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Original Article

The -4 kilobase promoter region of the winged helix transcription factor HNF-3α gene elicits transgene expression in mouse embryonic hepatic and intestinal diverticula

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ABSTRACT Murine hepatocyte nuclear factor-3α (HNF-3α) protein is a member of a large family of developmentally regulated transcription factors that share homology in the winged helix/*fork head* DNA binding domain and participate in embryonic pattern formation. HNF-3α also mediates cell-specific transcription of genes important for the function of hepatocytes, intestinal, pancreatic and bronchiolar epithelium. We have previously determined that -520 nucleotides upstream of the rat HNF-3α gene were sufficient to elicit hepatoma-specific expression in transfection assays and reported on a novel HNF-3α expression pattern in the renal pelvis urothelium of the embryonic and adult kidney. We also showed that retinoic acid mediated activation of the HNF-3α gene required -4 kb of the HNF-3α promoter region in F9 teratocarcinoma transfections. In order to determine regulatory sequences mediating the HNF-3α cellular expression pattern in developing mouse embryos, we created transgenic mice bearing the -4 kb HNF-3α promoter region driving expression of the β-galactosidase transgene. Embryonic analysis of two transgenic mouse lines demonstrated that the -4 kb HNF-3α promoter sequences were sufficient to elicit transgene expression in the developing liver, intestine, esophagus, nasal epithelial cells and floorplate of the neurotube, but not in the mesodermal notochord or in the lung bud. One of the transgenic lines also exhibited proper expression in the mesonephric ducts and metanephric diverticulum, suggesting that the -4 kb HNF-3α promoter region contained a subset of the regulatory sequences necessary for HNF-3α expression in the developing kidney.

KEY WORDS: *winged helix/fork head transcription factor, cell-specific promoter, transgenic mouse embryo, hepatic diverticulum, floorplate, intestine*

Introduction

The liver performs essential functions in the body by uniquely expressing hepatocyte-specific genes encoding plasma proteins, and enzymes involved in gluconeogenesis and glycogen storage, glucose metabolism, cholesterol homeostasis and synthesis of bile salts (Cereghini, 1996). Functional analysis of numerous hepatocyte-specific promoter and enhancer regions reveals that they are composed of multiple *cis*-acting DNA sequences that bind different families of hepatocyte nuclear factors (HNF) (Costa, 1994; Cereghini, 1996). One of these regulatory families is composed of the rodent hepatocyte nuclear factor (HNF)-3α, -3β, and -3γ proteins (Lai *et al.*, 1990,1991), which share homology in the winged helix DNA binding domain (Clark *et al.*, 1993) and collaborate with other liver transcription factors to coordinately regulate hepatocyte-specific gene transcription (Costa, 1994; Cereghini, 1996). During gastrulation and organogenesis in the mouse embryo, HNF-3β is expressed in the node, mesodermal notochord, floorplate of the neurotube, definitive and foregut endoderm and its expression continues in the liver and lung primordium (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Ruiz i Altaba *et al.*, 1993; Sasaki and Hogan, 1993; Zhou *et al.*, 1996). Consistent with HNF-3β expression during gastrulation, homozygous null mutant mouse embryos for the HNF-3β gene die in utero and lack node and notochord, causing defects in the formation of neurotube, somites and gut endoderm (Ang and Rossant, 1994; Weinstein *et al.*, 1994). Mouse genetic studies have demonstrated that mutations in other winged helix genes also elicit pronounced defects in organ morphogenesis during mouse embryogenesis (Ang and Rossant, 1994; Weinstein

Abbreviations used in this paper: HNF-3, Hepatocyte nuclear factor 3; PCR, polymerase chain reaction; X-gal, 5-Bromo-4-Chloro-3-indolyl-β-D-Galactoside (Lacz); SV40, simian virus 40; mP1, mouse protamine 1.

