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Down-Regulation of Thyroid Transcription Factor-1 Gene Expression in Fetal Lung Hypoplasia Is Restored by Glucocorticoids*

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ABSTRACT

The thyroid transcription factor (TTF)-1 has an essential role in lung morphogenesis and development. It is involved in the transcription of surfactant proteins (SP), which are critical in respiratory function. Neonates with congenital diaphragmatic hernia die of respiratory failure caused by pulmonary hypoplasia with associated biochemical immaturity. To gain new insights into the causes of this disorder and the effect of prenatal hormonal treatment on reducing mortality in these infants, we evaluated the expression of TTF-1 as marker of lung morphogenesis and SP-B as marker of lung maturity. Using a rat model of lung immaturity, we show that TTF-1 and SP-B messenger RNA (mRNA) levels are drastically reduced in congenital lung hypoplasia. Interestingly, prenatal dexamethasone (Dex) treatment increased both TTF-1 and SP-B mRNAs over control levels when administered to rats with lung hypoplasia, but it had no effect on TTF-1 or a moderate effect on SP-B mRNA when administered to control rats. TRH alone also increases TTF-1 and SP-B mRNA levels but to a lesser extent than Dex. When administered together with Dex, TRH counteracts the induction observed with the glucocorticoid. The decrease in TTF-1 mRNA levels in lung hypoplasia is paralleled by a down-regulation of TTF-1 protein levels, as well as by a decrease in the TTF-1/DNA complex when the TTF-1-binding site of the SP-B promoter was used as a probe. Both parameters were reestablished after glucocorticoid treatment. Moreover, the regulation of TTF-1 gene expression described in this report is accompanied by the same regulation in its promoter activity, as demonstrated in transfection experiments performed in H-441 human lung-derived adenocarcinoma cells. In conclusion, our data demonstrate, for the first time, that lung hypoplasia and the associated respiratory dysfunction caused by SP-B deficiency are caused, in part, by down-regulation of TTF-1 gene expression. The observations that prenatal glucocorticoid treatment induces the expression of TTF-1 supports routine *in utero* glucocorticoid treatment of patients expected to have lung hypoplasia. (*Endocrinology* **141:** 2166–2173, 2000)

LUNG MORPHOGENESIS and development are known to require the presence of transcription factors (1) that activate or repress the expression of specific genes. In both processes, members of the forkhead family, HNF-3α and HNF-3β (hepatocyte nuclear factors 3 α and β), the homeodomain proteins Hoxa5 and TTF-1 (thyroid transcription factor 1), and members of the nuclear receptor superfamily (including receptors for glucocorticoids, thyroid hormones, and retinoids) are involved. TTF-1 is a homeodomain-containing transcription factor (2), expressed in two of the many structures derived from the foregut endoderm, the thyroid, and the lung (3). TTF-1 messenger RNA (mRNA) is detectable within the ventral migrating edge of the lung bud on embryonic day (E) 9.5 in the rat (3). In E11.5, a strong signal can be detected in both branches of the primitive bronchi, and from E13.5–E15.5, TTF-1 mRNA is expressed in the bronchial epithelium. In late gestation stages (E17.5), TTF-1 is present in epithelial cells of the bronchioli. TTF-1 is involved in the transcription of pulmonary surfactant proteins (SP-A, SP-B, and SP-C) and clara cell secretory protein (4–7), as well as in thyroid-specific genes such as thyroglobulin, thyroid peroxidase, TSH receptor, and Na^+/I^- symporter (8–14). Surfactant protein B (SP-B) is a hydrophobic protein that enhances the spreading and stability of surfactant phospholipids in the alveolus. Ablation of the SP-B gene in transgenic mice demonstrates the critical role of SP-B in the initiation of lung inflation at birth. Homozygous SP-B (−/−) mice died of respiratory failure immediately after birth (15), and their lungs, normally developed, remained atelectatic. An essential role for TTF-1 in lung and thyroid morphogenesis has been established, because homozygous TTF-1 null mutant mice show dilated sac-like structures in the pleural cavity rather than normal lungs (16). These structures had a rudimentary bronchial tree (but no bronchioli, alveoli, or pulmonary parenchyma) and an abnormal epithelium. This mutant died of respiratory distress immediately after birth and lacked the thyroid; the hypothalamus was severely affected (16) and have defects in tracheoesophageal morphogenesis (17). Other transcription factors are required for normal lung morphogenesis, such as HNF-3α and HNF-3β, both of which are expressed in endoderm derivatives during embryogenesis and lung development (18, 19) and are involved in regulation of TTF-1 gene expression (20). The role during

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development of the glucocorticoid receptor (GR) was investigated by generation of GR-deficient mice (21). GR null mice show respiratory distress at birth and die within a few hours. The lungs are severely atelectatic, and development is impaired from day 15.5 of gestation. These observations suggest: 1) a possible role for these transcription factors in the pathogenesis of pulmonary hypoplasia; and 2) prompt research into how their expression is altered in this disorder during embryogenesis.

