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Genomic structure and promoter characterization of the human *Sprouty4* gene, a novel regulator of lung morphogenesis

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Ding, Wei, Saverio Bellusci, Wei Shi, and David Warburton. Genomic structure and promoter characterization of the human *Sprouty4* gene, a novel regulator of lung morphogenesis. *Am J Physiol Lung Cell Mol Physiol* 287: L52–L59, 2004. First published February 20, 2004; 10.1152/ajplung.00430.2003.—The expression of *Sprouty4* (*Spry4*), an intracellular FGF receptor antagonist, shows a temporally and spatially restricted pattern in embryonic lung and is induced by ERK signaling. To clarify the molecular mechanisms regulating *Spry4* transcription, the genomic structure of the human *Sprouty4* (*hSpry4*) gene was first determined by using the GenomeWalker kit. The *hSpry4* gene spans > 14 kb and is organized in three exons and two introns. Multiple transcription start sites were subsequently mapped by 5′-rapid amplification of cDNA ends. Analysis of up to 4 kb of sequence in the 5′-flanking region of the gene showed the presence of multiple potential transcription factor binding sites but no TATA or CAAT boxes. Transient transfection using luciferase reporter gene constructs with progressive deletions of the *hSpry4* 5′-flanking region revealed that the core promoter activity is located within the proximal 0.4-kb region, whereas the minimal ERK-inducible promoter activity is between −69 and −31. Homology analysis further showed that the core promoter region of the *hSpry4* gene exhibits significant similarity to the 5′-flanking region of the mouse gene.

lung development; transcription initiation site; homology analysis; reporter gene assay; transcription factor binding site

NORMAL FORMATION of three-dimensional tubular networks like the insect tracheal system and the mammalian lung requires a delicate balance between positive and negative regulators involved in cell growth, migration, and differentiation (41). Because fibroblast growth factor (FGF) receptors and their ligands function as critical positive signals in this phase of embryonic development, their strength and duration must be strictly modulated by negative mechanisms (1, 32). Recently, Sprouty (Spry) was identified by genetic studies as such an inhibitor (10, 39).

Spry was originally described as an antagonist of the Breathless FGF receptor during tracheal development in *Drosophila*, since loss of function mutations of *Drosophila Spry* (*dSpry*) led to overactive FGF signaling and excessive tracheal branching (10). Subsequent studies have shown that dSpry inhibits signaling mediated not only by the FGF receptor but also by several other receptor tyrosine kinases (RTK), such as epidermal growth factor receptor, Sevenless, and Torso (3, 15, 29); thus it may function as a general inhibitor of RTK signal transduction.

Although *Drosophila* has only one *Spry* gene, at least four *Spry* homologs (*Spry1–4*) have been cloned in the human as well as mouse (6, 10, 22). The various Spry isoforms have divergent NH_2-terminal sequences but share a well-conserved cysteine-rich domain in their COOH termini. This shared domain has been shown to be sufficient for inducible translocation of Spry proteins to the plasma membrane (19).

Among these four isoforms, the human *Sprouty4* (*hSpry4*) gene has been mapped to human *chromosome 5* and mouse *Sprouty4* (*mSpry4*) to the central region of *chromosome 18*, respectively (22). Comparison of the mouse and human *Spry4* sequences demonstrates 88% homology at the protein level and 87% at the DNA level, indicating that the *Spry4* gene has been highly conserved during vertebrate evolution (17).

By Northern analysis, the ERK pathway has been shown to upregulate the expression of *Spry4* (24). Subsequent studies further demonstrate that *Spry4* is expressed in highly restricted patterns during mouse and chick embryonic lung development (4, 20). One important aspect of these expression patterns is that *Spry4* expression is colocalized and induced upon FGF signaling, which suggests that, like its insect ortholog, the mammalian Spry4 may also serve as an FGF feedback inhibitor in normal respiratory organogenesis. In fact, ectopic expression of *Spry4* in mouse fetal lung epithelium inhibits branching morphogenesis and results in severe pulmonary hypoplasia (26). Moreover, the antagonistic action of Spry4 on FGF-mediated lung formation seems to be temporally restricted, since the inhibitory effects of Spry4 on lung branching can be substantially reversed by cessation of *Spry4* expression before, but not after, embryonic *day 13.5* (26). Similarly, selective overexpression of *Spry4* in the developing vascular endothelium of the 9.0-day mouse embryo resulted in the repression of angiogenesis, another form of branching morphogenesis (16). Therefore, a full understanding of the functional role that Spry4 plays during embryonic development must include knowledge of regulatory mechanisms governing the temporal and spatial expression of *Spry4*. Thus we initiated the present work, which has resulted in cloning and characterization of the promoter of the *hSpry4* gene.

MATERIALS AND METHODS

Cell culture. Human A549 (lung carcinoma) epithelial cells, human fetal lung WI-38 VA-13 fibroblasts (SV40 virus-transformed derivative of WI-38), and human HeLa (cervix carcinoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supple-

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mented with 10% fetal calf serum (FCS). WI-38 VA-13 cells were obtained from American Type Culture Collection (Manassas, VA).