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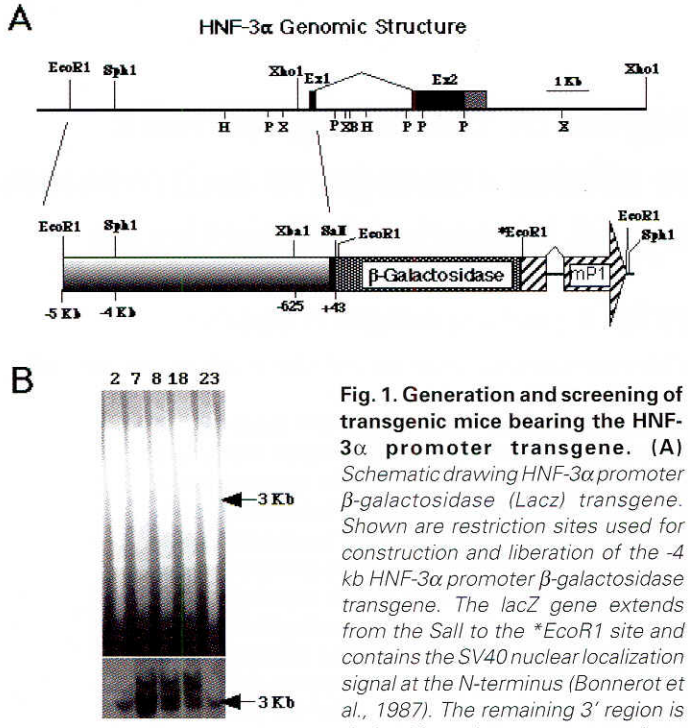

Fig. 1. Generation and screening of transgenic mice bearing the HNF-3α promoter transgene. (A) *Schematic drawing HNF-3α promoter β-galactosidase (Lacz) transgene. Shown are restriction sites used for construction and liberation of the -4 kb HNF-3α promoter β-galactosidase transgene. The lacZ gene extends from the SalI to the *EcoR1 site and contains the SV40 nuclear localization signal at the N-terminus (Bonnerot et al., 1987). The remaining 3' region is derived from the mouse protamine 1 (mP1) gene and includes an intron, 3' untranslated, and poly (A) addition sequences (Peschon, 1987). To create the transgenic mouse lines, the 7.6 kb transgene was isolated from the vector sequences by digestion with SphI containing -4 kb of the HNF-3α promoter region driving expression of the β-galactosidase gene.* **(B)** *Southern blot analysis of the five 7.6 kb transgenic mouse lines. Equal quantities of genomic DNA from the founder mice (lines 2,7,8,18 and 23) were digested with EcoR1 and electrophoretically separated on an agarose gel (top panel), transferred to a nylon membrane and hybridized with a digoxigenin labeled lacZ DNA fragment (bottom panel). Hybridization was detected by alkaline phosphatase-conjugated anti-digoxigenin antibody, and Lumi-Phos 530 (Lumigen, Inc.) chemiluminescence according to manufacturer's protocols (Boehringer Mannheim). The lacZ probe also hybridized to larger molecular weight bands with DNA from transgenic mouse lines 7,8 and 18, which may be due to partial DNA digestion with the EcoR1 enzyme.*

et al., 1994; Xuan *et al.*, 1995; Hatini *et al.*, 1996; Nehls *et al.*, 1996; Dou *et al.*, 1997; Kaestner *et al.*, 1997; Labosky *et al.*, 1997; Winnier *et al.*, 1997).

