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Combinatorial Action of HNF3 and Sp Family Transcription Factors in the Activation of the Rabbit Uteroglobin/CC10 Promoter*

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It has been reported that respiratory epithelium-specific transcription is mediated by thyroid transcription factor 1 and members of the HNF3/forkhead family of transcription factors. Here, we show that the uteroglobin/Clara cell 10-kDa promoters from rabbit and man are regulated by HNF3α and HNF3β but not by HFH-4 and TTF-1. We have identified two HNF3-responsive elements in the rabbit uteroglobin/CC10 promoter located around 95 and 130 base pairs upstream of the transcriptional start site. Both elements contribute to promoter activity in H441 cells expressing uteroglobin/CC10 and HNF3α. Gene transfer experiments into *Drosophila* Schneider cells that lack many mammalian transcription factor homologs revealed that HNF3α and HNF3β on their own cannot activate the uteroglobin/CC10 promoter. However, HNF3α and HNF3β strongly enhanced Sp1-mediated promoter activation. Synergistic activation by HNF3α and Sp1 was absolutely dependent on the integrity of two Sp1 sites located at around −65 and −230. We show further that multiple activation domains of Sp1 are required for cooperativity with HNF3α. These studies demonstrate that transcription from the rabbit uteroglobin/CC10 promoter in lung epithelium is controlled by the combinatorial action of the cell-specific factor HNF3α and the ubiquitous factor Sp1.

The level and pattern of expression of a gene is determined mainly by the combinatorial action of transcription factors binding to distinct promoter and enhancer elements. In the last years, we have started to define DNA elements and transcription factors responsible for the expression of the rabbit uteroglobin/CC10 (Clara cell 10-kDa protein) gene, which is expressed in several ontogenetically unrelated epithelial tissues. Highest expression of the uteroglobin/CC10 gene is observed in endometrium and lung (1, 2). In endometrium, expression of the gene is restricted to the glandular and luminal epithelium (1), where it is induced by progesterone and estradiol (3). In lung, Clara cells lining the respiratory epithelium express constitutively uteroglobin/CC10 at high levels. In these cells, glucocorticoids modulate the uteroglobin/CC10 mRNA level only slightly (4, 5).

DNase I protection, promoter deletion, and linker-scanning analyses revealed that at least six distinct regions contribute to the activity of the promoter in the epithelial endometrium cell line Ishikawa and and the lung cell line H441 (6, 7). Some of the transcription factors acting through these elements have been identified in the last years. Regions VI and II located 230 and 65 bp[1] upstream of the transcription start site are noncanonical binding sites for the closely related transcription factors Sp1 and Sp3 (8). Region V spans at least 60 nucleotides between −208 and −148 and probably contains several DNA elements that have not been characterized further. Regions III (around −95) and IV (around −130) are characterized by two A/T-rich nucleotide stretches. Region I contains a noncanonical TATA box motif (TACA box) which is bound specifically by two factors, the TATA core factor and the TATA palindrome factor (9). Both are different from the TATA box-binding protein. TATA palindrome factor has recently been identified as the transcription factor Yin Yang 1 (YY1) (10).

Studies of other genes specifically expressed in lung epithelium (surfactant protein A, surfactant protein B, and murine CC10) provided evidence that thyroid transcription factor-1 (TTF-1), which is expressed in addition to the thyroid and the brain also in lung type II and Clara cells (11, 12), is a major player involved in the expression of genes in the respiratory epithelium (13–17). In addition, hepatocyte nuclear factor 3 (HNF3) family members and the HNF3/forkhead homologs (HFH), which are also expressed in lung epithelia (18, 19), are involved in expression of surfactant protein A (14) and surfactant protein B (13, 15, 18) genes as well as of murine CC10 genes (16, 17, 20–24).

In the present study, we analyzed various different human lung cell lines for the expression of lung epithelium-specific marker genes. We show that H441 cells express human uteroglobin/CC10 mRNA in addition to several other lung epithelial cell-specific mRNAs. We demonstrate that the rabbit uteroglobin/CC10 gene promoter responds to the transcription factors HNF3α and HNF3β, but not to TTF-1 and HFH-4. Promoter deletion and linker-scanning analysis identified two functional elements for HNF3α/HNF3β around −130 and −95. The factors present in H441 cells and acting through these elements are HNF3α and Oct-1. Transfection studies into *Drosophila* Schneider cells that lack many mammalian transcription factor homologs revealed that HNF3α and HNF3β on their own cannot activate the uteroglobin/CC10 promoter, but that both proteins strongly enhance Sp1-mediated promoter activation. This study also demonstrates that synergistic activation by HNF3α and Sp1 is absolutely dependent on the integrity of the two Sp1 sites located at around −230 bp and −65 bp. In addition, we show that several activation domains of Sp1 are required for cooperativity with HNF3α.

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[1] The abbreviations used are: bp, base pair(s); LS, linker-scanning mutant; DLS, double linker-scanning mutant; LDL, low density lipoprotein; SREBP, sterol receptor element-binding protein; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; HFH, HNF3/forkhead homolog; RSV, Rous sarcoma virus; SP, surfactant protein.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The construction of the wild-type rabbit uteroglobin/CC10 promoter construct pUG(−395)CATSV as well as the linker-scanning mutants LS(−92/−99), LS(−127/−132), LS(−64/−72), and LS(−222/−229) fused to the CAT gene have been described (7). The double linker-scanning mutants DLS(−92/−127) and DLS(−64/−222) were generated by polymerase chain reaction-based mutagenesis using appropriate oligonucleotides. The human CC10 promoter-CAT construct (hCC10-CATSV) was obtained by replacing the 410-bp *Bam*HI rabbit UG promoter fragment in pUG(−395)CATSV by a 405-bp *Hin*fI fragment containing the human CC10 promoter sequences from −395 to +10 (25). An appropriate rat CC10 promoter-CAT construct (ratCC10-CATSV) was obtained by cloning a 350-bp *Hpa*II fragment containing rat CC10 sequences from −300 to +60 (26) into the blunted *Bam*HI site of pUG(−395)CATSV. During the course of our experimental studies, we recloned the various uteroglobin/CC10 promoters and linker-scanning mutants thereof as *Hin*dIII-*Xho*I fragments into the luciferase reporter vector pGAW, which is a self-made derivative of the pGL3 basic vector (Promega). In pGAW the single *Bam*HI site of pGL3 is destroyed by blunt end religation and the polylinker of pGL3 is replaced by a polylinker containing single recognition sequences for the restriction enzymes *Pst*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, *Bgl*II, *Xho*I, *Sma*I, *Nhe*I, *Sac*I, and *Kpn*I. The luciferase reporter plasmids were used for transfections in *Drosophila* Schneider cells (SL2 cells).