Determination of genomic organization of hSpry4. Genomic sequences that could not be determined by comparison with known sequences were obtained using the GenomeWalker kit (Clontech), following the protocol recommended by the manufacturer. Briefly, this kit contains four human genomic DNA libraries, each containing 5′-"adaptor"-ligated products from digestion of human genomic DNA with one of four restriction enzymes (*Eco*RV, *Dra*I, *Pvu*II, or *Ssp*I). Sequences of interest were obtained by two rounds of PCR amplification per library. The first PCR amplification used the outer adaptor primer (AP1) provided in the kit and an outer, gene-specific primer (GSP). "Nested" GSP and nested adaptor primer (AP2) were used in sequential PCR to amplify genomic DNA within the region of interest. All GSP sequences were based on the sequence of the previously published *hSpry4* cDNA and the sequence of a ~1,200-bp fragment obtained after the initial two-round PCR with GSP1 and GSP2 (see below).

GSP1 5′-GAAGGGGCTGGACCATGACTGAGTTG-3′, GSP2 5′-CCTGGACTGTACGGAGAAACAGGCTTCTAG-3′, GSP3 5′-ATCATGAAGTCAATGACTTAATGCATATGGTGC-3′, GSP4 5′-GGTGCTTAGGAGAGTACCTCCATCTTAAGTGC-3′, GSP5 5′-TGTGCTCAGCCCAGACTCTGGTCAACTA-3′, GSP6 5′-CTACCACTGCACGAATGAGGACGATGAG-3′, GSP7 5′-TGTTCAAGAACATCCATACATCCGTCACA-3′, GSP8 5′-CAACTGTGGCAAGGGTAGCTACACGAATC-3′.

For the primary PCR (AP1 and outer GSP as primers), there were seven cycles of 25 s at 94°C and 4 min at 72°C, followed by 32 cycles of 25 s at 94°C and 4 min at 67°C, with a final extension at 67°C for an additional 4 min. The primary PCR products were diluted 1:50, and 1 μl was used for the secondary amplification with primers AP2 and inner GSP. Cycling parameters in the secondary PCR were the same as those used during the primary PCR except that the first and second steps consisted of five and 22 cycles, respectively.

PCR products were TA cloned and subsequently sequenced.

Rapid amplification of cDNA ends. Transcription start sites were identified by 5′-rapid amplification of cDNA ends (RACE) following the protocol from the GeneRacer kit (Invitrogen). Human fetal lung poly(A)$^+$ RNA was purchased from Clontech (Palo Alto, CA). Human fetal lung poly(A)$^+$ RNA (250 ng) was first treated with calf intestinal phosphatase to dephosphorylate non-mRNA and truncated mRNA. Thereafter, the 5′-cap structure from full-length mRNA was removed by incubation with tobacco acid pyrophosphatase at 37°C for 1 h. This treatment left a 5′-phosphate on the decapped, full-length mRNA, so it was then ligated to 0.25 μg of GeneRacer RNA Oligo (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) at 37°C for 1 h in a solution containing 5 units of T4 RNA ligase. Next, reverse transcription was carried out at 42°C for 50 min using 200 units of SuperScript II reverse transcriptase to create RACE-ready cDNA. Subsequently, the *hSpry4*-specific primer Y4RACE (5′-CTTCACCTGGTCAATGGGTAGGATGGTGAGT-3′) and GeneRacer 5′-primer (5′-CGACTGGAGCACGAGGACACTGA-3′) were used in the primary PCR reaction in combination with the Advantage-GC Genomic Polymerase Mix (Clontech). The PCR conditions consisted of one cycle of 2 min at 95°C, followed by 45 cycles of 30 s at 95°C, 1 min at 65°C, and 2 min at 72°C. A single band of ~400 bp was obtained after amplification and cloned into the pCR-Blunt II-TOPO vector (Invitrogen). Twenty-five clones were picked for sequencing analysis.

Construction of reporter plasmids for promoter analysis. Progressive deletion of the *hSpry4* 5′-flanking region was accomplished by unidirectional cloning of PCR fragments into the *Kpn*I/*Nhe*I site of the promoterless and enhancerless firefly luciferase reporter vector pGL$_2$-Basic (Promega). These PCR fragments were generated using a common reverse primer and 13 different forward primers. The *hSpry4*-specific sequences for these primers were taken from the *Homo sapiens chromosome 5* working draft sequence segment NT_006447.6 (in uppercase, see below). All these forward primers were preceded by an arbitrary sequence including *Kpn*I restriction site (in lowercase, see below), whereas the reverse primer was designed to include *Nhe*I site at its 5′-end. The numbers indicated after the primer sequences correspond to the distance in nucleotides from the 5-end of the sequence in uppercase to the 5′-most transcription start site determined by 5′-RACE.

Forward (Fwd) 1 5′-ggtaccGAAACAGCTGAAACGCTGAACCGGA-3′ (−4,446), Fwd2 5′-ggtaccAAAAACAAATCCAGCACAGACCTACCGG-3′ (−3,058), Fwd3 5′-ggtaccCCCAGACTCTGCTCTTATCCACGCATC-3′ (−1,366), Fwd4 5′-ggtaccAACGCCACCATCATTAACAGCG-3′ (−1,182), Fwd5 5′-ggtaccGAAAGGCGCCTACAACAGCGAAGG-3′ (−979), Fwd6 5′-ggtaccTCTGGCTGAGTTGCCAGAATCG-3′ (−774), Fwd7 5′-ggtaccAATCCTCACCCCACCCTTTGAT-3′ (−625), Fwd8 5′-ggtaccGGTGGCCTTTACCTCCTCTCGCAG-3′ (−342), Fwd9 5′-ggtaccCGCCGCCGCCCTCCATTCATTAAA-3′ (−273), Fwd10 5′-ggtaccACCCCTCTTTGTAAAAGAACGACGGC (−208), Fwd11 5′-ggtaccGGAGCTTGAGCCAGTCCCAGCAAT (−174), Fwd12 5′-ggtaccCATTCATAGAATCGCGAACGGCAC (−69), Fwd13 5′-ggtaccCGCTCCCTTCCCTCCCATTCATAA (−31), reverse 5′-gctagcCTGTCAGCTCAGCTCGCTACCTCCG-3′ (+56).