During gastrulation and organogenesis stages of mouse embryogenesis, HNF-3α expression is restricted to definitive endoderm, the anterior notochord, and the entire gut endoderm including the hepatic and lung diverticulum and the midbrain floorplate (Monaghan *et al.*, 1993; Ruiz i Altaba *et al.*, 1993; Sasaki and Hogan, 1993). However, HNF-3α exhibits weak signals in the posterior notochord and in the floorplate of the posterior neural tube (Sasaki and Hogan, 1993). Interestingly, between 12.5 and 15.5 days of gestation, a transient decrease in HNF-3α and HNF-3β expression is observed in the developing liver and intestine (Monaghan *et al.*, 1993; Rausa *et al.*, 1997). At later stages of mouse embryogenesis, HNF-3α and HNF-3β expression continues in gut endoderm derived epithelial cells of the esophagus, trachea, salivary gland, lung, stomach, intestine and pancreas (Monaghan *et al.*, 1993; Kaestner *et al.*, 1994; Cockell *et al.*, 1995; Peterson *et al.*, 1997; Rausa *et al.*, 1997; Vaisse *et al.*, 1997). However, exclusive expression of HNF-3α is observed in epithelial cells of the embryonic and adult renal pelvis, prostate gland, bladder and urinary tract (Monaghan *et al.*, 1993; Peterson *et al.*, 1997; Kopachik *et al.*, 1998). Their expression patterns also diverge in the adult intestine, with HNF-3α localized to the epithelial cells throughout the crypt/villus axis, whereas HNF-3β transcripts are restricted to the crypt epithelium (Ye *et al.*, 1997). In order to determine regulatory sequences mediating the cellular expression pattern of HNF-3α in the developing mouse embryo, we created transgenic mice bearing the -4 kb HNF-3α promoter region driving expression of the β-galactosidase transgene. Embryonic analysis of two transgenic mouse lines demonstrated that the -4 kb HNF-3α promoter sequences were sufficient to elicit transgene expression in the developing liver, intestine and floorplate of the neurotube, but not in the mesodermal notochord or in the lung bud.

Results

Generating transgenic mice containing the -4 kb HNF-3α promoter driving expression of the β-galactosidase gene

Transfection studies in F9 embryonic carcinoma cells demonstrated that retinoic acid (RA) mediated activation of HNF-3α expression required -4 kb of the HNF-3α promoter region (Jacob *et al.*, 1994). Although the -520 rat HNF-3α promoter region is sufficient to elicit expression in hepatoma cell transfections (Peterson *et al.*, 1997), we wanted to include these RA response element(s) in our HNF-3α transgene. Transgenes were constructed in which the -5 kb HNF-3α upstream region was fused to the bacterial β-galactosidase (*LacZ*) gene containing the SV-40 nuclear localization signal (Bonnerot *et al.*, 1987). To generate transgenic mice, we used SphI digestion to excise the transgene fragment containing the -4 kb HNF-3α promoter region driving expression of the nuclear localizing β-galactosidase transgene (Fig. 1A). Five independent transgenic lines were obtained carrying the -4 kb HNF-3α promoter transgene as evidenced by PCR screening with primers made in the *lacZ* gene. Equal quantities of genomic DNA from the founder mice (lines 2,7,8,18 and 23) were digested with EcoR1, which cleaves within the *LacZ* gene (Fig. 1A), electrophoretically separated on an agarose gel and then subjected to Southern blot analysis with a lacZ DNA hybridization probe (Fig. 1B). These analyses revealed that the transgenic mouse lines 2 and 23 contained relatively fewer copies of the integrated transgene compared to the other lines. Two of the five transgenic lines (8 and 23) were chosen for further analysis because their embryos exhibited specific β-galactosidase enzyme staining with X-gal substrate.

Transgenic mouse embryos carrying the -4 kb HNF-3α promoter transgene exhibit expression in the hepatic and intestine diverticula but not the developing lung

To determine the expression pattern of the transgene, we stained day 10 transgenic mouse embryos (line 8) for β-galactosidase enzyme activity with X-gal substrate. This analysis demonstrated that the -4 kb HNF-3α regulatory region was sufficient to elicit transgene expression in the foregut region including the hepatic and lung diverticula, along the neurotube, in the branchial arch region and in the allantois (Fig. 2A and B). A transverse

section of these embryos at the level of the forelimb reveals that the neurotube X-gal staining was restricted to the floorplate region (Fig. 2C). In order to further localize the transgene expression pattern in the foregut region, X-gal stained day 11 transgenic mouse embryos (line 8) were paraffin embedded, microtome sectioned, dewaxed and the β-galactosidase enzyme expression was visualized using dark field microscopy. This dark field illumination revealed intense β-galactosidase enzyme staining (pink color) in the hepatic diverticulum and intestinal epithelial cells (Fig. 2D), but not in the lung bud, which expresses the endogenous HNF-3α during this period of lung development (Monaghan *et al.*, 1993). Higher magnification allowed visualization of the nuclear X-gal staining pattern in the liver primordium and intestine, demonstrating that β-galactosidase enzyme activity in these tissues is derived from the transgene and not from endogenous lysosomal enzyme (Fig. 2E and F). These studies show that the -4 kb HNF-3α promoter region is sufficient to drive β-galactosidase enzyme expression in the developing liver, intestine and the floorplate region but insufficient to elicit transgene expression in the developing lung.