Newborns with congenital diaphragmatic hernia, a malformation that occurs in up to 1:2200 births, still have a poor prognosis, in spite of recent therapeutic progress. This high mortality rate is attributable mainly to neonatal respiratory failure caused by the associated severe pulmonary hypoplasia, with physiological and biochemical immaturity. Aiming at better understanding the pathophysiology of pulmonary hypoplasia, a reproducible animal model was developed in neonatal rats by feeding nitrofen (NF) (2,4-dichlorophenyl-p-nitrophenyl ether) to timed-mated pregnant rats in an appropriate embryological window (22). Lung hypoplasia (23) and immaturity (24), observed in rat fetuses born to exposed females, are very similar to those found in the human disorders. In this rat model, the most severe pulmonary defects were observed only after exposure to NF between E9–E11, and not before this stage (22). Nitrofen is detected in the embryonic compartment, 3 h after administration, with a peak, 72 h later, that corresponds to E12–E14 (25). Nitrofen is thus present in the embryo at the time at which TTF-1 is expressed in lung. These observations suggest a possible alteration of TTF-1 in lung hypoplasia. Using the above rat model, we determined TTF-1 expression as a marker of adequate lung morphogenesis, and SP-B expression as an indirect marker of lung maturity. We report here that expression of TTF-1 is severely decreased in lung hypoplasia.

Glucocorticoids are used clinically to induce lung maturation and surfactant production in human fetuses expected to be delivered prematurely. This antenatal hormonal therapy is known to accelerate pulmonary development in premature neonates and has decreased the incidence of respiratory distress syndrome and pulmonary complications of preterm birth (26, 27), but the mechanism remains unclear. The addition of TRH therapy is no more beneficial than glucocorticoids alone (28). We investigated the effect of this antenatal hormonal therapy on TTF-1 expression. Our results show that glucocorticoid therapy to animals with lung hypoplasia restores the expression of this transcription factor, supporting routine *in utero* glucocorticoid treatment for patients expected to suffer lung hypoplasia.

Materials and Methods

Animals

Sprague Dawley rats, maintained in environmental conditions with controlled temperature and an artificial dark-light cycle, were used throughout the study. Animals were fed *ad libitum* with a standard laboratory diet. We used animals weighing approximately 250 g. Females were caged with fertile males overnight, and mating was confirmed by the presence of spermatozoa in a vaginal plug (day 0). All animals were treated according to an experimental protocol approved by our institutional research committee that ensures that all requirements for animal research (EC86/liter609) are followed.

Experimental design

A scheme representing the animal treatment appears in Fig. 1 and was carried out as follows. After light ether anesthesia, time-mated pregnant rats received intragastrically, on day 9.5 of gestation, either 100 mg NF (NF group) (Wako Chemicals, Neuss, Germany) diluted in pure olive oil (1) or the same volume of vehicle (control group). Dams of both groups also received different hormonal therapies to accelerate pulmonary development. The dexamethasone (Dex) and NF + Dex groups received 0.4 mg/kg Dex ip on days 19 and 20. The TRH and NF + TRH groups were implanted ip on day 18 with an osmotic minipump (Alzet, model 2ML2; Alza Corp., Palo Alto, CA) containing 100 μg/kg TRH, after which 25 μg/kg TRH were ip injected. Other groups of animals were treated with combined hormonal treatment (Dex + TRH and NF + Dex + TRH groups), receiving Dex and TRH as described for Dex and TRH groups. To reproduce all manipulations, animals not treated with TRH had an Alzet minipump containing saline buffer, and animals not treated with Dex received ip saline buffer. TRH and Dex were purchased from Sigma (St. Louis, MO). The fetuses were recovered on day 21 by cesarean section, examined, weighed, and dissected under a binocular operating microscope for diaphragm inspection and lung recovery. Rat fetuses were weighed and their lungs immediately frozen for subsequent RNA and nuclear protein extraction. Fetal lung weight/BW ratio (mg/g) was significantly smaller ($P < 0.05$) in NF-treated rats (16.68 $\pm$ 1.02), compared with control groups (31.33 $\pm$ 1.15), indicating that NF clearly produces lung hypoplasia, thus validating our model of study. Dex reverts the effect of NF on lung hypoplasia (24.05 $\pm$ 1.95), making its effect statistically significant ($P < 0.05$).

Cell culture

The human lung-derived adenocarcinoma cell line H-441 (or NCI-H441, ATCC HTB 174) was obtained from American Type Culture Collection (Manassas, VA) and was maintained in RPMI 1640 medium supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD). These cells express TTF-1 (29, 30) and pulmonary-specific SPs A and B (30, 31).