We used the Advantage Genomic PCR kit (Clontech) to amplify these promoter fragments. The reaction conditions were chosen according to the manufacturer's recommendations. The human genomic DNA was used as template. The PCR products were first cloned into a TA cloning vector (Invitrogen) and then excised by *Kpn*I + *Nhe*I digestion and subcloned into the pGL$_2$-Basic vector.

The deletion construct pGL(−418/+56) was generated by digestion of the existing reporter plasmids with the restriction enzyme *Sma*I, followed by self-ligation, since this restriction site is naturally present at position −418 in the *hSpry4* promoter region.

Every construct was sequenced from both ends to ensure the correct orientation and fidelity.

Transient transfection. A549, WI-38, and HeLa cells were seeded on six-well tissue culture plates in DMEM with 10% FCS and grown for 16–24 h to 80% confluence. Next, cells were transiently transfected with the individual luciferase reporter plasmid by using LipofectAMINE (Invitrogen) following the manufacturer's instructions. The pSV-β-galactosidase control vector (Promega) was cotransfected with various luciferase reporter plasmids into cells to normalize the variations in transfection efficiency.

Each plasmid was transfected in triplicate in at least three independent experiments.

Reporter gene luciferase assay. Cells were lysed 48 h posttransfection by one freeze-and-thaw cycle in reporter lysis buffer containing 25 mM Tris-phosphate, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2 mM DTT, 10% glycerol, and 1% Triton X-100, pH 7.8. The lysates were centrifuged at 12,000 *g* for 2 min to remove cell debris. The supernatant was used for both luciferase and β-galactosidase activity assays. Luciferase activity was determined by using a luciferase assay system (Promega) according to the manufacturer's protocol and measured in a luminometer. The β-galactosidase activity was assayed using the β-galactosidase enzyme assay system (Promega) following the manufacturer's instructions. We normalized variations in transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The promoterless pGL$_2$-Basic vector was used as negative control, and the pGL$_2$-control vector (which has SV40 promoter and enhancer to drive the luciferase gene) was used as positive control for each transfection assay.

RESULTS

Genomic organization of the hSpry4 gene. Elucidating possible mechanisms governing transcription of the *hSpry4* gene requires knowledge of its genomic organization. We therefore

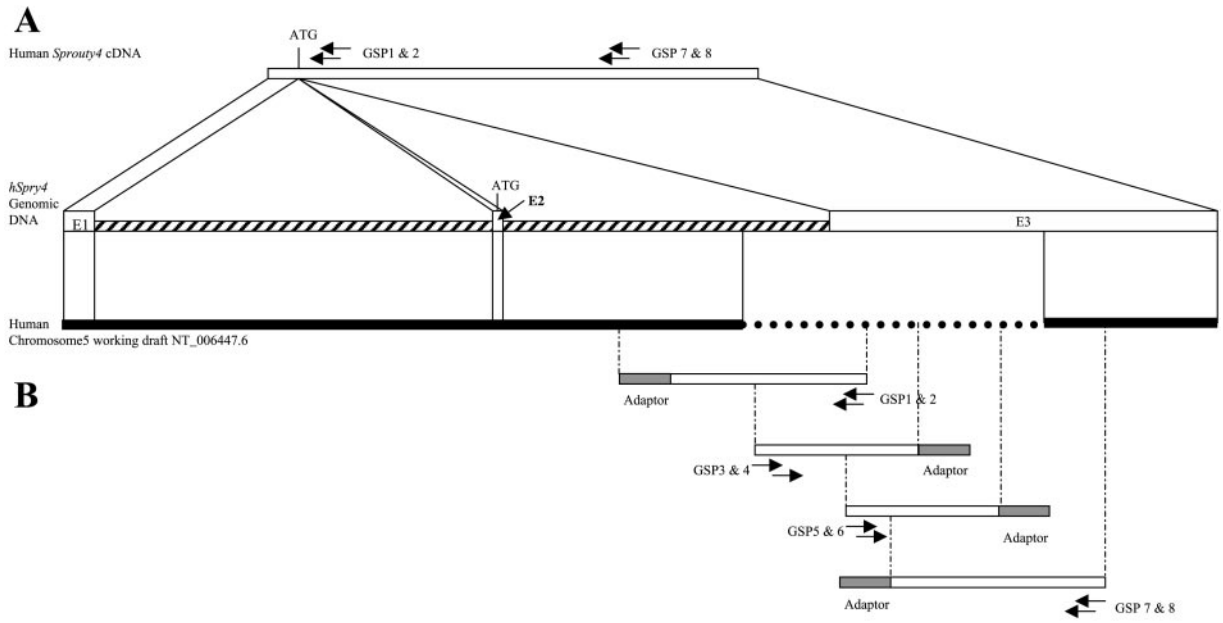

Fig. 1. Genomic organization of the human *Sprouty4* (*hSpry4*) gene. *A*: genomic organization of the *hSpry4* gene was deduced based on a comparison of the published *hSpry4* cDNA sequence and the working draft sequence of human *chromosome 5* (NT_006447.6). At the genomic DNA level, exons are shown as open bars, and introns are shown as hatched bars. Two regions, which are indicated by thick lines, showed complete matches between sequences from NT_006447.6 and *hSpry4* cDNA. Because the contig sequence of human *chromosome 5* is incomplete, a gap, represented by the dashed line, is present between two matching segments. *B*: schematic diagram of the strategy and positions of PCR primers used to clone the missing region in the genomic sequences. Nested PCR amplification reactions using adaptor primers (represented by gray bars) and gene-specific primers (GSP, represented by arrows) were conducted on the human GenomeWalker library from Clontech.

