cultured embryo fibroblasts (23). In this previous study by Odenwald et al. (23), they observed that numerous protein forms of Hoxa-5 were generated by multiple posttranslational modifications with the primary mechanism of multiple phosphorylations, particularly of the slower migrating forms. Several transcript sizes are synthesized from the Hoxa-5 gene, and these transcripts may also generate proteins of various sizes. Continuously high gene and protein expression throughout gestation in the lung may indicate that Hoxa-5 has an important role in developing lung.

We found that Hoxa-5 gene expression was strongly detected in untreated fibroblasts in the mesenchyme of the developing lungs, in agreement with previous tissue culture and in situ hybridization studies (1, 5). We studied the regulation of Hoxa-5 gene expression in these fetal lung fibroblasts by using RA, a known morphogen that plays a role in differential regulation of Hox genes (29, 30). These results showed that RA could regulate Hoxa-5 gene expression in fetal mouse lung fibroblasts. With the high doses of RA, Hoxa-5 expression was stimulated and sustained through at least a 48-h treatment, whereas the expression was stimulated and then decreased with the lower doses. It previously has been shown that with higher doses and prolonged exposure times with RA, Hox genes within a cluster not only become activated sequentially in the 3’ to 5’ direction, but the expression of early-response genes is also higher and sustained (30). It is possible that with high doses of RA and longer exposure times, the late-response Hox genes function to sustain the expression of Hoxa-5. The higher and more prolonged RA treatment also may allow continuous synthesis of new proteins that mediate Hoxa-5 expression and overcome repression.

In contrast to the fibroblasts, Hoxa-5 mRNA in untreated MLE-12 cells was only minimally detectable by Northern blot, with double the amount of total RNA used. The observed differences between the cell types in the magnitude of Hoxa-5 gene stimulation and in the kinetics of peak RA-induced Hoxa-5 gene expression suggested that RA mediated the induction of Hoxa-5 gene expression in fetal lung fibroblasts and in MLE-12 cells through different mechanisms. To determine whether synthesis of short-life protein intermediate(s) mediated RA-induced Hoxa-5 gene stimulation, the cells were treated with a protein synthesis inhibitor CHX. In lung fibroblasts treated with both RA and CHX, CHX significantly inhibited the stimulation of gene expression, indicating that the stimulatory effect of RA required new protein synthesis. New protein synthesis also was required in MLE-12 cells for RA-induced gene expression. However, in MLE-12 cells, an additional effect was seen. Hoxa-5 mRNA in the CHX-only treated group was significantly elevated more than twofold compared with that in the control group. This suggested that CHX inhibited the synthesis of a repressive protein factor, allowing Hoxa-5 to be expressed. We speculate that a repressor of baseline Hoxa-5 gene expression may normally be present in lung epithelial cells, resulting in the restriction of Hoxa-5 gene expression to fibroblasts. This repressor in epithelial cells can be overcome by RA to stimulate Hoxa-5 mRNA levels. Thus the induction of Hoxa-5 by RA may be delayed in MLE-12 cells because it not only required de novo protein synthesis but overriding of the repressor. However, this speculation is made with caution because it is possible that this observation may have resulted from a nonspecific, cumulative effect of CHX on protein and RNA synthesis. Further studies to identify a repressor are necessary.

Data supporting a requirement for synthesis of new protein in RA-mediated Hoxa-5 gene induction come also from RA induction studies of Hox clusters in which RA-responsive genes in the middle of the cluster required protein synthesis, whereas those responding more rapidly in the anterior 3’ region did not (30). The proteins that require new synthesis may involve transcription factors or accessory proteins that form part of the transcription initiation complex of Hoxa-5. RA receptors are additional possibilities, although no RA response elements have been identified so far in association with the Hoxa-5 gene. Contrasting data regarding the mechanism of RA stimulation of Hoxa-5 expression have been reported. Bernacki et al. (2) found that RA-induced stimulation of Hoxa-5 in human bronchial fibroblasts did not require new protein synthesis. The differences in findings may be intrinsic to the cell-specific differences in the molecular control of Hoxa-5 expression. The data support the likelihood that Hoxa-5 expression and the factors that control it may be different among different cell types within and across species.

