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Molecular Determinants of *NOTCH4* Transcription in Vascular Endothelium

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The process whereby the primitive vascular network develops into the mature vasculature, known as angiogenic vascular remodeling, is controlled by the Notch signaling pathway. Of the two mammalian Notch receptors expressed in vascular endothelium, Notch1 is broadly expressed in diverse cell types, whereas Notch4 is preferentially expressed in endothelial cells. As mechanisms that confer Notch4 expression were unknown, we investigated how *NOTCH4* transcription is regulated in human endothelial cells and in transgenic mice. The *NOTCH4* promoter and the 5′ portion of *NOTCH4* assembled into an endothelial cell-specific histone modification pattern. Analysis of *NOTCH4* primary transcripts in human umbilical vein endothelial cells by RNA fluorescence in situ hybridization revealed that 36% of the cells transcribed one or both *NOTCH4* alleles. The *NOTCH4* promoter was sufficient to confer endothelial cell-specific transcription in transfection assays, but intron 1 or upstream sequences were required for expression in the vasculature of transgenic mouse embryos. Cell-type-specific activator protein 1 (AP-1) complexes occupied *NOTCH4* chromatin and conferred endothelial cell-specific transcription. Vascular angiogenic factors activated AP-1 and reprogrammed the endogenous *NOTCH4* gene in HeLa cells from a repressed to a transcriptionally active state. These results reveal an AP-1–Notch4 pathway, which we propose to be crucial for transducing angiogenic signals and to be deregulated upon aberrant signal transduction in cancer.

The development and remodeling of blood vessels, which are termed vasculogenesis and angiogenesis, respectively, require integration of diverse cellular signals (79, 109). The Notch signaling pathway, in conjunction with secreted growth and differentiation factors, is critical for angiogenic vascular remodeling (29, 36), the process whereby the primitive vascular network is sculpted into mature vasculature. The binding of Notch ligands to transmembrane Notch receptors induces a proteolytic cascade that liberates the intracellular domain of Notch (NIC) from the plasma membrane (87). NIC translocates into the nucleus and forms a complex with the transcriptional repressor C promoter binding factor 1 (CBF1)/RBPJκ/suppressor of hairless/Lag-1 (CSL) (32, 37, 61, 84). Analogous to nuclear receptor-mediated coactivator-corepressor switches (107), NIC binding induces coactivator recruitment, precluding CSL interactions with corepressors (27, 33, 48, 100, 112, 113). Through its sequence-specific DNA binding function, CSL determines Notch target gene specificity. This canonical mechanism allows Notch to stringently control an ensemble of target genes and thus many developmental processes. NIC mutants defective in CSL binding retain certain activities, consistent with CSL-independent Notch signaling (69). However, such mutants can be tethered into CSL-containing complexes by the coregulator mastermind (38).

Loss-of-function and gain-of-function studies have provided evidence that two of the four mammalian Notch receptor subtypes expressed in vascular endothelium, Notch1 and Notch4, regulate vascular angiogenic remodeling (47, 96). Targeted deletion of the broadly expressed murine *Notch1* gene yielded multiple developmental defects, including impaired vascular angiogenic remodeling (47, 91). Whereas targeted deletion of the *Notch4* gene, which is expressed preferentially in endothelial cells (97), did not reveal overt phenotypes, the *Notch1-Notch4* double knockout caused a more severe disruption of vascular angiogenic remodeling than the *Notch1* single knockout (47). The genetic interaction of *Notch4* with *Notch1* provides strong evidence that physiological levels of Notch4 signaling control vascular development. Overexpression of NIC-1 and NIC-4 in transgenic mice and in cultured cells either inhibits (56, 60) or promotes (59, 92, 95) vascular morphogenesis and, in certain contexts, is oncogenic (3, 6, 10, 23, 76, 101).

Given the endothelial cell specificity of *Notch4* expression (97), the *Notch1-Notch4* genetic interaction (47), and the lack of nonvascular phenotypes in *Notch4*-null mice (47), Notch4 appears to have committed vascular functions. This attribute distinguishes Notch4 from other Notch subtypes, which are expressed more broadly (51, 102, 103). The vascular endothelium consists of functionally distinct endothelial cell subtypes (13), and *Notch4* is not expressed in all endothelium. In situ

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hybridization analysis showed that *Notch4* transcripts were highest in the vascular endothelium of embryonic day 9 (E9) and E13.5 mouse embryos and were detected in the pulmonary capillaries of adults (97). Another study reported that *Notch4* transcripts were detected in arteries, but not veins, of the E13.5 embryo (99). Zebra fish *notch3* is preferentially expressed in the dorsal aorta during embryogenesis and controls arterial-venous differentiation (52).