In 9 day transgenic mouse embryos from line 23, the -4 kb HNF-3α promoter region elicited X-gal staining in the first and second pharyngeal clefts, the foregut which gives rise to the hepatic diverticulum and in the mesonephric ducts (Fig. 3A and B). This is consistent with the expression pattern of the endogenous HNF-3α gene in the developing liver, intestine and kidney (Monaghan *et al.*, 1993; Sasaki and Hogan, 1994; Peterson *et al.*, 1997). By 12.5 days of gestation, the transgene expression pattern (Fig. 3C) is observed in the nasal epithelium, olfactory epithelium and branchial arch derivatives, which is consistent with the expression pattern of the endogenous HNF-3α gene (Monaghan *et al.*, 1993). However, ectopic transgene expression is observed in chondrogenetic centers (Fig. 3C) of all four limbs and the rib primordia, which may represent aberrant activation of the HNF-3α promoter by retinoic acid signaling (Gudas, 1994; Sucov *et al.*, 1996; Smith *et al.*, 1998). Further staining is seen along the neurotube and the crescent of stain at the dorsal/caudal edge of the liver corresponds to the mesonephric ducts (Fig. 3C). The intense X-gal staining of the mesonephric ducts (MD) and metanephric diverticulum (M) is revealed using a sagittal section of day 12.5 transgenic embryo (Fig. 3D) and with a higher magnification of the tail region of the transgenic mouse embryo (Fig. 3E). Also apparent in this sagittal section is X-gal staining of the nasal epithelium, esophagus and branchial arch derivatives, but not of the developing liver (Fig. 3D), which is no longer expressing the endogenous HNF-3α gene in the 12.5 day mouse embryo (Monaghan *et al.*, 1993). A transverse section of these embryos at the level of the forelimb shows paired ventrolateral X-gal staining in the floorplate of the neural tube and in the esophagus and branchial arch derivatives (Fig. 3F). This view of the embryo also shows that the -4 kb HNF-3α promoter sequences did not elicit appropriate expression of the transgene in the ventral mesodermal notochord (Monaghan *et al.*, 1993; Sasaki and Hogan, 1994). Taken together these studies suggest that the -4 kb HNF-3α promoter sequences are capable of driving transgene expression in the hepatic and intestine diverticula, and in the floorplate neuroepithelium, but not in the embryonic lung and notochord. In one of the transgenic mouse lines (number 23), the HNF-3α promoter sequences were also able to drive appropriate kidney expression in the mesonephric ducts and metanephric diverticulum.