The mammalian expression plasmids for TTF-1, HNF3α, HNF3β, and HFH-4 were generous gifts of R. DiLauro (CMV-TTF-1) and R. H. Costa (CMV-HNF3α, CMV-HNF3β, and CMV-HFH-4). Transcription factor expression in *Drosophila* Schneider cells was driven by the *Drosophila* actin 5C 5′-flanking region, and the constructs were generated as follows. The plasmids pPacHNF3α, pPacHNF3β, and pPacTTF-1 were obtained by cloning 1.6-kilobase pair *Eco*RI fragments from CMV-HNF3α or CMV-HNF3β or a 1.4-kilobase pair *Hin*dIII-*Xba*I fragment from CMV-TTF-1 into pPac (supplied by R. Paro, ZMBH Heidelberg) via *Bam*HI linkers. The expression plasmids for human Sp3 (pPacUSp3 in Ref. 27), Sp1 (pPacSp1), and Sp1 derivatives have been described (27–29). The Sp1 and Oct-1 expression plasmids pPacSp1 and pPacOct-1 were generously provided by R. Tjian and C. Möws, respectively.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Analysis (EMSA)—Nuclear extracts of mammalian and transfected SL2 cells were prepared according to Andrews and Faller (30). The sequences of the oligonucleotides used for EMSAs are presented in the appropriate figures. After annealing of the single-stranded oligonucleotides the double-stranded DNA was separated from unannealed strands by gel electrophoresis on a native 12% polyacrylamide gel in Tris borate-EDTA buffer. Double-stranded DNA was labeled by filling in the ends with [α-^{32}P]dCTP and Klenow fragment (20 ng of DNA in 20 μl). Labeled DNA was separated from free [α-^{32}P]dCTP by filtration through ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech). EMSAs were performed by preincubating 1–3 μl of nuclear extract with 1.5 μg of unspecific competitor poly(dI-dC) in a buffer containing 10 mM HEPES (pH 7.9), 150 mM KCl, 1 mM dithiothreitol, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 8.5% glycerol for 10 min on ice. Subsequently, 0.1 ng of labeled double-stranded oligonucleotide was added to a final volume of 20 μl, and samples were incubated for another 20 min on ice.

Antisera used for supershift assays were described previously (αSp1 and αSp3 in Ref. 31) or generously provided by C. Scheidereit (αOct-1), R. DiLauro (αTTF-1), and W. Schmid and G. Schütz (αHNF3α and αHNF3β). Usually, 1 μl of the appropriate antiserum was added to the binding reaction, and incubation was continued at room temperature for another 20 min. In the case of αOct-1, the antiserum was added prior to the labeled oligonucleotide, because the antiserum prevents binding of Oct-1 to DNA.[2] For competition experiments, unlabeled oligonucleotides were added in excess before the labeled oligonucleotide was added. Samples were analyzed on 4% native polyacrylamide gels in 45 mM Tris, 45 mM boric acid, 1.6 mM EDTA. Gels were transferred to Whatman no. 3MM paper, dried under heat and vacuum, and exposed to x-ray films overnight.

Cell Culture and Transfections—Ishikawa cells were grown as monolayers in minimum essential medium containing 10% fetal calf serum. The human lung adenocarcinoma cell-line H441 was maintained in RPMI medium containing 4% fetal calf serum. SL2 cells (32) were grown in Schneider medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (insect cell qualified; Life Technologies, Inc.) at 25 °C. All media were supplemented with L-glutamine and antibiotics.

[2] C. Scheidereit, personal communication.

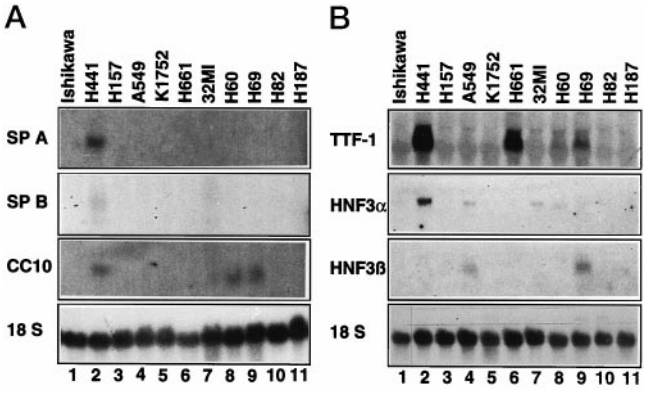

FIG. 1. **Northern analysis of uteroglobin/CC10, SP A, SP B, TTF-1, HNF3α, and HNF3β transcripts in human lung cell lines.** Total RNA (10 μg) from the endometrial cell line Ishikawa and the lung cell lines H441, H157, A549, K1752, H661, 32MI, H60, H69, H82, and H187 was subjected to electrophoresis on 1.2% (*A*) and 0.8% (*B*) agarose gels containing 2.2 M formaldehyde, and transferred to nylon hybridization membranes. The filters were successively hybridized with cDNA fragments of human uteroglobin/CC10, SP A, SP B, TTF-1, HNF3α, and HNF3β as indicated. As a control, the filters were probed with an 18 S ribosomal RNA specific oligonucleotide.

Ishikawa cells were transfected on 60-mm dishes at 50–70% confluence by the DEAE-dextran method as described (27) with 6–8 μg of reporter plasmid, 0.5–3 μg of expression vector and 2 μg of RSV-Luc or RSV-βgal plasmids as internal controls. H441 cells were transfected on 35 mm dishes at 50–70% confluence by using 2 μl of Lipofectin reagent (Life Technologies, Inc.) with 2 μg of reporter plasmid, 0.5–1 μg of expression vector, and 2 μg of RSV-Luc, RSV-CAT, or RSV-βgal plasmids as internal controls. SL2 cells were transfected on 60-mm dishes as described (27) or six-well microtiter plates using the calcium phosphate method described by DiNocera and Dawid (33). One day prior to transfection, cells were plated at a density of 1.5×10^6 cells/well. Every well received 2.5 μg of DNA including 1 μg of reporter plasmid and 1 μg of the β-galactosidase expression plasmid p97b as an internal reference. Variable amounts of expression plasmids were compensated with the plasmid pPac. Transfected cells were incubated for 24 h prior to changing media.

Ishikawa and H441 cells were harvested after a total of 72 h and SL2 cells after a total of 48 h. The cells were lysed by four freeze-thaw cycles and centrifuged at 4 °C in a microcentrifuge for 5 min. Chloramphenicol acetyltransferase was assayed as described (34). Chloramphenicol and its acetylated derivatives were separated by thin-layer chromatography and visualized by autoradiography. The percentage of acetylation was quantitated by using a Packard System 200 imaging scanner. Expression of CAT enzymatic activity was normalized to luciferase or β-galactosidase activity. Luciferase and β-galactosidase assays were carried out as described (35, 36).

Northern Blot Analysis—Total RNA from various cell lines was provided by M. Kalff-Suske. For Northern blots, the RNA was separated on 0.8% and 1.2% agarose gels containing 2.2 M formaldehyde and blotted to nylon membranes. Prehybridization was carried out in 5 × SSC, 5 × Denhardt's solution, 25 mM sodium phosphate, pH 6.4, 0.1% SDS, 250 μg/ml sonicated denaturated herring sperm DNA, 25 μg/ml poly(A), and 50% formamide at 42 °C for 4 h. For hybridization, 10% dextran sulfate and appropriate ^{32}P-labeled cDNA probes for SP A, SP B, SP C, uteroglobin/CC10, TTF-1, HNF3α, HNF3β, and HFH-4 (specific activity of 1×10^9 cpm/μg) were included prior to overnight incubation. Control hybridizations of 18 S ribosomal RNA have been performed as described (37) using a ^{32}P-labeled single-stranded oligonucleotide probe that has the sequence 5′-ACGGTATCTGATCGTCTTCGAACC-3′.