Besides endothelial cell-specific expression, Notch4 levels are dynamically regulated via cell signaling. Infection of human umbilical vein endothelial cells (HUVECs) with adenoviruses expressing vascular endothelial growth factor 121 ($VEGF_{121}$) and fibroblast growth factor 2 (FGF-2) increased *NOTCH4* mRNA levels (59). This effect was not specific for *NOTCH4*, as *NOTCH1* mRNA was also induced. Treatment of rheumatoid arthritis synovial fibroblasts, but not normal synovial fibroblasts, with tumor necrosis factor induced *NOTCH4* mRNA (1). Despite the selective expression and dynamic regulation of *NOTCH4* in vascular endothelium, mechanisms underlying the endothelial cell specificity of *NOTCH4* transcription have not been defined. Mechanisms controlling transcription of other Notch subtypes are also largely unknown.

Cell-type-specific transcription patterns can be achieved via utilization of a limited number of cell-type-specific transcription factors or through combinatorial interactions between multiple factors. Nearly all erythroid cell-specific genes are activated by the erythroid cell-specific factor GATA-1 (22, 93). While an endothelial cell-specific factor equivalent to GATA-1 that regulates most endothelial cell-specific genes has not been identified, GATA factors (41, 63, 65, 108), Ets factors (64), NF-κB (63), Egr1 (5), Vezf1 (105), HoxB5 (104), Sp1/Sp3 (11, 75), RTEF-1 (89), NFAT (111), and AP-1 (5, 41, 108) interact with and regulate promoters of endothelial cell-specific genes in transfection assays. No consensus has arisen regarding a single factor that might universally confer endothelial cell-specific transcription. Furthermore, no comprehensive studies of the native nucleoprotein structure (chromatin structure and bound *trans*-acting factors) of any endothelial cell-specific gene within its endogenous chromatin domain have been conducted.

The native nucleoprotein structures of endogenous loci reveal mechanistic insights that cannot be predicted from in vitro analysis (9, 39). Chromatin immunoprecipitation (ChIP) analysis has shown that the most abundant interactor in vitro is often not the actual endogenous interactor. Whereas c-*myc* occupies E-boxes in cells, the related factor USF (upstream stimulatory factor) is the predominant binder in vitro (8). GATA factors recognize simple (A/T)GATA(A/G) motifs distributed abundantly within chromosomal DNA (46, 62). However, ChIP analysis has revealed that the majority of such motifs are inaccessible to GATA factors in cells (28, 40, 73).

We used ChIP and functional assays to dissect the mechanism controlling *NOTCH4* transcription in vascular endothelium. This analysis revealed an endothlelial cell-specific histone modification pattern localized in a highly restricted manner at the *NOTCH4* promoter and the 5′ portion of *NOTCH4*. Analysis of *trans*-acting factors that confer *NOTCH4* transcription in endothelial cells revealed an important role of cell-type-specific, signal-dependent AP-1 complexes. These results are discussed vis-à-vis a model of how signaling networks established by angiogenic factors target AP-1 to control *NOTCH4* transcription and how disruption of the AP-1–Notch4 axis by aberrant signaling in cancer might facilitate tumor progression.

MATERIALS AND METHODS

Cell culture. Primary HUVECs (Cascade Biologics) were maintained in Medium 200 (Cascade Biologics) containing 1% penicillin-streptomycin (Gibco/BRL) and Low Serum Growth Supplement (Cascade Biologics). HeLa cells, which were derived from an ovarian carcinoma and have epithelial properties, were maintained in Dulbecco's modified Eagle medium (DMEM) (Biofluids) containing 1% antibiotic-antimycotic (Gibco/BRL) and 10% fetal bovine serum (FBS).