used the 4,934-bp published cDNA sequence as virtual probes to perform a basic local alignment search tool comparison. Upon searching the Human Genomic Sequence database at the National Center for Biotechnology Information (NCBI, Bethesda, MD), we found that the 5′-end 210-bp and 3′-end 1,898-bp cDNA sequence showed perfect matches with two discontinuous segments of the *H. sapiens chromosome 5* working draft sequence (NT_006447.6). This enabled us to deduce the *exon 1*, *intron 1*, *exon 2*, and a section of *intron 2*. However, since the genomic sequence was unfinished at that time, there was a gap between those two genomic segments, and a large fragment of *hSpry4* cDNA could not be found in the genomic sequence database. Therefore, we set out to clone the missing genomic region using the GenomeWalker kit from Clontech. The overall strategy and relevant experimental results are shown in Fig. 1. In the initial nested PCR, antisense gene-specific primers (GSP1 and GSP2) directed to the 5′-region of unmatched *hSpry4* cDNA were used to walk upstream. This method generated a ~1,200-bp fragment that was cloned and sequenced. Comparing this sequence to the above genomic database, which also exactly overlapped with NT_006447.6, we were able to identify the full *intron 2*. Subsequently, using sequences from *intron 2* and *hSpry4* cDNA, we designed additional primers. These were used to again amplify downstream and upstream sequences in the genomic libraries provided with the kit. On the basis of alignment results of all these amplified DNA sequences, the genomic structure of *hSpry4* was determined and is shown in Fig. 1. The *hSpry4* genomic sequences were assembled into a ~14-kb organizational unit that contains three exons and two introns. *Exon 1* and the first 29-bp of *exon 2* make up the entire 5′-untranslated region (5′-UTR), whereas *exon 2* also contains the beginning of the coding region to the amino acid *residue 7*. *Exon 3* encodes the remaining 947 bp of the open-reading frame for hSpry4 protein and the whole 3′-UTR (3769 bp). Sequences for intron-exon splice junctions (Table 1) match with the "GT/AG" rule (35).

The whole nucleotide sequence of the *hSpry4* gene has been deposited in the NCBI GenBank with an accession number of AY538661. Subsequently, in the process of preparing this manuscript, we searched the more updated version of human genome sequence database and found out that the nucleotide sequence we had cloned perfectly matched with a portion of the updated human *chromosome 5* contig (NT_029289). This

Table 1. *Exon-intron boundaries of the hSpry4 gene*

Exon	Exon Size, bp	Exon-Intron Boundary Sequence	Intron	Intron Size, bp
1	158	CTCCTCAAAgtaagtcca.....tcttttcagCCAGCCTGT	1	5,049
2	51	TCCCCACAGgtacaaatg.....cattctcagGGCCCCTAG	2	4,563
3	4,716			

All sequences for splice junctions reveal no deviation from the consensus 5′-donor and 3′-acceptor sites and follow the GT/AG rule. Consensus splice sites are underlined. Exon and intron sequences are shown in uppercase and lowercase letters, respectively. *hSpry4*, human *Sprouty4*.

confirmed our genomic sequence and structure proposed in Fig. 1.

Identification of the transcription start sites of hSpry4. We used 5′-RACE to localize the position of the *hSpry4* transcription start site(s). 5′-RACE was performed using poly(A)$^+$ RNA from fetal human lungs and an *hSpry4*-specific primer (designed to be a complement to the region from *position 358* to *388* of *hSpry4* cDNA). A strong single band of ~400 bp was obtained after amplification. This 5′-RACE product was subsequently subcloned and sequenced. From a total of 25 isolates, 24 clones contained sequences homologous to *hSpry4*. Four different end points were detected, suggesting the existence of multiple transcription initiation sites. The 5′-most end point (T1) was an adenine residue, which was located 39 nucleotides upstream from the start of the published cDNA sequence (GenBank accession no. NM_030964). The sequence around this transcription start site completely matches the consensus initiator element sequence (14, 36). The other sites were detected 12 (T2), 16 (T3), and 21 (T4) bases downstream from the site T1, which now is numbered as +1. A summary of the *hSpry4* transcription start sites is shown in Fig. 2.

Cloning and analysis of the hSpry4 gene 5′-flanking sequence. To assess the promoter activity of the 5′-flanking region of the *hSpry4* gene, we analyzed the 5′-flanking sequence of the *hSpry4* gene using PromoterInspector software (Genomatix). As shown in Fig. 3, the 5′-flanking region contains several consensus recognition sequences (core similarity 1.000) for putative transcription factors such as hepatic nuclear factor 1, stimulating protein 1 (SP1), activator protein 2, Elk-1, E47/Thing 1, CCAAT-enhancer binding protein-β, WT1, sterol regulatory element binding protein (SREBP), Myc associated zinc finger protein (MAZ), PBX-1, zinc-finger protein ZF5, signal transducer and activator of transcription 5 (STAT5) and hypoxia-inducible factor 1 (HIF-1). No TATA box or CAAT box was found in this region, which is a common phenomenon found primarily in housekeeping genes and is often associated with multiple transcription start sites. This analysis suggests that a TATA-less promoter is used for *hSpry4* expression, which is likely regulated by multiple transcription factors.