RESULTS

Identification of Cell Lines Expressing Lung Epithelial Marker Genes—To identify cell lines that express lung epithelium-specific marker genes, we performed Northern blot analysis of RNA from different human lung cell lines. As a negative control, we used RNA from Ishikawa cells, a cell line derived from an endometrium tumor (Fig. 1). Surfactant protein A and B (SP A and SP B) mRNAs were present exclusively in H441 cells whereas surfactant protein C mRNA was not detectable in

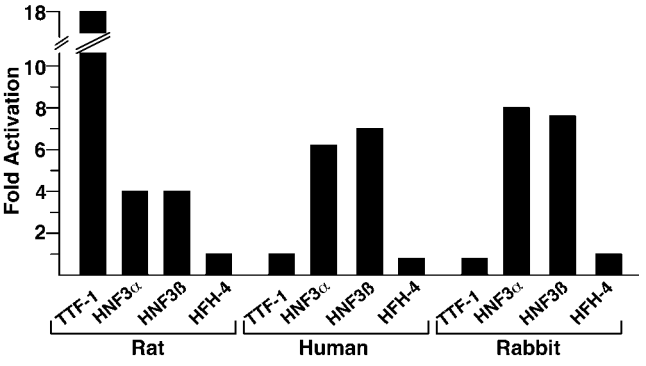

FIG. 2. **Activation of CC10 promoters by various transcription factors.** A total of 8 μg of reporter plasmids containing promoter regions of the rat (−300 to +60), human (−395 to +10), or rabbit (−395 to +14) uteroglobin/CC10 genes were transfected into Ishikawa cells along with 2 μg of CMV-driven expression vectors for the transcription factors TTF-1, HNF3α, HNF3β, and HFH-4 as indicated. The cells were lysed subsequently, CAT activities determined as described under "Experimental Procedures," and the -fold activation was calculated. The values represent the average of at least three independent determinations.

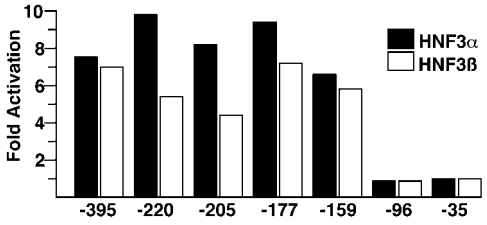

FIG. 3. **Identification of the rabbit uteroglobin/CC10 promoter region responsible for activation by HNF3α and HNF3β.** A total of 8 μg of CAT reporter plasmids containing the wild-type uteroglobin/CC10 promoter or 5′-promoter deletion mutants designated by the deletion end points relative to the transcriptional start site were transfected into Ishikawa cells in the absence or presence of 2 μg of expression constructs for HNF3α or HNF3β as indicated. CAT activities were assayed as described under "Experimental Procedures," and the -fold activation was calculated.

any of the cell lines (data not shown). Human uteroglobin/CC10 mRNA was expressed in H441 cells, in addition to H60 and H69 cells (Fig. 1*A*). This finding was surprising inasmuch as it has been reported previously that H441 cells do not express the Clara cell marker protein CC10 gene (16, 38).

It has been reported that the transcription factors TTF-1 and members of the HNF3/forkhead family of transcription factors are involved in SP A, SP B, and uteroglobin/CC10 gene expression. Thus, we also analyzed the expression pattern of the TTF-1, HNF3α, HNF3β, and HFH-4 genes (Fig. 1*B*). TTF-1 mRNA was present in H441, H661, and H69 cells with the highest amount in H441 cells. HNF3α mRNA was detected in H441, A549, 32M1, and H60 cells and HNF3β mRNA in A549 and H69 cells. In none of the cell lines was HFH-4 mRNA detectable (data not shown). Taken together, H441 cells, which express three different lung epithelial marker genes (SP A, SP B, and uteroglobin/CC10), coexpress TTF-1 and HNF3α but not HNF3β and HFH-4 mRNA.

Activation of the Uteroglobin/CC10 Promoters from Rabbit, Rat, and Man by HNF3α and HNF3β—Since H441 cells expressed uteroglobin/CC10 as well as TTF-1 and HNF3α, we asked whether TTF-1 and members of the HNF3/forkhead family of transcription factors could activate transcription from the uteroglobin/CC10 promoters of various species. The promoters of the rat, human, and rabbit uteroglobin/CC10 genes were fused to the CAT gene and cotransfected along with CMV promoter-driven expression constructs for TTF-1, HNF3α, HNF3β, and HFH-4 into Ishikawa cells lacking these transcription factors (see Fig. 1*B*). TTF-1 enhanced transcription from the rat uteroglobin/CC10 promoter up to 18-fold but had essentially no influence on the homologous human and rabbit gene promoters (Fig. 2). In contrast, both HNF3α and HNF3β stimulated transcription from all three homologous uteroglobin/CC10 promoters up to 8-fold. Cotransfection of an HFH-4 expression plasmid did not alter uteroglobin/CC10 promoter activities (Fig. 2). Thus, it appears that TTF-1 can activate the uteroglobin/CC10 promoter only in certain species. HNF3α and HNF3β, however, can stimulate uteroglobin/CC10 promoters from all species tested. These results suggest that the presence of HNF3α or HNF3β in respiratory epithelial cells is indispensable for the expression of uteroglobin/CC10 genes in mammals.

Identification of Rabbit Uteroglobin/CC10 Promoter Elements Responsible for Activation by HNF3α and HNF3β—To identify the promoter elements in the rabbit uteroglobin/CC10 promoter responsible for the activation by HNF3α and HNF3β, we cotransfected a series of 5′-promoter deletion-CAT constructs (7) along with the expression constructs for HNF3α and HNF3β in Ishikawa cells (Fig. 3). Promoter deletion mutants with 5′ end points at −395, −220, −205, −177, and −159 were all activated by HNF3α and HNF3β. Promoter mutants that contain only 96 or 35 nucleotides upstream of the transcription start site, however, were not stimulated by these two transcription factors. Thus, the DNA elements that are essential for stimulation by HNF3α and HNF3β lie within −159 bp and −96 bp upstream of the transcription start site.

Close inspection of this region revealed two A/T-rich sequence stretches (previously designated elements III and IV) (7) (Fig. 4), which coincide with the consensus sequence of HNF3α and HNF3β binding sites (VAWTRTTKRYTY) (39). Moreover, these A/T-rich sequences are conserved between the corresponding uteroglobin/CC10 promoter regions of human, rat, and mouse.

Consequently, we tested whether these two A/T-rich regions are responsible for activation of the promoter by HNF3α or HNF3β. For this purpose, linker-scanning mutants that destroy either the distal (LS(−127/−132)), the proximal (LS(−92/−99)), or both sequence elements (DLS(−92/−127)) were cotransfected along with HNF3α or HNF3β. Mutation of the proximal A/T-rich region (in LS(−92/−99)) reduced inducibility of the uteroglobin/CC10 promoter approximately 2-fold. Mutations in the linker-scanning mutant LS(−127/−132) and in the double mutant DLS(−92/−127) completely abolished the inducibility by HNF3α and HNF3β (Fig. 4). These results show that both A/T-rich elements are necessary for full promoter activation by HNF3α and HNF3β; however, the distal element appears to be more important.

HNF3α and Oct-1 Are the Nuclear Factors Binding to the A/T-rich Elements III and IV of the Uteroglobin/CC10 Gene in H441 Cells—The experiments described so far were performed with Ishikawa cells. In similar cotransfection experiments with H441 cells, we could not detect a significant activation of the rabbit CC10/uteroglobin promoter by HNF3α and HNF3β. This observation is likely due to the presence of high levels of endogenous HNF3α (Fig. 1; see below). However, transfection of the wild-type promoter and the linker-scanning mutants LS(−92/−99), LS(−127/−132), and DLS(−92/−127) revealed that both elements are necessary for full promoter activity in H441 cells (Fig. 5). Mutation of either the distal or proximal HNF3-responsive element (in LS(−127/−132) and LS(−92/−99)) reduced promoter activity 2–3-fold. Mutation of both elements (in DLS(−92/−127)) diminished promoter activity at least 5-fold.