Plasmids. The bacterial artificial chromosome containing human *NOTCH4* genomic DNA was generously provided by Monica Dors, Institute for Systems Biology. The pGL3basic and pGL3promoter (pGL3pro) reporter plasmids were obtained from Promega. Other luciferase reporter constructs for transient transfection assay were generated by the following methods. For pGL3 N4-pro, the human *NOTCH4* promoter was amplified by PCR using the primer pair (5′ to 3′) TGACTCTCGAGACCAAGATTTCCCCAAAACC and CAGTCAAGCTTCA GGCAGGGACCCTC. The PCR product was digested with XhoI and HindIII and then inserted into the pGL3basic vector. For pGL3 N4-proIN1, intron-1 of *NOTCH4* was amplified by PCR using the primer pair (5′ to 3′) AGTTAGGA TCCTCAGTGGTCAGACCCAGAGG and TAGGTGTCGACATTGGCACA GGGTTCTGG. The PCR product was digested with SalI and BamHI and then inserted into pGL3 N4-pro construct. For pGL3 N4-CRpro, the *NOTCH4* upstream conserved region was amplified by PCR using the primer pair (5′ to 3′) CTGATGCTAGCTACAGTGGCCTATTGCC and TCAGTCTCGAGCATGT TTAGGTGGGTCTC. The PCR product was digested with NheI and XhoI and then inserted into the pGL3 N4-pro construct. For pGL3 N4-UPpro, the *NOTCH4* upstream region was amplified by PCR using primer pair (5′ to 3′) CTGATGCTAGCTACAGTGGCCTATTGCC and CAGTCAAGCTTCAGG CAGGGACCCTC. The PCR product was digested with NheI and HindIII and then inserted into the pGL3basic vector. A two-step PCR strategy was used to generate the AP-1 binding motif-mutated construct pGL3 N4-pro(mtAP-1) using primers (5′ to 3′) TGACTCTCGAGACCAAGATTTCCCCAAAACC, TT GTGGCTAGACGGAAACAGCTCAGACGTG, CACGTCTGAGCTGTTTC CGTCTAGCCACAA, and CAGTCAAGCTTCAGGCAGGGACCCTC. The PCR product was digested with XhoI and HindIII and then inserted into the pGL3basic vector.

The p4xAP-1 reporter and the AP-1 reporter plasmid pGL2AP-1, containing a collagenase promoter fragment (−73 to +67) with a single AP-1 binding motif in the luciferase reporter vector pGL2basic, were generously provided by Nancy Colburn, National Cancer Institute, National Institutes of Health. The expression vector encoding dominant-negative AP-1, A-Fos (71), was generously provided by Charles Vinson, National Cancer Institute, National Institutes of Health.

The β-galactosidase reporter constructs for transient transgenic analysis were generated by the following methods. For pSVβ N4-pro, the *NOTCH4* promoter was amplified by PCR using the primer pair (5′ to 3′) TGACTGAATTCAAG ACCAAGATTTCCCCAAAACC and CTGACCTCGAGGCAGGGACCCTC AGAGCT. The PCR product was digested with EcoRI and XhoI and then inserted into the pSVβ vector (Clontech), which lacks a promoter (72). The pSVβ vector contains the full-length β-galactosidase gene with a poly(A) site in a 6.9-kb plasmid. For pSVβ N4-UPpro, the *NOTCH4* upstream region was amplified by PCR using the primer pair (5′ to 3′) CTGATGCTAGCTACAGT GGCCTATTGCC and CTGACCTCGAGGCAGGGACCCTCAGAGCT. The PCR product was digested with NheI and XhoI and then inserted into the pSVβ vector. For pSVβ N4-proIN1, intron 1 of *NOTCH4* was amplified by PCR using primer pair (5′ to 3′) AGTTAGTCGACTCAGTGGTCAGACCCAGAGG and TTGCTAAGCTTGGCACAGGGTTCTGGG. The PCR product was digested with SalI and HindIII and then inserted into pSVβ N4-pro. For pSVβ N4-pro(mAP-1)IN1, the AP-1 binding motif-mutated *NOTCH4* promoter was amplified by PCR from pGL3 N4-pro(mtAP-1) using the primer pair (5′ to 3′) TGACTGAATTCAAGACCAAGATTTCCCCAAAACC and CTGACCTCGA GGCAGGGACCCTCAGAGCT. The PCR product was digested with EcoRI and XhoI and then substituted within the promoter region of pSVβ N4-proIN1. The Tie-2–LacZ construct (Tie-2-LacZ) containing the *Tie-2* enhancer and promoter was generously provided by Tom Sato, University of Texas Southwestern Medical Center.