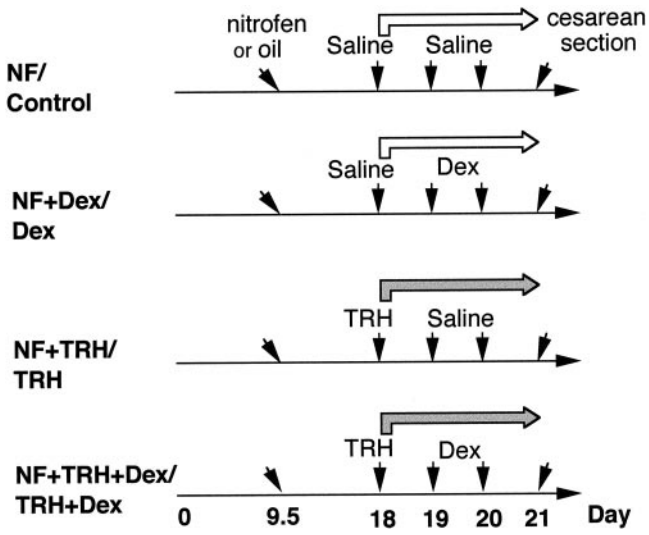

FIG. 1. Experimental design. Adult female Sprague Dawley rats were mated with fertile males. The gestational ages are indicated by numbers (vaginal plug positive, day 0; full-term, day 22). *Left diagonal arrows*, Intragastrically fed with NF (NF-treated groups) or with oil (Control groups). Dams of both groups were subdivided into different groups, according to the subsequent treatment received. The groups are indicated in the *left* portion of the figure. *Vertical arrows*, ip injection of the hormonal therapies (or the placebo solution) on the corresponding gestational day; *shadow long arrows*, infusion with osmotic minipump from day 18 with TRH or with the placebo solution (*open long arrows*); *right diagonal arrows*, cesarean section performed 1 day before term; Dex + TRH, combined hormonal treatment.

RNA isolation and Northern blot analysis.

Fetal lungs from different groups were pooled separately, and total RNA was isolated with guanidine isothiocyanate and cesium chloride centrifugation (32). Total RNA was electrophoresed on a formaldehyde-1% agarose gel and transferred to nytran membrane (Schleicher & Schuell, Inc., Keene, NH). Hybridization was carried out using a 0.6-kb *Eco*RI fragment of rat TTF-1 (2) and a 0.5-kb *Pst*I fragment of rat SP-B (33). All probes were isolated and labeled with [α-^{32}P] deoxycytidine triphosphate (ICN Biochemicals, Irvine, CA.) by random oligopriming to a specific activity of 1×10^9 cpm/DNA. Northern blot images were analyzed quantitatively using a scanning densitometer and image software and normalized with the methylene blue-stained 18S ribosomal RNA.

Transfection assays

Ten micrograms of a luciferase-linked chimeric construct, spanning 2.8 kb of the human TTF-1 5′-flanking region subcloned in the reporter vector pSV0AL-A△5′ (34), were transiently transfected into H-441 cells by the calcium phosphate precipitation method (35). The plasmid RSV-CAT (2 μg) (36) was also transfected and used to normalize transfection efficiency. After transfection, cells maintained under different conditions were harvested. Luciferase and CAT activities were measured as described (35, 37). In the experiments indicated, 2 μg of the TTF-1 expression vector (pCMV-THA) (38) or the corresponding empty vector (pRC-CMV) were cotransfected.

Electrophoretic mobility shift assays

Nuclear extracts from lungs were prepared, following the method of Gorski *et al.* (39). Protein concentration was determined with the Bio-Rad Laboratories, Inc. kit (Bio-Rad Laboratories, Inc., Richmond, CA) and BSA as standard. Gel shift assays were performed with the TTF-1-binding site of human SP-B promoter as probe (4). The oligonucleotide SPB-f1, derived from positions −113 to −90 of human SP-B promoter, was labeled with T_4 polynucleotide kinase and [γ-^{32}P] ATP (ICN., Irvine, CA) and annealed as described (40). For binding reactions, 10 μg of nuclear proteins were preincubated in a binding reaction mixture containing 40 mM HEPES (pH 7.9), 200 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, 5% Ficoll, and 3 μg poly (dI-dC) for 15 min on ice. In competition experiments, the unlabeled oligonucleotide was added in excess (100×). Labeled oligonucleotide was added to the mixture and incubated for 30 min at room temperature. For supershift assay, 1 μl anti-TTF-1 antibody (3) was added before the addition of the probe and was incubated for 3 h. As a control of supershift specificity, a preimmune serum was used and incubated in the same conditions. The resulting DNA-protein complexes were separated from free DNA on a 5% polyacrylamide gel (29:1, acrylamide-bisacrylamide). Gels were resolved at 20 mA in a cold room in 0.5 × TBE [1 × TBE is 90 mM Tris, 90 mM boric acid, and 1 mM EDTA (pH 8)] before being vacuum dried and exposed to x-ray film at −70 C.

Immunoblotting analysis

Nuclear protein extracts (40 μg) were separated in SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Inc.), in a buffer containing 25 mM Tris, 200 mM glycine, and 20% methanol. After blocking the membranes with 10% low-fat dried milk in Tris-buffered saline containing 0.05% Tween-20, immunodetection was performed using the antibody anti-TTF-1 (3). After probing with the antibodies, membranes were incubated with a streptavidin-conjugated antirabbit-specific secondary antibody. Immunoreactive bands were visualized using the luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To assess specificity and equivalent loading of the samples, the same blots were reprobed with a specific anticonstitutive transcription factor anti-CTF/NF-1 antibody (sc-870) (Santa Cruz Biotechnology, Inc.).