We next asked whether the nuclear protein in H441 cells that binds to the HNF3-responsive elements is indeed HNF3α

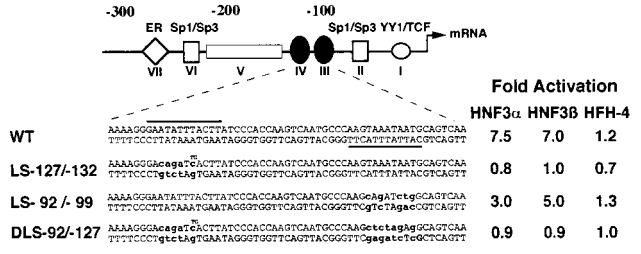

FIG. 4. **Elements III and IV of the rabbit CC10/uteroglobin promoter are the HNF3α/HNF3β-responsive promoter elements.** The rabbit uteroglobin/CC10 promoter structure and nucleotide sequences of the region containing elements III and IV in the wild-type promoter (*WT*) and the corresponding linker-scanning promoter mutants LS(−92/−99), LS(−127/−132), and DLS(−92/−127) are shown. Seven regions (*I–VII*) that contribute to the overall activity of the promoter have been identified previously (7). Identified transcription factors binding to these elements are the estrogen receptor (*ER*), the specificity proteins 1 and 3 (*Sp1/Sp3*), the transcription factor Yin Yang 1 (*YY1*), and the TACA box core factor (*TCF*) (9, 10). A total of 8 μg of reporter plasmids containing the rabbit uteroglobin/CC10 wild-type promoter construct or the linker-scanning mutants LS(−127/−132), LS(−92/−99), and DLS(−92/−127) were transfected into Ishikawa cells along with 2 μg of expression vectors for the transcription factors HNF3α, HNF3β, and HFH-4 as indicated. The cells were lysed subsequently, CAT activities determined as described under "Experimental Procedures," and the -fold activation was calculated.

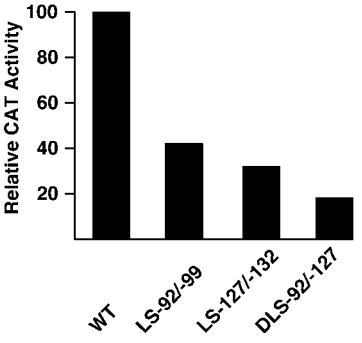

FIG. 5. **Elements III and IV of the rabbit CC10/uteroglobin promoter are essential for full promoter activity in H441 cells.** A total of 8 μg of CAT reporter plasmids containing the wild-type (*WT*) uteroglobin/CC10 promoter or the linker-scanning mutants LS(−92/−99), LS(−127/−132) and DLS(−92/−127), respectively, were transfected into H441 cells. The cells were lysed subsequently and CAT activities determined. The values represent the average of three independent transfections.

or whether additional transcription factors may recognize these elements. EMSA experiments were performed with crude nuclear extracts from H441 cells and oligonucleotides containing either the distal or the proximal HNF3-responsive element (oligonucleotides OEIV and OEIII in Fig. 6*A*). Two specific complexes, a strong fast migrating complex and a weak slow migrating complex, were observed when we used the OEIV oligonucleotide as probe (Fig. 6*B*). Essentially the same migration pattern was observed with the OEIII oligonucleotide (Fig. 6*C*). Both bands were specifically competed with oligonucleotides containing elements III or IV, but not with an unspecific oligonucleotide or an oligonucleotide that contained the same mutations as in the linker-scanning mutants (Fig. 6, *B* and *C*). Moreover, oligonucleotides containing the mutations present in the linker-scanning mutants LS(−127/−132) and LS(−92/−99) did not bind any nuclear protein from H441 cells (Fig. 6*D*). In the presence of an HNF3α antiserum, the strong fast migrating complex was shifted (Fig. 6*E*, *lane 4*). Antisera against HNF3β and HNF3γ did not affect migration of the complex (data not shown). Thus, the major transcription factor in H441 cells binding to elements IV and III is HNF3α.

Previous experiments showed that elements III and IV can also bind octamer transcription factors *in vitro*.[3] Therefore, we tested whether the slow migrating complex could be Oct-1. When an anti-Oct-1 serum raised against the DNA-binding domain was present in the binding reaction, the slow migrating complex disappeared, but the fast migrating complex was unaltered (Fig. 6*E*, *lane 6*). If antisera against HNF3α and Oct-1 were present in the binding reaction (Fig. 6*E*, *αH/O*, *lane 7*), both bands disappeared. In the presence of the Oct-1 antiserum, fast migrating complexes appeared, which probably reflect proteolytic degradation products of the Oct-1 protein bound by antibodies. Nevertheless, this experiment demonstrated that other factors distinct from HNF3α and Oct-1, which may bind to element III and IV, are not present in nuclear extracts from H441 cells, suggesting that HNF3α and/or Oct-1 are responsible for the transcriptional activity of both promoter elements in H441 cells.

An oligonucleotide spanning the uteroglobin/CC10 promoter sequence from −86 to −139 (OEIII/IV in Fig. 6*A*) and thus spanning both HNF3/Oct-1 sites was also used in EMSAs (Fig. 6, *F* and *G*). These experiments revealed that other transcription factors binding to this promoter region are not present in H441 cells. Notably, the HNF3α-containing complex obtained with the OEIII/IV oligonucleotide migrated much faster compared with the complexes obtained with the short OEIII or OEIV oligonucleotides (Fig. 6*G*, compare *lanes 1* and *8*). It is likely that this behavior is due to bending of the DNA by HNF3α (40) and might have mechanistic implications for promoter activation (see "Discussion").

HNF3α and HNF3β on Their Own Are Not Sufficient to Activate the Uteroglobin/CC10 Promoter—To further substantiate the conclusion that HNF3α and HNF3β or Oct-1, but not TTF-1, contribute to the level of transcription from the rabbit uteroglobin/CC10 promoter in lung, we performed gene transfer experiments into the *Drosophila melanogaster* Schneider cell line (SL2 cells) that lacks many mammalian transcription factor activities (Fig. 7). For these experiments, luciferase constructs instead of CAT reporter constructs that we generated in the course of our studies were used. The uteroglobin/CC10 promoter fused to the luciferase gene was cotransfected along with plasmids expressing Oct-1, TTF-1, HNF3α, or HNF3β, specifically designed for insect cells (pPacOct-1, pPacTTF-1, pPacHNF3α, and pPacHNF3β). Cotransfection of pPacOct-1 along with the uteroglobin/CC10-luciferase gene led to a 2–3-fold increase of luciferase activity. As expected, TTF-1 did not activate the uteroglobin/CC10 promoter, confirming the results of the cotransfection experiments obtained with mammalian cell lines. Surprisingly, HNF3α as well as HNF3β did also not stimulate transcription from the uteroglobin promoter (Fig. 7*A*). Control experiments showed, however, that all proteins were expressed in SL2 cells after transfection and that they specifically bind their appropriate promoter elements in electrophoretic mobility shift assays (Fig. 7*B*). This result suggested that HNF3α or HNF3β on their own are not sufficient to activate the uteroglobin/CC10 promoter. Additional transcription factors or coactivators not present in SL2 cells might be necessary for stimulation of the uteroglobin/CC10 promoter by HNF3α and HNF3β.