Antibodies. The antibodies used in ChIP analysis were as follows. Rabbit anti-di-acetylated histone H3 (anti-di-acH3) (06-599), anti-tetra-acH4 (06-866), and anti-histone H3 methylation at lysine 4 (anti-H3-meK4) (07–030) antibodies were obtained from Upstate Biotechnology; rabbit anti-polymerase II (Pol II)

(N-20) was obtained from Santa Cruz Biotechnology; rabbit immunoglobulin G (IgG) (Sigma) and preimmune serum (Covance) were used as controls. The AP-1 antibodies rabbit anti-c-Fos (K-25), anti-c-Fos (H-125), anti-FosB (102), anti-Fra-1 (R-20), anti-Fra-2 (L-15), anti-ATF2 (N-96), anti-c-Jun (D), anti-c-Jun (H-79), anti-c-Jun (N), anti-JunB (N-17), and anti-JunD (329) were obtained from Santa Cruz Biotechnology. Mouse antitubulin (Ab-1) antibody was obtained from Oncogene Research Products. The secondary antibodies goat-anti-rabbit IgG-horseradish peroxidase (HRP) and goat-anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology.

Quantitative real-time RT-PCR. Total RNA was purified from cell cultures with Trizol (Invitrogen). cDNA was synthesized by annealing RNA (2 μg) with 50 ng of random hexamer and 200 ng of oligo(dT) primer (Promega) at 68°C for 10 min. After denaturation, the samples were incubated with Moloney murine leukemia virus reverse transcriptase (10 U/μl; Invitrogen) combined with 20 mM dithiothreitol, 1 mM deoxynucleoside triphosphates, and RNasin (2 U/μl; Promega) at 42°C for 1 h. The reaction mixture was heat inactivated at 95 to 100°C for 5 min and diluted to a final volume of 200 μl. The samples were analyzed by quantitative real-time reverse transcription-PCR (RT-PCR) (ABI Prism 7000) with primers designed with Primer Express 1.0 software (PE Applied Biosystems) to amplify regions of 50 to 150 bp. Quantitative real time RT-PCR mixtures (25 μl) contained 2 μl of cDNA, 12.5 μl of SYBR Green (Applied Biosystems), and the indicated primers. Product accumulation was monitored by the levels of SYBR Green fluorescence. Relative expression levels were determined from a standard curve of serial dilutions of cDNA samples. Analysis of product denaturation curves postamplification showed that primer pairs generated single products. Forward and reverse primers for real-time RT-PCR (5′-3′) were as follows: NOTCH4, GAGGACAGCATTGGTCTCAAGG and CAACTCCATC CTCATCAACTTCTG; NOTCH4-2, CAGCCCAAGCAGATATGTAAGGA and CGTCCAACCCACGTCACA; NOTCH1, CTGCATGCGGCTGTGTCT and CTCGGTTCCGGATCAGGAT; TSBP, AAAGTGCTAGACTTCTGGAC TATGAGG and TGATATGCATGTCGGGATCCT; G18, CTTTACAGCACT ATCCTCAGTCACCA and GCTCTGACCGCTGGGCT; HPRT, ATTGGTG GAGATGATCTCTCAACTTT and GCCAGTGTCAATTATATCTTCCA CAA; eNOS, AATCAACGTGGCCGTGCT and ACGATGGTGACTTTGGC TAGCT; vWF, CCTCAAAGGCGGTGGTCAT and CCAATAGGGAACACT GTCACTCTG; and Flk-1, GAGGAGAAGTCCCTCAGTGATGTAG and CC TTATACAGATCTTCAGGAGCTTCC.