Statistical analysis

Statistical significance between different treatments was determined using Student's *t* test. Differences are considered significant at $P < 0.01$.

Results

TTF-1 and SP-B gene expression is down-regulated in lung hypoplasia, and glucocorticoids rescued their expression over control values

We studied whether TTF-1 gene expression is altered in lung hypoplasia. To do so, we performed Northern blot analysis with 30 μg total RNA extracted from control or hypoplastic lungs of fetuses after NF treatment. TTF-1 mRNA levels are strongly decreased in the hypoplastic lungs (NF group) (Fig. 2A). The values obtained after quantitation were 75% lower than the corresponding control (Fig. 2C, compare Control and NF groups). Based on the extensively use of Dex as a treatment to induce lung maturation, we next studied its effect in TTF-1 mRNA levels of control and hypoplastic fetal lungs. Pregnant rats, untreated or treated with

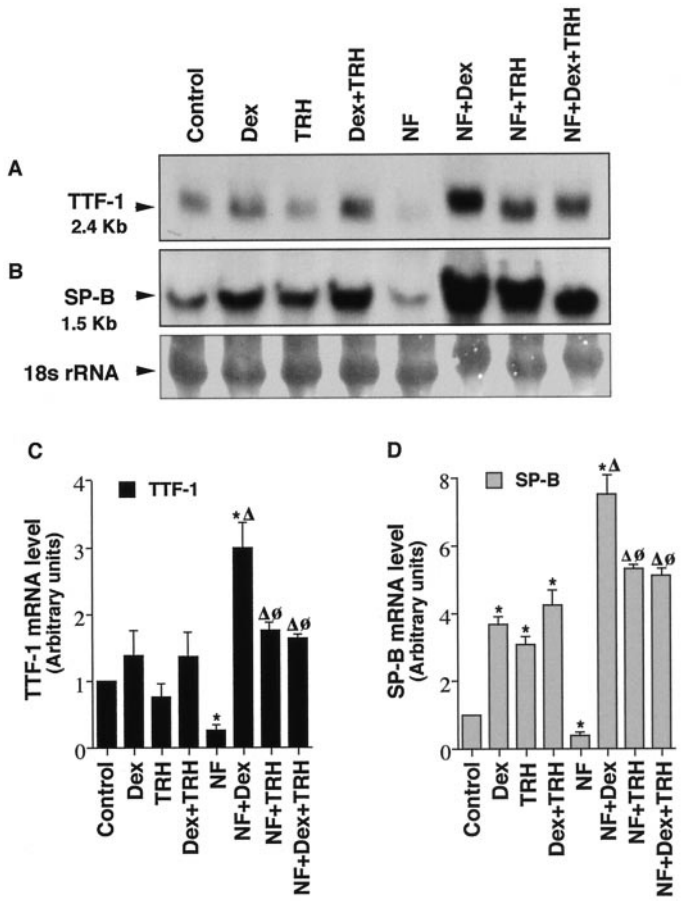

FIG. 2. Effect of NF, Dex, and TRH on TTF-1 and SP-B mRNA levels in rat fetal lungs. Total RNA (30 μg) was extracted from rats' fetal lungs, treated under the experimental protocol described in the Fig. 1 legend, then hybridized with the TTF-1 (A) or the SP-B probe (B). *Arrows*, mRNA size. The *lower panel* shows the methylene blue staining of 18S ribosomal RNA (rRNA) for loading control. C and D, Quantitation of TTF-1 and SP-B mRNAs, respectively, after correction with the 18 S, by densitometer scanning of autoradiograms from three independent experiments. The TTF-1 and the SP-B mRNA levels, expressed as arbitrary units, refers to its control (= 1) in each case. The data are mean ± SEM of three independent experiments. Significant differences among groups are indicated, and means. *, $P < 0.01$ (one group *vs.* control group); △, $P < 0.01$ (one group *vs.* NF group); ø, $P < 0.01$ (one group *vs.* NF + Dex group).

NF, received Dex, as described in *Materials and Methods*. Total RNA was extracted from fetal lungs, and the TTF-1 mRNA levels were analyzed by Northern blot. Interestingly, Dex treatment markedly increased (10-fold) TTF-1 mRNA levels when it was administered to NF-treated rats (Fig. 2, A and C, compare NF with NF + Dex groups), but it had no significant effect on control rats (Fig. 2, A and C, compare Control with Dex groups). The observation that Dex treatment of hypoplastic lungs not only rescues the TTF-1 mRNA levels but also increases these levels over control values (3-fold) (Fig. 2, A and C, compare Control with NF + Dex groups) is very important. It has been suggested that combined antenatal glucocorticoid and TRH treatment result in synergistic improvement of lung function of premature neonates (41). We studied the role of this combined treatment in lung hypoplasia. TRH was administered alone or with Dex to pregnant mothers, untreated or previously treated with NF, as described in *Materials and Methods*. The results obtained in Northern blot analysis show that TRH alone increases TTF-1 mRNA levels (5-fold) when given to NF-treated rats (Fig. 2, A and C, compare NF and NF + TRH groups), but it has no a significant effect when given to control rats (Fig. 2, A and C, compare Control and TRH groups). It is important to mention that the increase obtained with TRH was approximately half of that obtained with Dex and that, when both hormones were coadministered to NF rats, TRH decreased by 2-fold the marked effect obtained with Dex on TTF-1 mRNA levels (Fig. 2, A and C, compare NF + Dex with NF + Dex + TRH groups). The combined Dex and TRH treatment did not induce a significative effect on TTF-1 mRNA levels when administered to control rats (Fig. 2, A and C, compare Control and Dex + TRH groups).