HNF3α and HNF3β Strongly Enhance Sp1-mediated Activation of the Rabbit Uteroglobin/CC10 Promoter—Previously, we have shown that Sp1 and Sp3 are also regulators of uteroglobin promoter activity (8). Thus, we asked next whether HNF3α, HNF3β, or TTF-1 may stimulate the uteroglobin/CC10 promoter in combination with these two ubiquitous transcription factors. Sp1 on its own activated the uteroglobin promoter in a

[3] H. Braun and G. Suske, unpublished results.

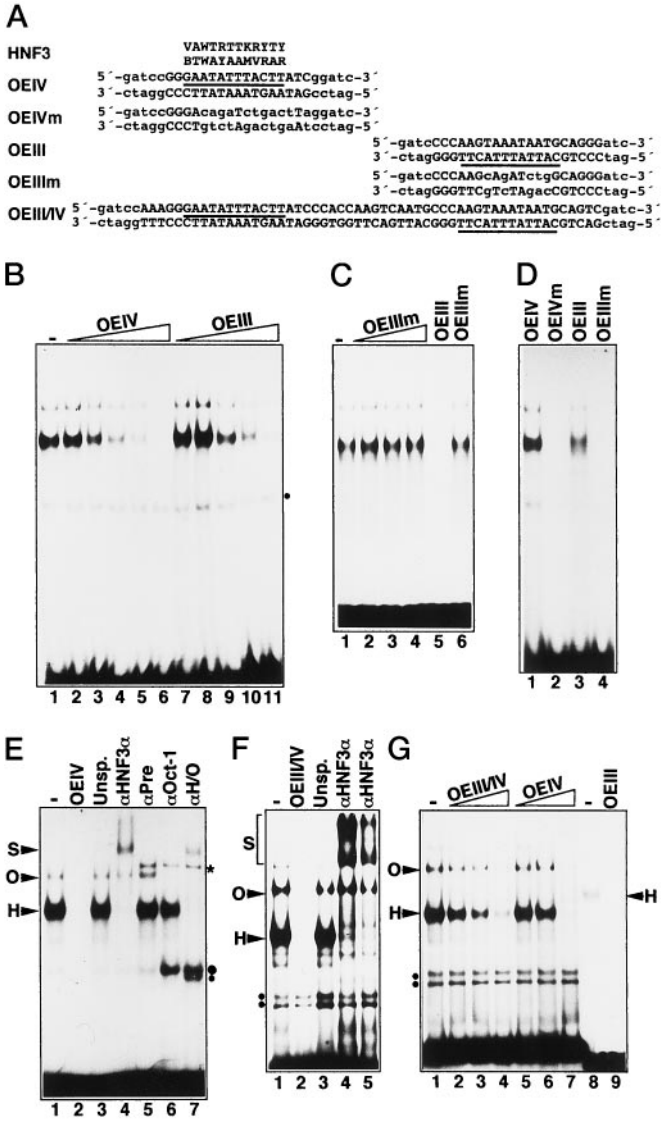

FIG. 6. **The nuclear factors in H441 cells binding to elements III and IV of the rabbit uteroglobin/CC10 promoter are HNF3α and Oct-1.** *A*, oligonucleotides used for the EMSAs. Sequences present in the wild-type uteroglobin promoter are written in *uppercase letters*. The HNF3 binding sites in the oligonucleotides OEIV, OEIII, and OEIII/IV are *underlined*. The oligonucleotides OEIVm and OEIIIm contain the same mutations as the linker-scanning mutants LS(−92/−99) and LS(−127/−132). The HNF3 consensus recognition sequence (39) is shown at the *top* (HNF3). *B*, elements IV and III bind the same nuclear factors with similar affinities. Crude nuclear extracts from H441 cells (3 μg of protein) were incubated with labeled OEIV oligonucleotide in the absence (*lane 1*) and presence of 1-fold (*lanes 2* and *7*), 3-fold (*lanes 3* and *8*), 10-fold (*lanes 4* and *9*), 30-fold (*lanes 5* and *10*), or 100-fold (*lanes 6* and *11*) molar excess of OEIV or OEIII oligonucleotide as indicated. The *dot* depicts an unspecific complex that could not be competed. *C*, mutated element III does not compete with the wild-type element III for nuclear factor binding. Labeled OEIII oligonucleotide was incubated with nuclear extracts from H441 cells in the absence (*lane 1*) and presence of 5-fold (*lane 2*), 20-fold (*lane 3*), 50-fold (*lane 4*), or 250-fold (*lane 6*) molar excess of OEIIIm oligonucleotide. In *lane 5*, competition was performed with a 50-fold molar excess of the wild-type OEIII oligonucleotide. *D*, mutated elements IV and III do not bind nuclear factors. Oligonucleotides OEIV, OEIVm, OEIII, and OEIIIm were labeled, and an EMSA was performed with nuclear extract from H441 cells as described above. *E*, identification of nuclear factors in H441 cells binding to elements III and IV. Nuclear extracts from H441 cells were incubated with labeled OEIV oligonucleotide in the absence (*lanes 1, 4, 5, 6*, and *7*) and presence of 50-fold molar excess of OEIV oligonucleotide (*lane 2*), or a nonspecific (*Unsp.*, *lane 3*) oligonucleotide. Serum (1 μl) against HNF3α (*αHNF3α*, *lane 4*), serum against Oct-1 (*αOct-1*, *lane 6*), the corresponding preimmune serum (*αPre*, *lane 5*), or a mixture of sera against HNF3α and Oct-1 (*αH/O*, *lane 7*) were included in the binding reactions. Specific complexes for HNF3α (*H*), Oct-1 (*O*), and the supershift obtained in the presence of αHNF3α (*S*) are indicated on the *left*. A nonspecific complex, which is also seen with the preimmune serum, is depicted by an *asterisk*. Two fast migrating complexes, which were generated by the Oct-1-specific serum, are indicated by *dots*. *F*, HNF3α and Oct-1 are the major proteins binding to the uteroglobin/CC10 promoter region −86 to −139. Nuclear extracts from H441 cells were incubated with labeled OEIII/IV oligonucleotide in the absence (*lanes 1*, *4*, and *5*) and presence of 50-fold molar excess of OEIII/IV oligonucleotide (*lane 2*) or a nonspecific (*Unsp.*, *lane 3*) oligonucleotide. A total of 1 μl (*lane 4*) or 2 μl (*lane 5*) of serum against HNF3α were included in the binding reactions. Specific complexes for HNF3α (*H*), Oct-1 (*O*), and the supershift obtained in the presence of αHNF3α (*S*) are indicated on the *left*. Two nonspecific complexes are indicated by *dots*. *G*, proteins binding to the uteroglobin/CC10 region −86 to −139 were competed by element OEIV. Labeled OEIII/IV oligonucleotide (*lanes 1–7*) was incubated with nuclear extracts from H441 cells in the absence (*lane 1*) and presence of 5-, 20-, or 50-fold unlabeled OEIII/IV oligonucleotide (*lanes 2*, *3*, and *4*, respectively) or 1-, 5-, and 50-fold molar excess of unlabeled OEIV oligonucleotide (*lanes 5*, *6*, and *7*, respectively). In *lanes 8* and *9*, the OEIII oligonucleotide was used as a probe in the absence (*lane 8*) or presence (*lane 9*) of 50-fold molar excess of cold OEIII oligonucleotide. Two nonspecific complexes are indicated by *dots*.