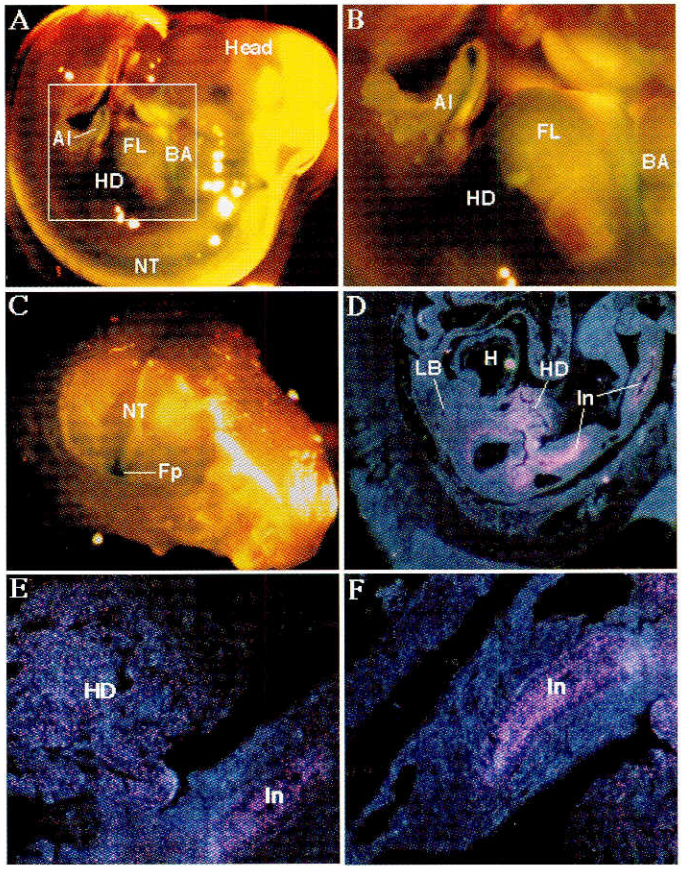

Fig. 2. The -4 kb HNF-3α promoter drives transgene expression in floorplate and in the liver diverticulum and intestine of transgenic mouse line 8. *Transgenic mouse embryos from line 8 were fixed and stained for β-galactosidase enzyme activity using X-gal substrate.* **(A)** *Whole-mount X-gal staining for β-galactosidase enzyme activity of day 10 transgenic mouse embryo. β-galactosidase enzyme staining of the day 10 transgenic mouse embryo is visible in the region of the foregut including hepatic and lung diverticula, in branchial arch, in allantois and in the neurotube.* **(B)** *High magnification of the β-galactosidase enzyme staining region (indicated in Panel A) from day 10 transgenic mouse embryos.* **(C)** *Spinal chord view of day 10 transgenic mouse embryo demonstrating β-galactosidase enzyme staining in the floorplate of the neurotube.* **(D)** *Dark field microscopy of sagittal sections of X-gal stained mouse embryos reveals expression in hepatic diverticulum and intestine but not in the lung bud. Dark field microscopy of xylene dewaxed sectioned day 11 transgenic embryos was used to visualize the β-galactosidase staining regions (pink color).* **(E-F)** *Higher magnification of sagittal mouse embryo sections stained for β-galactosidase enzyme activity depicting expression in hepatic diverticulum and intestine. Abbreviations are as follows: Allantois (Al), brachial arch (BA), floorplate (Fp), Forelimb (FL), heart (H), hepatic diverticulum (HD), intestine (In), lung bud (LB), and neural tube (NT).*

Discussion

Embryonic expression of HNF-3α is restricted to the anterior notochord, the midbrain floorplate, developing kidney and the entire gut endoderm including the hepatic and lung diverticulum (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Ruiz i Altaba *et al.*, 1993; Sasaki and Hogan, 1993; Peterson *et al.*, 1997). In the present study, we show that the -4 kb HNF-3α promoter region is sufficient