Because of the low TTF-1 mRNA levels in hypoplastic lungs and their reversion over the control values with Dex, we determined the SP-B mRNA levels. The same Northern blots were hybridized with the SP-B probe. The results show a decrease in SP-B mRNA levels in hypoplastic lungs (NF), when compared with control values (60% lower than controls) (Fig. 2, B and D). The hormone therapy has an action on the SP-B mRNA levels similar to that obtained for TTF-1 transcript, although some differences have been observed. Thus, Dex markedly increased SP-B mRNA levels, both in NF-treated (18-fold) and control (7-fold) rats (Fig. 2, B and D). TRH, alone or combined with Dex, increased SP-B mRNA levels more than TTF-1 mRNA levels, although to a lesser extent than the glucocorticoid alone. The effect was observed in both NF-treated and untreated (control) rats. It is important to comment on two observations: 1) the maximum effect on SP-B mRNA levels was obtained in hypoplastic lungs treated with Dex, with increases of 7- to 8-fold over control values; and 2) the TRH effect counteracts the Dex effect when administered to hypoplastic lungs. From these data, we conclude that TTF-1 and SP-B are strongly decreased in lung hypoplasia. Furthermore, SP-B is under hormonal regulation in both normal and hypoplastic lungs, whereas TTF-1 is regulated only in hypoplastic lungs. These results suggest that SP-B is regulated by TTF-1-dependent and -independent mechanisms and that TTF-1-mediated regulation is an important mechanism of control in lung hypoplasia.

Because Dex treatment induces the maximal effect on TTF-1 mRNA levels, when administered to rats with hypoplastic lungs, we next determined whether this effect is correlated with protein levels. We assayed TTF-1 protein, by immunoblotting analysis, in nuclear protein extracts (Fig. 3) and found that TTF-1 protein levels are drastically reduced in hypoplastic lungs (NF), as compared with the control group. Again, Dex has no effect when administered to control animals (Dex), but it increased TTF-1 protein levels, over the control, when administered to NF-treated rats (NF + Dex). The effect is specific, as demonstrated by reprobing the same Western blots with the constitutive CTF/NF-1 transcription factor, also expressed in lungs (42). These results suggest that lung hypoplasia is caused by down-regulation of the homeotic transcription factor TTF-1. The possible mechanism of the therapeutic action of glucocorticoids in lung hypoplasia, inducing lung maturation and surfactant production, may be explained, at least in part, by an increase in TTF-1 gene expression.

The binding activity of nuclear factor TTF-1 to the SP-B proximal promoter is reduced in hypoplastic lungs and reestablished after glucocorticoid treatment

SP-B and TTF-1 are under TTF-1 control; thus, we hypothesized that the decreased levels of both mRNAs may reflect changes in TTF-1 protein bound to DNA. Gel shift assays were performed with an oligonucleotide derived from the TTF-1-binding site of the SP-B promoter (SPB-f1 probe) (4) and with nuclear extracts from control and NF-treated fetal lungs. The data in Fig. 4 show a retarded protein/DNA complex in the control groups (lane 2), which is consistently reduced in NF-treated rats (lane 8). The complex is specific, as demonstrated in competition experiments with a 100-fold excess of a related (lane 3) or an unrelated (lane 4) oligonucleotide. We also investigated the effect of hormonal therapy in the gel shift assay. Dex, TRH, or both hormones together did not modify the intensity of the DNA complex when nuclear extracts from control groups were studied (Fig. 4, compare lane 2 with lanes 5–7). Interestingly, the administration of Dex to NF-treated rats showed a marked increase in the intensity of the complex (Fig. 4, compare lanes 8 and 9), whereas TRH (lane 10) or TRH plus Dex (lane 11) increased it only slightly. Supershift assays were performed to confirm TTF-1 implication in complex decrease or increase (Fig. 5). The complex formed with nuclear extract from control lungs (lane 2) was recognized by a specific α-TTF-1

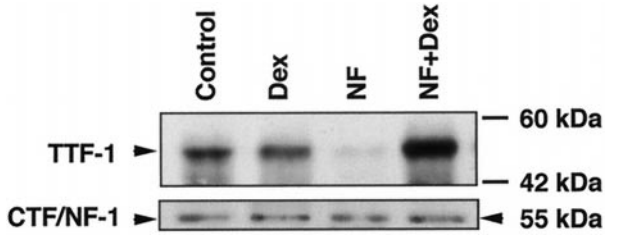

FIG. 3. TTF-1 protein levels in control and hypoplastic fetal lungs, untreated or Dex-treated. Representative Western blot was performed with 40 μg of nuclear proteins extracted from fetal lungs of control and NF groups, untreated or treated with Dex, and probed with specific anti-TTF-1 antibody. CTF/NF-1 protein was used as the loading control.