dose-dependent manner (13-fold in Fig. 7). Under these conditions, Sp3 also stimulated the uteroglobin/promoter to a certain extent (2.5-fold). TTF-1 had no effect on Sp-mediated activation, and Oct-1 in combination with Sp1 enhanced Sp1-mediated activation 2-fold. Most significantly, cotransfection of HNF3α or HNF3β along with Sp1 or Sp3 strongly increased uteroglobin/CC10 promoter activity (Fig. 7*A*). Thus, the presence of Sp1 or Sp3 appears to be essential for uteroglobin/CC10 promoter stimulation by HNF3 factor members. This conclusion was further substantiated by experiments in which variable amounts of Sp1 and HNF3α expression plasmids were transfected in the presence or absence of constant amounts of HNF3α or Sp1, respectively (Fig. 8). Increasing amounts of transfected Sp1 expression plasmid activated the uteroglobin/CC10 promoter in a dose-dependent manner (Fig. 8*A*), whereas HNF3α alone was inactive even at highest DNA levels (Fig. 8*B*). HNF3α stimulated Sp1-mediated activation under all conditions. Highest stimulation was obtained with 50 ng of Sp1-expression plasmid and 100 ng of HNF3α expression plasmid (up to 12-fold over the value obtained with Sp1 alone). Similar titration experiments were performed also with TTF-1 or Oct-1 and Sp1. These experiments revealed that TTF-1 does not at all influence Sp1-mediated activation whereas Oct-1 enhanced Sp1-mediated activation 2–3-fold (data not shown). Thus, Oct-1 and Sp1 act in an additive manner, whereas HNF3α and Sp1 strongly synergize with each other.

Next, we asked whether the enhancement of Sp1-mediated activation by HNF3α requires the above identified HNF3 binding sites of the promoter or whether protein-protein interactions may be sufficient for synergy. Linker-scanning mutants, which destroy either the distal, the proximal, or both HNF3-binding sites, were used to address this question (Fig. 9). Linker-scanning mutants that destroy either the proximal or distal HNF3 binding sites of the uteroglobin/CC10 promoter (LS(−92/−99) and LS(−127/−132)) still stimulated Sp1 activation although at lower levels. Mutation of both HNF3 binding sites (in (DLS(−92/−127)) further reduced the stimulatory effect of HNF3α. Nevertheless, HNF3α still enhanced Sp1-mediated activation up to 3-fold in the double mutant. At this stage, we cannot readily explain this HNF3α-dependent activity. An additional cryptic binding site for HNF3α present on the plasmid or, alternatively, DNA-binding independent HNF3α-Sp1 protein interactions may account for this residual activity.

Both Sp1-binding Sites Are Absolutely Necessary for HNF3α-mediated Activation of the Uteroglobin/CC10 Promoter—The

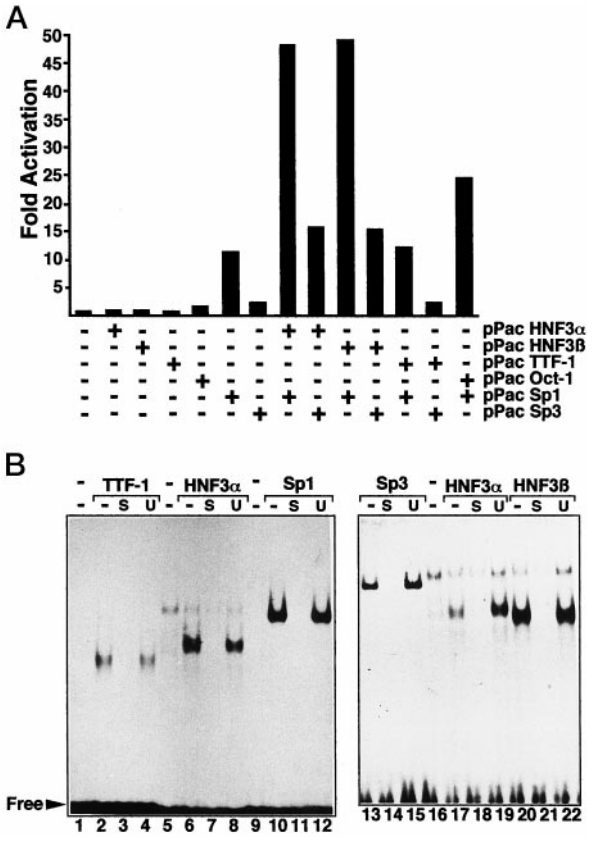

FIG. 7. **Activation of the uteroglobin/CC10 promoter by different transcription factors in SL2 cells.** *A*, 4 μg of a rabbit uteroglobin/CC10 promoter-luciferase reporter plasmid were transfected into SL2 cells plated on 6-cm plates along with 1 μg of pPacHNF3α, pPacHNF3β, pPacTTF-1, pPacOct-1, pPacSp1, or pPacSp3 as indicated. The cells were lysed subsequently, and luciferase activities determined. *B*, transient expression of TTF-1, HNF3α, HNF3β, Sp1 and Sp3 in SL2 cells. EMSAs were performed with crude nuclear extracts from SL2 cells on 6-cm plates transfected with 8 μg of expression plasmids for TTF-1 (*lanes 2–4*), HNF3α (*lanes 6–8* and *17–19*), HNF3β (*lanes 20–22*), Sp1 (*lanes 10–12*), Sp3 (*lanes 13–15*), or mock DNA (pPac plasmid) (*lanes 1*, *5*, *9*, and *16*). Reactions contained 2.5 μg of protein extract and 0.1 ng of labeled GT oligonucleotide (31) for Sp1 and Sp3, OEIII oligonucleotide (see Fig. 6*A*) for HNF3α and HNF3β and an oligonucleotide containing the proximal TTF-1 binding site of the thyroglobulin promoter (oligonucleotide C in Ref. 47). In *lanes S* and *U*, a 50-fold molar excess of unlabeled specific (*S*) or nonspecific (*U*) oligonucleotide, respectively, was included in the binding reaction.

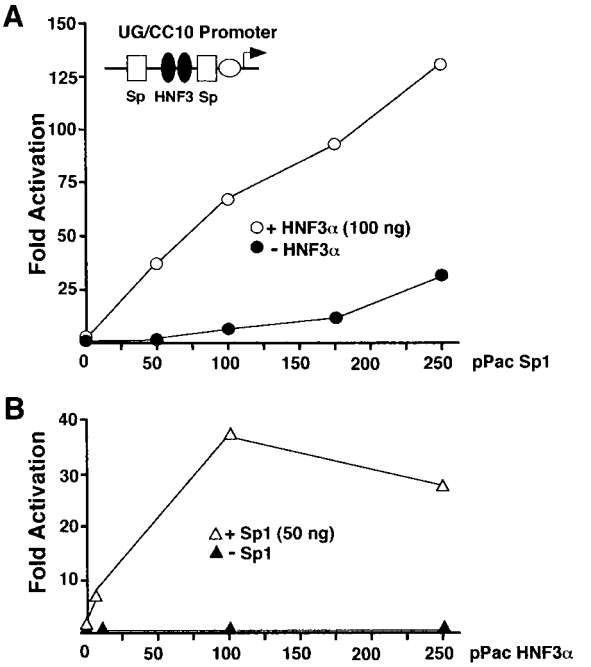

FIG. 8. **HNF3α strongly enhances Sp1-mediated activation of the uteroglobin/CC10 promoter.** *A*, 1 μg of the UG/CC10 promoter-luciferase reporter plasmid was transfected in SL2 cells plated on six-well microtiter plates along with variable amounts of pPacSp1 (0, 50, 100, 175, and 250 ng) in the absence (*black circles*) or presence (*open circles*) of 100 ng of pPacHNF3α. The cells were lysed subsequently and luciferase activities determined. The values represent the average of five independent determinations. *B*, SL2 cells were transfected with the UG/CC10 promoter-luciferase reporter construct along with variable amounts of pPacHNF3α (0, 10, 100, and 250 ng) in the absence (*black triangles*) or presence (*open triangles*) of 50 ng of pPacSp1.