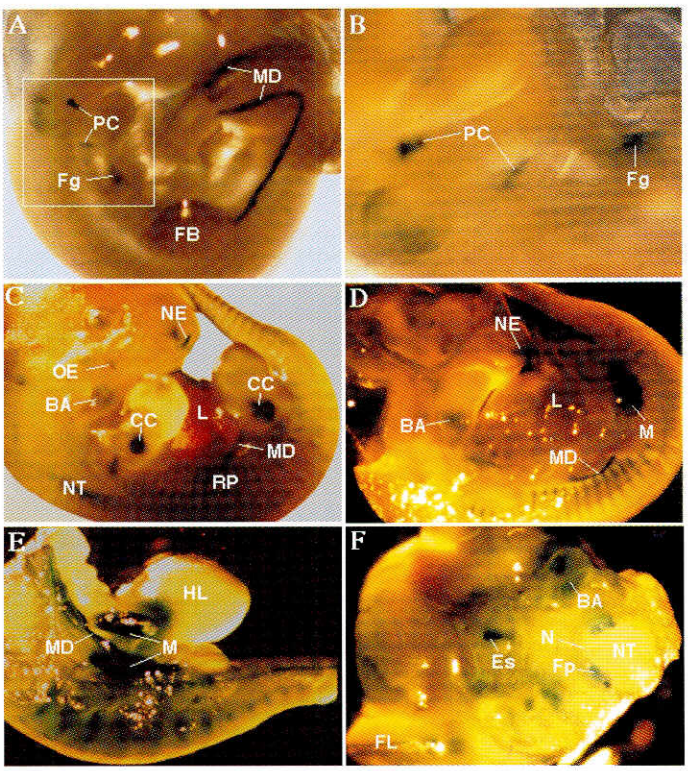

Fig. 3. The -4 kb HNF-3α promoter drives transgene expression in the foregut, esophagus, floorplate and metanephric diverticulum of transgenic mouse embryo line 23. (A) *X-gal stained day 9 transgenic mouse embryo. The -4 kb HNF-3α promoter region elicits β-galactosidase transgene expression in the foregut diverticulum, pharyngeal clefts and mesonephric ducts.* **(B)** *Higher magnification of X-gal staining in the foregut diverticulum and pharyngeal clefts.* **(C)** *X-gal stained day 12.5 transgenic mouse embryo. Staining is apparent in the nasal and lateral oral epithelium, branchial arch derivatives, the chondrogenetic centers of the limbs, along the neural tube, in the rib primordia, and in the mesonephric ducts.* **(D)** *A similar X-gal stained embryo (12 day) is cut in the coronal plane. X-gal staining is observed in mesonephric ducts, metanephric diverticulum, branchial arch derivatives and nasal epithelium.* **(E)** *Higher magnification of the caudal region of 12 day embryo showing staining in the mesonephric ducts and metanephric diverticulum.* **(F)** *A similarly X-gal stained transgenic mouse embryo cut transversely through the neck/upper thorax at the level of the forelimbs and viewed cephalo-caudally. Staining is evident in the floorplate of the neural tube, in branchial arch derivatives, and in the esophagus, but not in the notochord. Abbreviations are the same as in Figure 2 legend except: chondrogenetic centers (CC), esophagus (Es), foregut (Fg), forelimb bud (FB), hindlimb (HL), mesonephric ducts (MD), metanephric diverticulum (M), liver (L), nasal epithelium (NE), notochord (N), pharyngeal clefts (PC), olfactory epithelium (OE) and rib primordium (RP).*

to drive appropriate β-galactosidase transgene expression in the foregut endoderm, the developing liver, intestine and the floorplate neuroepithelium, nasal epithelium, esophagus and branchial arch derivatives (Figs. 2 and 3). However, these HNF-3α regulatory sequences were not sufficient to drive transgene expression in the mesoderm notochord and developing lung. Consistent with the endogenous HNF-3α expression (Monaghan *et al.*, 1993), we observed that transgene expression was extinguished in the liver and intestine of day 12.5 day embryos from transgenic line 23 (Fig. 3). HNF-3α is also expressed in the embryonic and adult renal pelvis urothelium where it potentially regulates a number of genes critical for kidney function (Peterson *et al.*, 1997). Consistent with this HNF-3α expression pattern in the developing kidney, the HNF-3α day 9 and 12.5 transgenic embryos from line 23 exhibited intense X-gal staining in both the mesonephric ducts and metanephric diverticulum (Fig. 3). The expression of the transgene in the developing kidney is also compatible with the fact that the -600 HNF-3α promoter region was recognized by several factors present in kidney nuclear extracts (Peterson *et al.*, 1997). The fact that only one of the transgenic lines exhibited expression in the developing kidney suggests that the -4 kb HNF-3α promoter region may contain only a subset of the necessary regulatory sequences necessary to elicit copy number and position independent transgene expression.

Comparison of the HNF-3α foregut and hepatic regulatory sequences with those found in the *HNF-3β* and *HNF-3γ* genes suggests that these regulatory regions are situated in different positions within each of the *HNF-3* gene loci. In contrast to the HNF-3α promoter region, transgenic analysis of the -17 kb to + 6 kb of the *HNF-3β* gene did not elicit transgene expression in the foregut endoderm, but did elicit node, notochord, floorplate and hindgut transgene expression (Sasaki and Hogan, 1996). Similarly, transgenic mouse analysis of the HNF-3γ promoter determined that the combination of the -8 kb upstream region plus sequences located in the 3' flanking region was required for transgene expression in the developing liver and gut (Hiemisch *et al.*, 1997). These transgenic mouse studies suggest that the regulatory elements necessary for foregut and hepatic expression have evolved in distinct regions of the *HNF-3* genes.