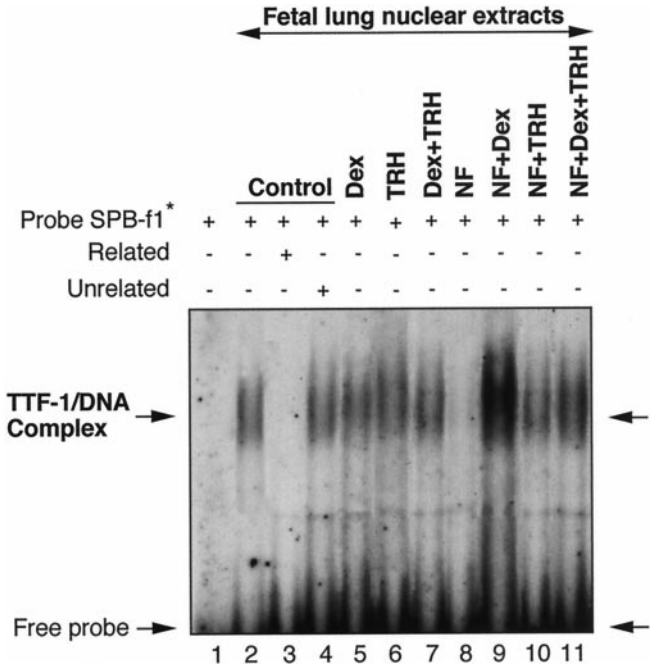

FIG. 4. TTF-1/DNA binding in rat fetal lungs after different treatments. Nuclear extracts from control (lane 2) or NF groups (lane 8) [untreated or treated with Dex (lanes 5 and 9), TRH (lanes 6 and 10), or both hormones together (lanes 7 and 11)] were incubated with a radiolabeled synthetic oligonucleotide (SPB-f1) derived from the TTF-1-binding-site of the SP-B promoter. Free and bound DNA were resolved in a gel shift assay. For competition, a 100-fold excess of unlabeled related SPB-f1 oligonucleotide (lane 3) or an unrelated oligonucleotide (lane 4) was used.

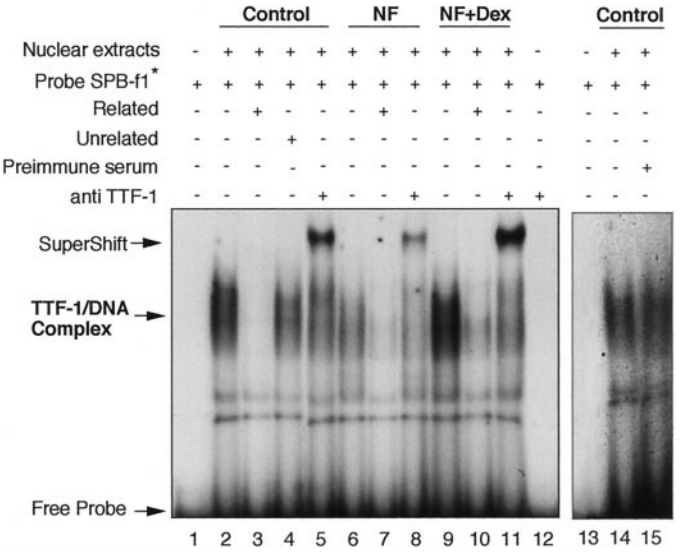

FIG. 5. TTF-1 supershift assay in control or hypoplastic fetal lungs, untreated or treated with Dex. Gel shift assay was performed as described in Fig. 4 [using nuclear extracts from control (lane 2), NF (lane 7), or NF plus Dex (NF + Dex) (lane 9) groups]. The specificity of the retarded complex was established by competition with a 100-fold excess of a related (lanes 3, 7, and 10) or unrelated (lane 4) oligonucleotide and by supershift with an anti-TTF-1-specific antibody (lanes 5, 8, and 11). The preimmune serum (lane 15) and the antibody alone, without nuclear extracts (lane 12), were used as control of supershift specificity.

antibody inducing a clear supershift (lane 5). The protein/DNA (lane 6) and TTF-1 supershift (lane 8) complexes decreased in NF-treated rats; and again, Dex restored both complexes (lane 9 and 11) over control levels when administered to hypoplastic lungs. The specificity of the complex was assayed with the corresponding 100-fold excess of the related (lanes 3, 7, and 10) and unrelated (lane 4) oligonucleotides. As a control of the supershift assay, the labeled oligonucleotide was preincubated with the α-TTF-1 antibody alone, but this failed to produce the supershifted band (lane 12). Furthermore, the incubation with a preimmune serum did not induce a supershift in control nuclear extracts (lanes 14 and 15). From these results, we conclude that the variation observed on TTF-1 mRNA levels in all the experimental approaches studied in this work parallels the variation found in TTF-1 protein levels and in its binding activity.