Functional characterization of the hSpry4 promoter region. To localize the DNA elements that are important for promoter activity, we carried out a series of unidirectional deletion analyses of the 4.5-kb 5′-flanking region of the *hSpry4* gene. Deletion fragments, having 5′-ends ranging from −4,446 to −31 and 3′-ends at +56, were generated by PCR and cloned into the promoterless pGL$_2$-Basic, a luciferase reporter vector. Each resulting recombinant construct was then transiently transfected into three different human cell types, A549, WI-38, and HeLa cells. After 48 h, cell extracts were prepared and luciferase activity was measured. As noted under materials and methods, cells were cotransfected with a constant amount of pSV-β-galactosidase control plasmid as an internal control for transfection efficiency. The resulting luciferase reporter gene activities were then normalized to β-galactosidase activities.

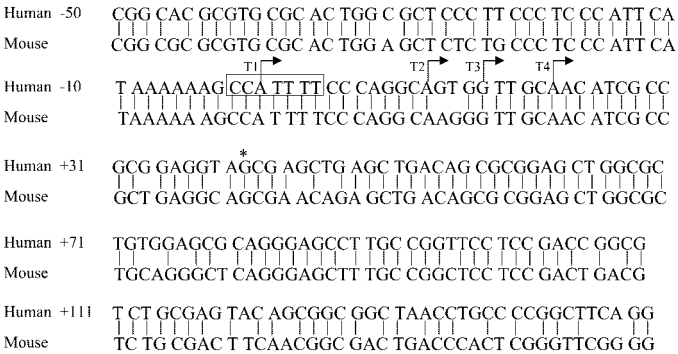

Fig. 2. Identification of *hSpry4* transcription start sites. Summary of *hSpry4* transcription start sites and comparison with corresponding murine sequences. T1, T2, T3, and T4 are transcription start sites determined by 5′-rapid amplification of cDNA ends (RACE). Position of T1 is designated +1. The boxed sequence is a direct match to the eukaryotic initiator element consensus sequence. *5′-Boundary of the previously published *hSpry4* cDNA sequence.

As shown in Fig. 4, the reporter gene expression levels showed no significant difference among three cell lines, suggesting that cell-specific elements may not be present in those sequences. In all cell lines, the construct pGL(−1,182/+56) was found to express the highest level of promoter activity. When the sequence length extended from −1,182 to −3,392, no alteration of luciferase activity was observed. Inclusion of more of the upstream 5′-flanking region resulted in a 35% decrease of luciferase activity, suggesting the presence of weak suppressor activity in this distal 5′-flanking region. A similar extent of reduction in promoter activity was observed for deletions between −1,182 and −418. It is important to point out that construct pGL(−418/+56) still exhibited significantly higher (~200-fold) luciferase activity than that of the pGL$_2$-Basic vector, but only a background level of luciferase activity was obtained with construct pGL(−31/+56), thus indicating that the fragment located between −418 and −31 nucleotides plays a critical role in promoter activity of *hSpry4*. Thus further experiments were focused on characterization of this functionally important region. We found that progressive deletion of this 387-bp region caused a gradual reduction in promoter activity, with one exception: truncation from −208 to −174 restored the promoter activity to a level similar to that of pGL(−418/+56), suggesting the presence of negative regulatory element(s) between −208 and −174. Deletion of 105 nucleotides from the 5′-end of pGL(−174/+56) resulted in the construct pGL(−69/+56), which showed a nearly 65% decrease of promoter activity relative to that of the pGL(−174/+56) construct. When an additional 38-nucleotide fragment, which contains putative STAT5 and HIF-1 binding sites, was removed from pGL(−69/+56), transcriptional regulatory activity was abolished.

Comparative analysis of the 5′-flanking regions of the hSpry4 and mSpry4 genes. The human and murine *Spry4* 5′-flanking region upstream of their transcription start sites were aligned for sequence comparison. Over the entire 1,182 bp of *hSpry4* promoter, there was ~80% homology with the *mSpry4* promoter. Less homology (~70%) was observed between −1,182 and −419 in the human sequence relative to the transcription start, but a higher degree of homology, with 87% nucleotide identity, was found between −418 and +1. As illustrated in Fig. 3, several putative transcription factor binding sites were also conserved between the two species. This high homology may indicate evolutionary conserved mechanisms of transcriptional regulation for *Spry4*.

Upregulation of hSpry4 promoter by the ERK pathway. The ERK pathway has been shown to positively regulate the expression of the *Spry4* gene (24). To identify the response elements implicated in the transcriptional regulation of *hSpry4*