uteroglobin/CC10 promoter contains two non-GC box binding sites for Sp1 located 65 and 230 bp upstream of the transcriptional start site (8). We wanted to know how these sites contribute individually to HNF3α stimulation. Linker-scanning mutants in which either a single (LS(−64/−72) and LS(−222/−229)) or both Sp1 binding sites (DLS(−64/−222)) are mutated were cotransfected in the presence or absence of HNF3α (Fig. 9). This series of experiments revealed that enhancement of Sp1-mediated activation by HNF3α is dependent on the integrity of both Sp1-binding sites of the uteroglobin/CC10 promoter. Mutation of either the distal (LS(−222/−229)) or the proximal binding site (LS(−64/−72)) reduced synergistic activation by HNF3α and Sp1 close to background levels (Fig. 9). Mutation of both Sp-binding sites (LS(−64/−222)) abolished activation completely. Thus, it appears that each Sp1-binding site flanking the HNF3-binding sites contributes similarly to HNF3α-mediated enhancement of activation. The essential contribution of the distal Sp1-binding site for HNF3α activation in addition to the proximal Sp1 site indicates that Sp1 does not act simply as a bridging factor between HNF3α and the basal transcription machinery. Additional mechanisms must be at work in the combinatorial activation of the rabbit uteroglobin/CC10 promoter by HNF3α and Sp1. It should be noted that this result is compatible with experiments performed previously with H441 cells (7). In H441 cells, the two linker-scanning mutants LS(−222/−229) and LS(−64/−72) reduced promoter activity 10- and 15-fold, respectively.

Multiple Activation Domains of Sp1 Are Required for Cooperativity with HNF3α—Sp1 contains a zinc finger DNA binding domain and four activation domains designated A, B, C, and D (28, 29). To identify those activation domains of Sp1 that are essential for cooperativity with HNF3α, we tested various Sp1 deletion derivatives (Fig. 10). All Sp1 mutants were poor activators of the rabbit/CC10 promoter, indicating that all four activation domains of Sp1 are involved in activation of the uteroglobin/CC10 promoter. Surprisingly, the activity of none of the mutants could be enhanced by HNF3α. This result suggests that several activation domains of Sp1 including the glutamine-rich activation domain A and B, the highly charged domain C, and the most C-terminal activation domain D are involved in transcriptional synergy with HNF3α.

DISCUSSION

Human Uteroglobin/CC10 Is Expressed in Some Lung Epithelial Cell Lines—H441 cells have been widely used to study lung epithelium-specific promoters from different species. This cell line was derived from a human adenocarcinoma with morphological characteristics of Clara cells lining the bronchiolar epithelium. Here, we show for the first time that this cell line as well as H60 and H69 cells express the Clara cell-specific marker gene uteroglobin/CC10. This finding was surprising because, in two previous reports, human CC10 could not be detected in H441 cells (24, 38). H441 cells express also surfactant protein A and B mRNAs. However, it should be noted that

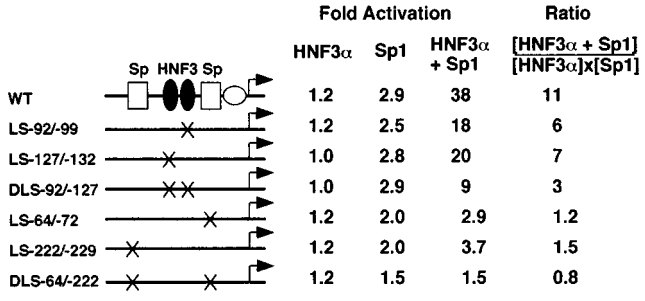

FIG. 9. **The integrity of both HNF3- and Sp-binding sites is essential for full activation by HNF3α and Sp1.** The wild-type uteroglobin/CC10 promoter-luciferase construct (*WT*) and appropriate linker-scanning mutants in which the binding sites for HNF3 (LS(−92/−99), LS(−127/−132), and DLS(−92/−127)) or Sp1 (LS(−64/−72), LS(−222/−229), and DLS(−64/−222)) are mutated are shown schematically. Reporter plasmid (1 μg) was transfected in SL2 cells along with 100 ng of pPacHNF3α (*HNF3α*), 50 ng of pPacSp1 (*Sp1*), or both expression plasmids (100 ng and 50 ng, respectively; *HNF3α + Sp1*). The values (-fold activation) represent the average of at least three independent determinations. The ratio of the activation by HNF3α plus Sp1 *versus* the total of HNF3α and Sp1 on their own is shown on the *right* (*Ratio*).

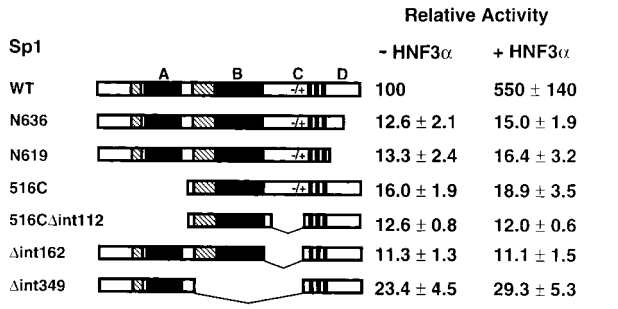

FIG. 10. **Multiple activation domains of Sp1 are required for cooperativity with HNF3α.** Sp1 and the deletion derivatives are shown schematically. The activation domains are labeled above the wild-type (*WT*) construct (*A–D*) according to Refs. 28 and 29. The *hatched* and *shaded boxes* represent serine/threonine-rich and glutamine-rich regions of the protein, respectively. The three zinc fingers are indicated as *black bars*. SL2 cells were transfected with 1 μg of uteroglobin/CC10 luciferase reporter plasmid along with 200 ng of expression plasmids for Sp1 mutants in the absence or presence of 100 ng of HNF3α expression plasmid as indicated. The cells were subsequently lysed and assayed for luciferase activities. The values represent mean values of at least three independent transfection experiments and are expressed relative to wild-type Sp1, which has been given the arbitrary value of 100.

we estimate the expression levels of all three mRNAs in these cells to be 3 orders of magnitude lower than *in vivo*. In addition, H441 cells express the transcription factors TTF-1 and HNF3α, which are involved in regulation of lung epithelium-specific genes (for a review, see Ref. 41). Thus, this cell line reflects in many aspects an *in vivo* situation.