Nitrofen decreases TTF-1 gene promoter activity

To elucidate the molecular mechanism that induces down-regulation of TTF-1 gene expression in our animal lung hypoplasia model, we used the H441 pulmonary cell line. This cell line is used extensively in studies of lung-specific gene expression (4, 29–31, 34), and we have shown that, in these cells (as well as in rat fetal lung), NF reduces TTF-1 mRNA levels in a dose- and time-dependent manner, with maximal inhibition using 1.5 μM NF after 72 h of treatment (43). The doses studied were calculated in accordance with pharmacokinetic and biotransformation data previously described for NF (25, 44). The dose used corresponded to the cellular absorption of NF found in the embryonic compartment (25, 44). To determine whether the down-regulation of TTF-1 gene expression described in this work correlates with a decrease in TTF-1 promoter activity, and whether Dex reverses this effect, a construct spanning 2.8 kb of the human TTF-1 5′-flanking region (34), linked to a luciferase reporter gene (Fig. 6A), was transiently transfected into H441 cells. In all transfections, the reporter vector pSV0AL-A$\triangle$5 (34) was transfected in parallel as control. After transfection, cells were maintained in medium containing only 0.2% FBS, with or without 1.5 μM NF, for the following 48 h. The low serum was used to detect the Dex effect more clearly; as in 10% serum, the hormone effect is masked by the endogenous glucocorticoid content of serum (45). The cells were then treated, or not, for another 24 h with 100 nM Dex. Nitrofen decreased TTF-1 promoter activity approximately 3-fold, relative to control cells (Fig. 6B). Dex treatment increased the promoter activity in NF-treated cells but not in control cells.

In light of the results obtained, we questioned whether the NF action on TTF-1 occurs by interference of this compound with the TTF-1 synthesis mechanism or by direct interference with its transcriptional activity. To approach this question, H441 cells were cotransfected with a 2.8-kb fragment of the human TTF-1 promoter and an expression vector for the TTF-1 complementary DNA (cDNA) (38). Overexpression of TTF-1 increased TTF-1 promoter activity 2-fold (Fig. 6C), because of the existence of multiple TTF-1-binding sites in this promoter (Fig. 6A) (34). The down-regulation of TTF-1 elicited by NF, as well as the reestablishment of activity induced by Dex, also occurred when the exogenous tran-

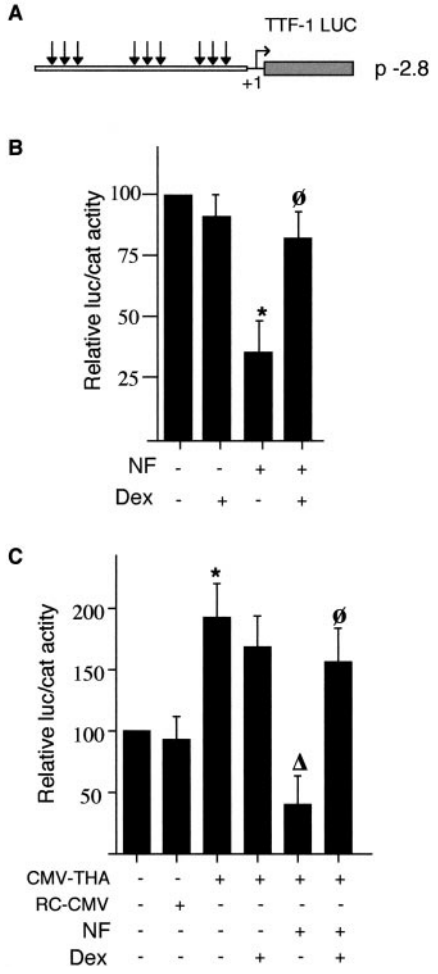

FIG. 6. Regulation of TTF-1 promoter activity by NF and Dex. A, Schematic diagram of the 2.8-kb human TTF-1 promoter linked to the luciferase (LUC) reporter gene. The *arrows* represent the TTF-1-binding sites. The TTF-1 promoter activity was obtained either after transfection of the above construct to H441 cells (B) or by cotransfection with the expression vectors RC-CMV with no insert or harboring the cDNA for TTF-1 (CMV-THA). Transfected cells were maintained for 48 h in 0.2% serum, plus or minus NF, then incubated alone or with Dex for another 24 h. Luciferase activity was determined as relative light units normalizing to CAT activity derived from the RSV-CAT transfected to adjustments of transfection efficiency. The data are mean ± SEM of three independent experiments. Significant differences among groups are indicated, and means: *, $P < 0.01$ (one group *vs.* control group); △, $P < 0.01$ (one group *vs.* CMV-THA group); ø, $P < 0.01$ (one group *vs.* NF group).

scription factor was cotransfected (Fig. 6C). These data suggest that NF may act through a mechanism that impairs the transcriptional activity of TTF-1 and that the glucocorticoids would counteract this mechanism.