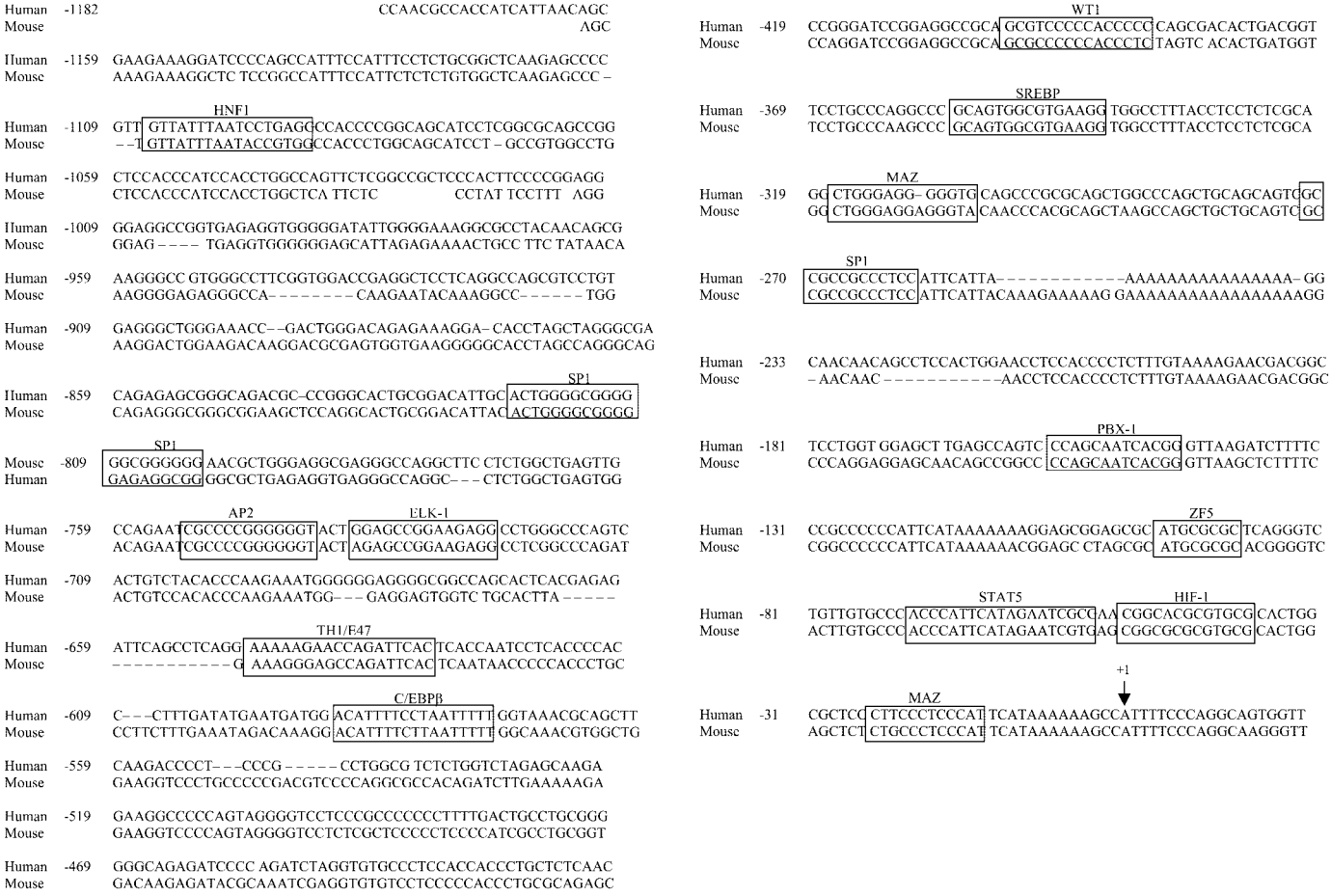

Fig. 3. Alignment of sequences in the 5′-flanking region of human and murine *Spry4* genes. The nucleotide sequences surrounding the transcription start site and the 5′-flanking region were compared. The putative binding sites for indicated transcription factors, which are conserved in both species, are boxed. A vertical arrow and position +1 indicate the 5′-most transcription start site of *hSpry4* as determined by 5′-RACE. HNF, hepatic nuclear factor; SP, stimulating protein; AP, activator protein; TH, thing; C/EBP, CCAAT-enhancer binding protein; SREBP, sterol regulatory element binding protein; MAZ, Myc-associated zinc finger protein; ZF, zinc finger; HIF, hypoxia-inducible factor.

by the ERK pathway, we transfected A549 and HeLa cells with the previously described *hSpry4* promoter-driven reporter constructs along with either the empty control vector or constitutive active RasV12 or its dominant-negative RasN17, since Ras is an upstream activator of the ERK signaling cascade. As shown in Fig. 5, the luciferase activities of the pGL_2-Basic vector and vector containing 31-bp of *hSpry4* 5′-flanking DNA were unaffected by coexpression of either Ras mutant. However, when the 5′-flanking sequence length extended to 69 bp, the promoter activity was modestly induced (twofold) by constitutively activated Ras. Conversely, dominant-negative Ras significantly inhibited the promoter activity by ~70%. Next, with each progressive addition of upstream sequences, all promoter constructs were found to be regulated by both Ras mutants to nearly the same degree compared with the construct pGL (−69/+56). Furthermore, cotransfection of a constitutively active form of ERK led to an induction of promoter activity comparable to that induced by active Ras (data not shown). All these results suggested that the ERK-induced upregulation of *hSpry4* transcription is likely to be mediated through the 5′-flanking region between nucleotide *positions −69* and *−31*, which contains putative STAT5 and HIF-1 binding sites. However, we cannot rule out that additional enhancer elements located further upstream in the genome or within introns may also contribute to the inducible activity and thus give rise to stronger regulation of *hSpry4* expression, as observed in the in vivo situation.

DISCUSSION

Previous studies have demonstrated regulation of *Spry4* gene expression via the ERK signaling pathway during the branching process in developing lung (20, 24). In the present study, we established the genomic organization of the *hSpry4* gene, identified its promoter, and began examining mechanisms of its regulation.