We show that the HNF-3α DNA regulatory sequences necessary for transgene expression in notochordal mesoderm and developing lung are not present in the -4 kb HNF-3α promoter region. The fact that regulatory sequences necessary for transgene expression in the notochord and hepatic diverticulum do not overlap is consistent with the transgenic analysis of the HNF-3β promoter region (Sasaki and Hogan, 1996). Similarly, different 3' HNF-3γ enhancers regions were required to elicit transgene expression in bone and gut endoderm (Hiemisch *et al.*, 1997). Although the endogenous *HNF-3γ* gene is not expressed in embryonic mouse lung, trachea or esophagus (Monaghan *et al.*, 1993), a single gut endoderm enhancer region (+16 kb) was sufficient to drive expression of the transgene in liver, pancreas, stomach and small intestine (Hiemisch *et al.*, 1997). We were therefore surprised that HNF-3α regulatory region was not capable of driving transgene expression in the developing lung even though development of the liver and lung is initiated from distinct mesenchyme induction of the foregut endoderm (Ten Have-Opbroek, 1991; Zaret, 1996). Furthermore, the lack of lung expression occurred despite the presence of two binding sites for thyroid transcription factor-1 (TTF-1) in the -4 kb HNF-3α promoter construct (Peterson *et al.*, 1997). As performed with the *HNF-3γ* (Hiemisch *et al.*, 1997) and the *HNF-4* genes (Zhong *et al.*, 1994), identification of DNase I hypersensitive sites in the *HNF-3α* gene in lung tissue may allow determination of regulatory sequences that are critical for its expression in bronchiolar epithelial cells. However, this divergent regulation of HNF-3α transcription in the liver and lung is consistent with their differential HNF-3α expression in response to lipopolysaccharide (LPS) induced inflammation (Qian *et al.*, 1995). Administration of LPS via intraperitoneal injection and nasal aspiration to induce the acute phase response, elicited a rapid decrease in hepatocyte expres-

sion of HNF-3α, whereas its lung expression levels were not influenced by this LPS treatment (Qian *et al.*, 1995).

The HNF-3α transgenic line 23 also exhibited ectopic expression pattern in the ribs and forelimbs, which is similar to patterns found with retinoic acid activation (Gudas, 1994; Sucov *et al.*, 1996; Smith *et al.*, 1998) and not unlike that seen for day 13.5 embryos carrying the ADH3-LacZ (retinol dehydrogenase) transgene (Zgombic-Knight *et al.*, 1994). Moreover, the -4 kb HNF-3α reporter construct has been shown to be stimulated during retinoic acid mediated differentiation of F9 teratocarcinoma cells, a response which reflects that of the endogenous gene as well (Jacob *et al.*, 1994). This activation of HNF-3α expression is a direct response to retinoic acid because HNF-3α expression is observed within two hours of retinoic acid mediated differentiation of P19 cells toward neuronal and astrocyte cell lineage (Jacob *et al.*, 1997). The -4 kb HNF-3α lacZ transgene, at least in the transgenic mouse line 23, might therefore be a marker for retinoic acid mediated activation.

In summary, embryonic analysis of two transgenic mouse lines demonstrated that the -4 kb HNF-3α promoter sequences were sufficient to elicit appropriate transgene expression in the developing liver, intestine, esophagus, nasal epithelium, floorplate of the neurotube, and branchial arch derivatives. These HNF-3α regulatory sequences did not drive appropriate transgene expression in the mesodermal notochord or in the lung bud.