Actinomycin D was used to inhibit mRNA synthesis and thereby test whether the differences in the timing of peak stimulation by RA between fibroblasts and
MLE-12 cells were due to altered mRNA half-life induced by RA. In the fibroblasts treated with actinomycin D, the mRNA half-life was not changed by RA, which indicated that RA did not alter message stability. This is similar to results described in human bronchial fibroblasts (2). In MLE-12 cells, RA shortened the mRNA half-life by nearly one-half. The reduction of message stability in MLE-12 cells may have a role in the delayed peaking of Hoxa-5 mRNA in RA-treated MLE-12 cells. Increased message stability did not appear to be the general mechanism by which RA stimulated the amount of Hoxa-5 mRNA in either cell type. We speculate that RA increased Hoxa-5 mRNA in both cell types by increasing the transcription rate. This speculation is supported in part by nuclear run-on experiments, which demonstrated an increased transcription rate of Hoxa-5 in RA-induced embryonal carcinoma cells (21, 22). Because there is decreased mRNA stability in MLE-12 cells when treated with RA, the transcription rate of Hoxa-5 must be high in order for the mRNA to accumulate. The short-lived proteins required for RA-induced Hoxa-5 expression may potentiate the transcription rate or stimulate other genes that may further increase Hoxa-5 expression. There may be multiple mechanisms through which RA may be functioning to increase Hoxa-5 expression. To test these speculations, complex detailed study and analysis are required.

There were potential problems associated with the use of primary cultures of type II cells. They rapidly dedifferentiate in some important aspects of gene expression and regulation in primary cultures (28, 33), dedifferentiate in some important aspects of gene expression and regulation in primary cultures (28, 33), making the reliability of their cell function ex vivo highly questionable for studies such as these. Additionally, most primary cultures of type II cells contain a small amount of fibroblast contamination, especially if they are in culture for >24–48 h. Because the expression of Hoxa-5 is strong in fibroblasts and weak or absent in epithelial cells, it is possible that what is generally acceptable as a small amount of fibroblast contamination would make the results difficult to interpret in our studies. Because the immortalized MLE-12 cells have been demonstrated to retain functional and morphological characteristics of bronchiolar and alveolar type II cells without contamination of fibroblasts (33), they are frequently used in surfactant and lung development studies in place of primary epithelial cell cultures (10, 12, 13, 31, 34). These cells were used in our study to examine cell-specific regulation of Hoxa-5 expression. Caution must be exercised, however, in extrapolating results from MLE-12 cells to fetal type II cells.

Our studies observed Hoxa-5 expression at very low levels in MLE-12 cells, which suggests that Hoxa-5 may be expressed in distal respiratory epithelium of fetal mouse lungs. However, in previous studies, Hoxa-5 expression was observed exclusively in the mesenchyme and not in the epithelial cells in E10.5, E12, E12.5, and E15.5 mouse embryos (1, 5). The apparent discrepancy between previous in situ hybridization studies and our data of epithelial expression of Hoxa-5 in Northern hybridization may be due partly to differences in methodology. Previous in situ hybridization studies used radioactive probes on whole mount embryo or lung sections. Visualization and localization of signals within specific cells require alternating between bright and dark fields, which may not permit easy identification of individual cells expressing the signal. Additionally, very strong expression signal seen throughout the mesenchyme may obscure a weak expression signal in epithelial cells. Although not as sensitive as ribonuclease protection assays, Northern hybridization of cell cultures readily allow evaluation and quantification of weaker signals, which may be difficult to accomplish on lung sections in in situ hybridization. Also, the in situ hybridization studies observed no Hoxa-5 expression in gestation day 18 mouse lung (5), whereas we and others (3) clearly detected expression by Northern hybridization. Other homeobox genes such as Hoxa-4 (25) and in particular Hoxb-5 (32), which is paralogous to Hoxa-5, have been demonstrated to have a low level of expression in distal or alveolar epithelium in near-term mice. For all these reasons, Hoxa-5 expression observed in MLE-12 cells at extremely small levels compared with fibroblasts in our study does not necessarily contradict existing data. The possibility that Hoxa-5 expression in lung epithelial cells may be development specific and may be present in near-term, infant, or adult lung needs further study.

In summary our studies demonstrate that Hoxa-5 gene and protein are strongly expressed in developing lungs from E13.5 to P2. The gene is highly expressed in fibroblasts but in very small amounts in the MLE-12 epithelial cell line. RA stimulated Hoxa-5 expression in both cell types in a dose- and time-dependent manner. Peak expression occurred later in MLE-12 cells than in fibroblasts. Cell-specific responses to RA were evaluated with protein and mRNA synthesis inhibitors. There was no RA-induced increase in Hoxa-5 expression in both cells when new protein synthesis was inhibited. Messenger RNA stability was not prolonged in either cell type. However, when MLE-12 cells were treated with protein synthesis inhibitor alone, there was a significant increase in Hoxa-5 expression. Our data suggest that a repressor protein may be present in epithelial cells to limit Hoxa-5 expression to mesenchymal cells and to mediate cell-specific response to RA. Dose- and time-dependent as well as cell-specific responses of lung cells to RA suggest that one of the numerous mechanisms through which RA acts in the developing lung may be through stimulation of Hoxa-5.

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REFERENCES

EFFECT OF RETINOIC ACID ON HOXA-5 IN DEVELOPING LUNG