The *hSpry4* gene is ~14.5 kb in length interrupted by two introns. *Exon 1* only encodes the 5′-UTR of the cDNA, whereas *exon 2* contains the translation initiation codon. The remainder of the open-reading frame for the protein as well as the entire 3′-UTR is encoded by the third exon. All the exon-intron boundaries possess consensus GT/AG splice junctions (35). Our earlier work had already determined that both human and mouse *Spry2* genes consist of two exons and one

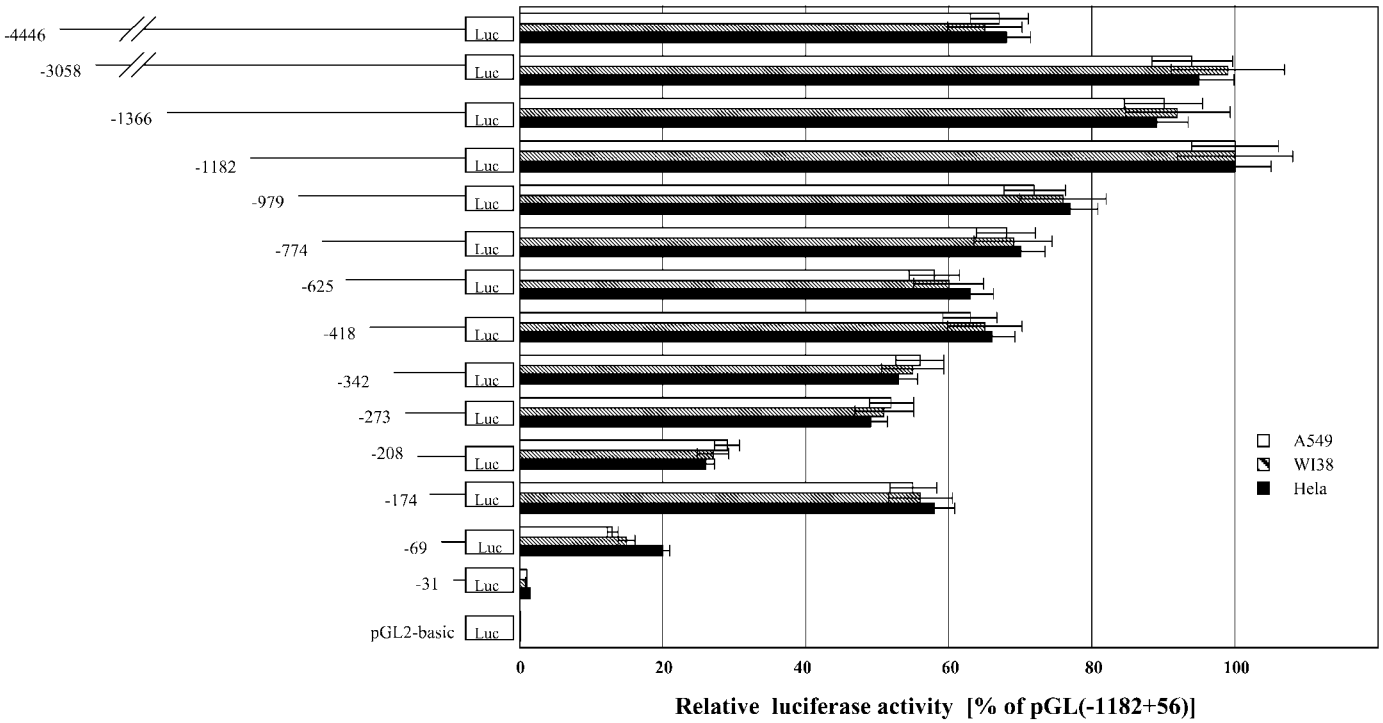

Fig. 4. Progressive 5′-deletion analysis of the 5′-flanking region of the *hSpry4* gene. The schematic diagram on the *left* represents a series of *hSpry4* promoter-luciferase gene constructs with variable 5′-ends (from −4,446 to −31) and the same 3′-end (+56). The constructs were all cloned into a luciferase (Luc) reporter vector (pGL$_2$-Basic). One microgram of each Luc construct and 0.05 μg of the internal control pSV-β-galactosidase expression plasmid were transfected into A549, WI-38 VA-13, and HeLa cells. Cells were lysed 48 h posttransfection. We measured and normalized the Luc activity for transfection efficiency by dividing the measurement of the firefly Luc activity by that of the β-galactosidase activity. The normalized Luc activities were evaluated as a percentage of the construct pGL(−1,182/+56), which was set at 100% activity, and are presented as means ± SE of 3 independent experiments.