Differential Activation of Uteroglobin/CC10 Promoters by TTF-1, HNF3α, and HNF3β—An essential role of TTF-1 for the activation of the rat and mouse uteroglobin/CC10 promoter regions, which contain several binding sites for TTF-1, has been reported (16). Our experiments confirmed this conclusion since we found a strong activation of the rat uteroglobin/CC10 promoter by TTF-1. However, neither the homologous rabbit nor the human uteroglobin/CC10 promoters were activated by TTF-1 although purified TTF-1 specifically recognized two sites of the rabbit/uteroglobin promoter (around −85 and −1) in DNase I protection experiments (data not shown). This result suggests species-specific differences in the role of TTF-1 for uteroglobin/CC10 transcription in lung. However, we cannot exclude that potential TTF-1 binding sites located far upstream of the uteroglobin/CC10 5′-flanking region could confer activation of the gene by TTF-1. In contrast to TTF-1, both HNF3α and HNF3β, but not HFH-4 could activate all three uteroglobin/CC10 promoters we have tested.

In mice, HNF3β is expressed in airway epithelial cells at the onset of lung morphogenesis (gestational day 10) and throughout fetal lung development (19). HNF3α and HFH-4 are coexpressed in adult lung in the bronchiolar epithelium. Uteroglobin/CC10 mRNA expression in embryonic lung starts at gestational day 17 (42). Thus, HNF3β and HNF3α expression in lung precedes and overlaps, respectively, expression of the uteroglobin/CC10 gene. This finding supports the idea that HNF3β and HNF3α are both directly involved in uteroglobin/CC10 gene expression in fetal and adult lung, respectively. It should be noted that the originally described expression of HNF3β mRNA in smooth muscle of adult lung (18) turned out to be an artifact.[4]

Two promoter elements of the rabbit uteroglobin/CC10 promoter that are responsible for activation by HNF3α and HNF3β have been mapped to two A/T-rich regions located around −95 and −130. Both regions contain consensus recognition sequences for HNF3 family members (39). In addition, both sites are necessary for full activation by HNF3α and HNF3β. Moreover, both sites contribute to the promoter activity in H441 cells which contain high levels of HNF3α protein. Alignment of the homologous rat, mouse, and human promoters shows that these sites are conserved between the different species. For the rat promoter, it has been demonstrated that both HNF3α and HNF3β can bind these sites (21).

Our finding that both factors, HNF3α and HNF3β, activated the uteroglobin/CC10 promoters from rabbit, rat, and man contradicts a previous report in which HNF3α and HNF3β mediated opposite responses on the rat uteroglobin/CC10 promoter (22). HNF3α stimulated and HNF3β inhibited rat uteroglobin/CC10 promoter activity. However, yet another group also reported activation of the rat CC10 promoter by HNF3β (20). A possible explanation for these discrepancies could be the usage of different constructs and cell lines. For instance, in the report where HNF3β had an inhibitory effect, an artificial construct containing multiple synthetic binding sites for HNF3 fused to a rat minimal promoter was used instead of the natural promoter (22).

HNF3α Synergizes with Members of the Sp Family of Transcription Factors—We found that HNF3α and HNF3β on their own cannot activate transcription from the uteroglobin/CC10 promoter in an insect cell line that lacks many mammalian transcription factor activities. However, both factors, HNF3α and HNF3β, strongly enhanced Sp1-mediated activation of the uteroglobin/CC10 promoter. Thus, it appears that HNF3α and Sp1 act in a synergistic manner. Interestingly, the surfactant protein B promoter also contains binding sites for Sp1/Sp3 adjacent to an HNF3 binding site. Moreover, both sites are necessary for lung specific activation of SP B gene transcription (15). Thus, the combinatorial action of Sp family transcription factors and the lung epithelial-specific transcription factors HNF3α and HNF3β might be a common theme in lung epithelial gene expression.

The synergistic activation of the rabbit uteroglobin/CC10 promoter by Sp1 and HNF3α resembles to some extent the synergistic activation of the low density lipoprotein (LDL) receptor promoter by the sterol receptor element-binding protein (SREBP) and Sp1 (43). The LDL receptor promoter contains binding sites for SREBP flanked by Sp1-binding sites. SREBP on its own cannot activate transcription from the LDL receptor

[4] R. H. Costa, personal communication.

promoter, but strongly enhances Sp1-mediated activation (43, 44). The domain of Sp1 that is responsible for synergistic activation with SREBP and very likely for a direct protein-protein interaction has been mapped to the highly charged Sp1 C-domain (44) located between the second glutamine-rich activation domain and the DNA-binding domain. Domain C of Sp1 is also required for cooperativity with HNF3α. However, the glutamine-rich domains of Sp1 and the most C-terminal D domain are also absolutely necessary to confer synergism with HNF3α. Thus, a different mechanism of synergistic activation by HNF3α and Sp1 may be at work.

How Might HNF3α Cooperate with Sp1 Mechanistically?—Several mechanisms underlying the synergistic activation of HNF3α and Sp1 have to be considered. One possibility would be that HNF3α on its own is transcriptionally inactive on the uteroglobin/CC10 promoter in the absence, but active in the presence of Sp1. In such a model, Sp1 bound to its proximal site could function as a bridging factor between HNF3α and the basal transcriptional machinery. Since HNF3α does have activation domains (45), a direct interaction of HNF3α and components of the basal transcription machinery seems possible. However, such a model would not explain why the distal Sp1 site is also essential for synergistic activation by HNF3α and Sp1.

Another possibility would be that HNF3α stimulates binding of Sp1 to its adjacent recognition site. We have performed EMSAs under various conditions with an oligonucleotide containing the proximal HNF3 and Sp1 sites of the uteroglobin/CC10 promoter and could not detect any differences in the binding of Sp1 in the presence or absence of recombinant HNF3α.[3] Thus, cooperative binding of HNF3α and Sp1 to adjacent sites seems not to account for synergistic activation.

Models that explain synergistic activation of the uteroglobin/CC10 promoter should consider that both Sp1-binding sites, one lying upstream (at −225) and the other downstream (at −65) of the HNF3 sites, are equally necessary to mediate HNF3α stimulation. Consistent with the crystal structure of the HNF3 DNA recognition domain bound to DNA (40), we have observed that binding of HNF3α to its sites on the uteroglobin/CC10 promoter at −95 or −130 bent the DNA. Thus, one could speculate that upon binding of HNF3 proteins the upstream and downstream Sp1 sites come in close proximity that allow protein-protein interactions of two Sp1 molecules, which in turn would lead to strong interactions with the basal transcription machinery. Such a model would be compatible with the finding that two Sp1 sites in close proximity activate transcription synergistically (29). Interaction analyses of HNF3α and Sp1 on the full-length promoter will be necessary to test such an hypothesis.

Directed DNA writhing to bring the two Sp1 sites together could also be achieved by a nucleosomal core, where DNA wraps around a histone octamer. Thus, another intriguing possibility could be that two Sp1 molecules bound to their sites at −225 and −65 are brought into juxtaposition by a nucleosome. In that context, it should be noted that the DNA-binding domain of HNF3 is highly similar to that of histone H5 (40), a variant of histone H1. Thus, one could speculate that HNF3 could bind to nucleosomal organized DNA and in turn position or alter nucleosomal structures. Remarkably, positioning of a nucleosome on the albumin enhancer is liver-specific and dependent on two adjacent HNF3 binding sites. Based on these findings, it has been suggested that HNF3 proteins play an active role in nucleosomal positioning on the albumin enhancer (46). Since we have not analyzed the nucleosomal structure of the uteroglobin/CC10 promoter in lung, thus far, a potential involvement of nucleosomal structures in the regulation of the uteroglobin/CC10 promoter by HNF3α and Sp1 remains a matter of speculation.

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