Materials and Methods

Generation and screening of transgenic mice bearing the HNF-3α promoter transgene

The HNF-3α genomic upstream region was cloned as *EcoRI*(-5 kb)-*SalI*(+43) in pGEM-1 (Promega). This plasmid was then fully digested with *SalI* and partially digested with *SphI* and the plasmid having only the vector *SphI* digested was purified and ligated with the *SalI-SphI* cut nlacZ gene (Fig. 1A). The plasmid was then grown in *E. coli* and purified by standard CsCl density gradient techniques (Qian and Costa, 1995). The injected fragment of the DNA was prepared by complete digestion with *SphI* and agarose gel purification by the Geneclean system (Bio 101), ethanol precipitation, and resuspension in 0.1xPBS. Swiss Webster mice were made transgenic (Hogan *et al.*, 1994) with a 7.6 kb DNA fragment containing approximately 4 kb of HNF-3α upstream sequence (*SphI* -4 kb to +43) driving expression of the bacterial β-galactosidase gene linked to the SV40 nuclear localization signal (Bonnerot *et al.*, 1987).

Tail samples of founder mice were digested in proteinase K at 55°C for at least 4 h, and the genomic DNA was purified using the QIAamp Tissue Kit (Qiagen) according to the manufacturers protocol. The purified DNA was then screened for the presence of the transgene by PCR and Southern blot.

For PCR, two oligonucleotides corresponding to sequences in the *lacZ* gene were synthesized [primer 1 (sense) 5'-ggg ttg tta ctc gct-3' and primer 2 (antisense) 5'-aca gtt tcg ggt ttt-3'] which generated a unique product of 541bp in the presence of a lacZ template. Reaction conditions for amplification were as follows: 1 μl (out of 400 μl) DNA sample (QIAGEN; QIAamp kit protocol for 1 cm tail), 5 μl 10xPCR buffer (Gibco BRL), 2 μl 50 mM MgCl2 (Gibco BRL), 1 μl 10 mM dNTP (Pharmacia), 600 ng each primers, 3 units Taq DNA Polymerase (Gibco BRL), and water to a final volume of 50 μl, then overlayed with light mineral oil (Sigma). Reaction mixes were denatured for 3 min at 94°C, then subjected to 33 cycles of 52°C hybridization, 72°C polymerization, and denaturation at 94°C for 1 min each, followed by a 10 min soak at 72°C using a Perkin-Elmer DNA Thermal Cycler. Products were visualized on an agarose gel containing ethidium bromide.

For Southern blots, 80 μl of the purified DNA sample was digested with EcoRI, ethanol precipitated, separated by 0.8% agarose gel electrophoresis, and transferred in 0.4 N NaOH to charged nylon membranes (Boehringer Mannheim) using a Stratagene Posiblotter. LacZ DNA was detected using digoxigenin labeled DNA probes, alkaline phosphatase-conjugated anti-digoxigenin antibody, and Lumi-Phos 530 chemiluminescence according to manufacturer's protocols (Boehringer Mannheim).

Analysis of the β-galactosidase transgene expression pattern during mouse embryogenesis

Positive founders were bred to non-transgenic Swiss Webster mice (Charles River), and positive offspring were sacrificed for embryonic studies. Embryos between day 9 and 12 were dissected from the uterus and extraembryonic membranes in PBS (pH 7.8 to inhibit endogenous β-galactosidase, which functions at acidic pH) and fixed directly. Fixation was done using an ice cold solution of 2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS (pH 7.8). Fixation was generally for one hour, with adjustment for larger or smaller embryos. After fixation, tissues were rinsed three times in PBS (pH 7.8) and incubated for 12 to 24 h at 37°C in a solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.02% NP-40, 0.01% sodium deoxycholate, and 1 mg/ml of X-gal (5-Bromo-4-Chloro-3-indolyl-β-D-Galactoside; from a stock solution of 40 mg/ml in dimethyl sulfoxide) in PBS (pH 7.8). Following staining, samples were rinsed three times in PBS, post-fixed in 4% paraformaldehyde for 2 h, and stored in 70% ethanol at 4°C. Prior to photography, some samples were dehydrated through increasing concentrations of ethanol or cleared in benzyl alcohol:benzyl benzoate (1:2 volume/volume). To further localize the transgene cellular expression pattern, transgenic mouse embryos were fixed, stained for β-galactosidase enzyme activity using X-gal substrate, paraffin embedded and sectioned with a microtome. Following removal of paraffin wax, β-galactosidase enzyme activity (pink color) was visualized using dark field microscopy (Hogan *et al.*, 1994).

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