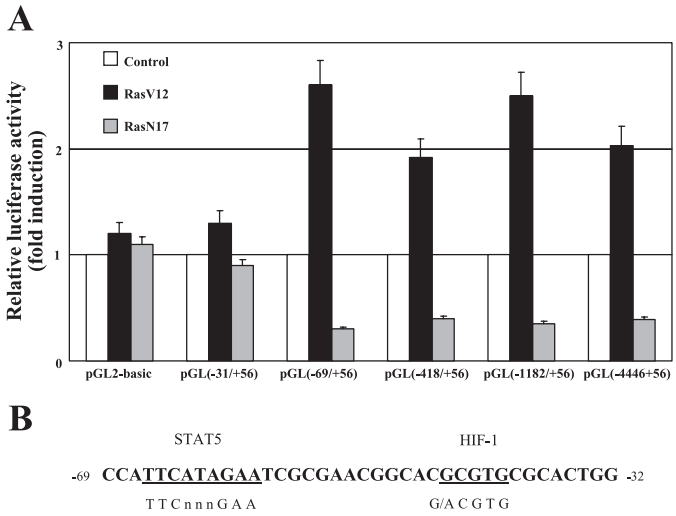

Fig. 5. Localization of *hSpry4* 5′-flanking sequences that mediate transcriptional response to ERK activation. *A*: indicated *hSpry4* promoter-luciferase reporter constructs were cotransfected into A549 cells with RasV12, RasN17, or empty control vector. The relative luciferase activities are represented as fold induction with respect to that obtained in cells transfected with the empty control vector. Data are given as means ± SE of 3 independent experiments performed in triplicate. *B*: sequence of the minimal ERK-inducible promoter region (−69 to −31) of *hSpry4* is compared with the consensus motifs of HIF-1 and STAT5 binding sites.

intron (7). These results, along with further elucidation of the gene structure of the other *Spry* isoforms, should be useful for better understanding the molecular evolution path of the members of the *Spry* gene family.

Multiple transcription start sites have been identified by 5′-RACE analysis. The 5′-flanking region of the *hSpry4* gene lacks a canonical TATA box or CAAT sequence within the expected proximity to the transcription start site. Such features were also found in the *hSpry2* promoter (7). Transient transfection studies revealed the presence of maximal basal promoter activity within the 1,182-bp 5′-flanking region upstream from the transcription initiation sites. Further addition of upstream sequences (up to 4 kb) or progressive deletion to *position −418* only moderately changed the transcriptional activity by <40%, suggesting that the proximal ~400-bp sequences 5′-flanking the transcription start site might contain key regulatory elements for driving high-level transcription of *hSpry4* and thus function as the core promoter region.

As further evidence demonstrating the importance of this core promoter region, a comparison of human and murine *Spry4* proximal promoter sequences showed significant homology with each other. Of particular interest was that the binding sites for transcription factors WT1, SREBP, MAZ, SP1, PBX-1, ZF5, STAT5, and HIF-1 were all conserved between the two species. The functional importance of each of these transcription factors with respect to modulation of *Spry4* expression will need to be addressed in future studies.

It is known that *Spry4* expression can be upregulated by ERK activation (24). In the present study, we found that a modest induction was mediated through regulatory elements located between *nucleotides −69* and *−31* in the proximal promoter region. Within this 38-bp fragment, the HIF-1 and STAT5 binding sites are two likely candidates for mediating the ERK-stimulatory effects.

HIF-1 is a heterodimer composed of the basic-helix-loop-helix-PAS domain containing the HIF-1α subunit and aryl hydrocarbon receptor nuclear translocator subunit (40). HIF-1 binding sites have been found in promoters or enhancers of many HIF-1-regulated target genes, all of which are involved in maintenance of oxygen homeostasis, such as vascular endothelial growth factor, erythropoietin, glucose transporter 1, transferrin, nitric oxide synthase 2, and others (33). Knockout studies further demonstrated that HIF-$1\alpha^{-/-}$ mice were deficient in vascularization and had cardiac and neuronal abnormalities (2, 13, 31). All these results clearly show the critical role that HIF-1 plays in a multitude of developmental and physiological processes, especially in neovascularization and embryogenesis. HIF-1 can be activated by hypoxia and many other signals including nitric oxide, cytokines, and growth factors (34). Although the molecular mechanisms underlying the activation of HIF-1 are currently unclear, it has been demonstrated that ERK kinases can phosphorylate and enhance the transcriptional activity of HIF-1 in many cell types (21, 30, 37). Conversely, specific inhibitors of the ERK pathway as well as dominant-negative ERK mutant can efficiently block HIF-1 activation (23).

Likewise, STAT5 may also contribute to the ERK inducibility of the *hSpry4* promoter. STAT5 is one of the members of the STAT family of transcription factors that are activated by a number of cytokines and growth factors (12). It has been well established that this activation is primarily mediated through tyrosine phosphorylation of STAT5 by Janus kinases (9, 38). However, the importance of serine phosphorylation of STAT5 protein has recently been acknowledged (25). STAT5 serine phosphorylation may in part be mediated by the MAPK cascade, as suggested by direct physical interactions between STAT5 and ERK1/2 (27, 28). Nevertheless, the consequence of MAPK phosphorylation on the transcriptional activity of STAT5 is still a controversial issue, and it may vary with the activating extracellular signal or with the target gene (5, 11, 42).

Another important feature of the pulmonary developmental expression pattern of *Spry4* is that it is mainly expressed in the lung mesenchyme (6, 26). However, the profile of *hSpry4* promoter activity within a deletion series of promoter constructs was similar between fibroblast WI-38 and epithelial A549 cells. These results suggest that major tissue-specific transcriptional activators of *hSpry4* expression either are not present in the cell lines used or could not transactivate the promoter fragments used in this analysis. It is not unusual for tissue-specific regulatory sequences to reside at a long distance from transcription start sites. Moreover, tissue-specific expression of *Spry4* in vivo may require proper chromatin structure or DNA methylation; thus the function of transgenes in transfected cells does not always mimic exactly the expression in the complex milieu of developing lung (8, 18). A more definitive test of promoter specificity is to express a reporter gene in vivo using transgenic methods, which will be an important area for our future studies.

The studies reported herein provide a starting point for examination of the regulation of the *hSpry4* gene in more detail. Further work is required to define factors and elements necessary for *hSpry4* activation in lung development.

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