Discussion

Hypoplastic lungs of infants with congenital diaphragmatic hernia seem to be delayed in their advancement through developmental stages. One consequence of this developmental delay is that the hypoplastic lungs have fewer bronchial branches and a delay in epithelial cell differentiation, with a clear SP deficiency. Teratogens, administered to pregnant rats on the appropriate gestational days, disturb organogenesis during early embryonic life and induce malformations that provide good models for several human disorders. Nitrofen-exposed rat fetuses have lung hypoplasia with biochemical immaturity. This fetal rat model is, therefore, the most appropriate for our study, because it reproduces the same lung morphological changes described in human (22–24).

Using the above model, we demonstrate that the homeodomain transcription factor TTF-1 has an essential role in the pathogenesis of lung hypoplasia (16, 29). This role has been also demonstrated in mice lacking TTF-1 expression. Mutant Hoxa-5 mice (46) also have defects in lung ontogeny caused by reduction of TTF-1 expression, inducing a significant decrease in SP proteins that cause respiratory dysfunction similar to the respiratory distress syndrome described in premature human infants. Our results show that hypoplastic lungs have severely decreased TTF-1, mRNA, and protein levels and a consequential reduction in TTF-1-binding activity to the proximal SP-B promoter. Moreover, the upstream DNA of the rat and human TTF-1 gene possesses many putative TTF-1-binding sites (34, 47), suggesting that TTF-1 gene may be autoregulated (34). In consequence, a reduction in TTF-1 binding to its own promoter should have an effect at the transcriptional level. We have shown that NF, the herbicide that induces lung hypoplasia in rats, downregulates the 2.8-kb fragment of the TTF-1 promoter activity, which includes the putative TTF-1-binding sites. In consequence, less transcription factor is synthesized. These results support the possible autoregulation of TTF-1, confirming that TTF-1 is a transcription factor essential in lung formation, and that its low expression is essential in the pathogenesis of lung hypoplasia.

Prenatal glucocorticoid therapy accelerates pulmonary development in premature infants (26, 27), and the addition of prenatal TRH therapy is no more beneficial than the use of glucocorticoids alone (28). Our results show that glucocorticoid treatment induces expression of TTF-1 mRNA and TTF-1 protein and, in consequence, induces an increase in TTF-1-binding activity to the SP-B proximal promoter, only in hypoplastic lungs. This increase is reproduced in TTF-1 promoter activity, although only in NF-treated cells. Glucocorticoids may thus restore TTF-1 and SP-B levels by increasing the TTF-1 autoregulatory mechanism. As a consequence, there is an increase in the TTF-1 protein level, which may then bind at a high ratio to its target promoter genes. We also have shown that treatment with TRH alone, or combined with Dex, had minimal consequences, as compared with the Dex effect in hypoplastic fetal lungs. These results are in agreement with a previous work that has shown that prenatal corticoid therapy improves pulmonary morphology in NF-treated rats, whereas TRH had minimal beneficial effects (48). Results also are in accordance with data on prevention of lung disease in human preterm infants (26).

Because NF induces a delay in lung development, the morphology of lungs from NF-treated fetuses is different from that of control, having proportionately more mesenchimal than epithelial cells (49). We believe that the effect of NF in fetal lung takes place mainly in epithelial cells affecting directly lung-specific genes expression. This idea is reinforced by the fact that the same results obtained in rats were

found in epithelial culture of H441 cells. The intrinsic molecular mechanism of NF and glucocorticoids on TTF-1 gene expression is unknown. TTF-1 regulation has been extensively studied in FRTL-5 cells, showing down-regulation by TSH via cAMP (13, 50). To our knowledge, however, there is no evidence that NF increases cAMP in lung cells. Interestingly, clear down-regulation of TTF-1-binding activity is induced by an oxidative state (51), and reduction/oxidation (redox) reactions are intimately involved in the control of biological processes, including modulation of transcription factor function, *e.g.* AP-1 and NF-kB (52, 53). Antioxidant compounds are also described to reverse fetal rat lung hypoplasia (54), and Dex increases antioxidant enzymes (55). In addition, no functional glucocorticoid response elements have been reported in the promoter region of TTF-1. It thus seems that glucocorticoid response element-mediated gene induction is not the basis of this regulation. Based on these observations, we hypothesized that a possible mechanism for down-regulation of TTF-1 by NF and its restoration by glucocorticoids may be elicited by redox mechanisms. This idea is supported by the fact that cellular glucocorticoid responsiveness is coordinately modulated by the redox state and thioredoxin levels (56). This plausible mechanism remains to be studied; however, our data, showing that activity of exogenous TTF-1 cotransfected to H441 cells is also regulated by both NF and glucocorticoids, reinforce this hypothesis.

In conclusion, the data reported in this study demonstrate that lung hypoplasia is induced, at least in part, by an alteration of one of the main transcription factors involved in lung organogenesis. This work also supports routine *in utero* glucocorticoid treatment for patients expected to have lung hypoplasia.

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