Quantitative ChIP analysis. HUVECs or HeLa cells were incubated in a culture plate with medium containing 0.4% (for analysis of histone modifications) or 1% (for analysis of AP-1 occupancy) formaldehyde for 10 min at room temperature. The cross-linking reaction was terminated by incubation with 0.125 M glycine for 5 min. Cells were harvested by scraping, collected by centrifugation at 400 × *g* for 8 min, and washed in phosphate-buffered saline (PBS). Real-time PCR-based quantitative ChIP analysis was preformed as previously described (35). Nuclei were isolated by a 10-min incubation in cell lysis buffer (10 mM Tris, 10 mM NaCl, 0.2% NP-40 [pH 8.0]) on ice, followed by centrifugation at 500 × *g* for 5 min. Nuclei were lysed in nucleus lysis buffer (50 mM Tris, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS] [pH 8.0]) for 10 min on ice. The lysate was diluted with IP dilution buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.01% SDS, 1% Triton X-100 [pH 8.0]) and sonicated with eight 30-s pulses at 50 to 60% of maximum power with a HeatWave Systems W185F sonicator (Ultrasonics, Inc., Plainview, N.Y.) equipped with a microtip. Sonicated chromatin fragments were an average size of ~300 to 400 bp. Soluble chromatin was precleared by the addition of 50 μl of preimmune serum, followed by 100 μl of protein A-Sepharose. Precleared chromatin (180 μl) was removed (input) and used in the subsequent PCR analysis. The remainder of the chromatin was aliquoted and incubated with the indicated antibodies in a final volume of 900 μl for 3 h at 4°C. Immune complexes were collected by incubation with 30 μl of protein A-Sepharose for 2 h at 4°C. Protein A-Sepharose pellets were washed twice with 500-μl aliquots of IP wash buffer 1 (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 [pH 8.0]), once with IP wash buffer 2 (10 mM Tris, 0.25 mM LiCl, 1 mM EDTA, 1% NP-40, 1% desoxycholate [pH 8.0]), and twice with TE (10 mM Tris, 1 mM EDTA [pH 8.0]). Immune complexes were eluted twice with 150 μl of IP elution buffer (0.1 mM $NaHCO_3$, 1% SDS). RNase A (0.03 mg/ml) and NaCl (0.3 m/liter) were added, and cross-links were reversed by incubation for 4 to 5 h at 65°C. DNA was then digested with proteinase K (0.24 mg/ml) for at least 2 h at 45°C and purified by two extractions with phenol-chloroform, followed by ethanol precipitation. Purified DNA was resuspended in 30 μl of water. Aliquots (1 μl) were analyzed by quantitative real-time PCR with the indicated primer pairs. The amounts of products were determined relative to a standard curve generated from a titration of input chromatin. Measurements were made under conditions in which signals were in the linear range, and analysis of denaturation curves postamplification showed that primer pairs generated single products (data not shown). Forward and reverse primers for real-time PCR (5′ to 3′) ChIP analysis were as follows: N4-1, CCTCTCTGTCTCC GTTGCAAT and GGCACTAGGCTGGAGCATGA; N4-2, ACCATTATAAA TGATGCTGGCTCAC and AGTACCAGTGTATCACATTTGGAAGC; N4-3, GTGTTTGTTACAGACAATTCAGACTGC and TGAACATAGTCTACCCT AAATTTTGCA; N4-pro1, CAGCCACCTTGCAATTCTCA and CAGCCCTG CTGTTTGTTGATC; N4-pro2, GGACATTGTGTGACTCAGGAAACA and CCTCGGCCTGCTGCAA; N4-in1, CTGTTGTCTTGCTTCCGAGAGAT and TTATTCTCTGGCCTCCCAAGTC; N4-ex3, TGGCTTCACTGGCGAG AGAT and GGCCCCTTTTGGAACAGAA; N4-in3, GGGTCCTCCAGACTT TTGCAT and ATGGCTCCCTCCACTCAGAAT; N4-in8, CAGACTCCTCAG GCAAGAAAAGA and TGGGATCAACCTCTGGACCTT; N4-ex11, GAAG GGCCACGCTGTCAA and CAACGGGACATGGGTCACTC; N4-ex18, GG ATTCCAAGGCAGCCTGT and CCTGGACTCACATGGGTTCAC; N4-in20, TCTCTGTTGCCCCCTATGCT and CCCTTCTGGGATTCCAACTGA; N4-ex30, CCTGCGATAATGCGAGGAA and AATCACAGGGCCAGTCATCC; cyclophilin pro, GTCTATAGGCCAGATGCACTGTCA and CCAATCGGGTCT GCGACTT; β-globin pro, AGTGCCAGAAGAGCCAAGGA and CAGGGTGA GGTCTAAGTGATGACA; IL-8 pro, GGGATGGGCCATCAGTTG and CCTC ATCTTTTCATTATGTCAGAGGAA; and N4 pro(AP-1), CCCCCATTACTAG GGTGTCCA and TGCTGCAAGCCTCACGTC.

Transient transfection assay. HUVECs or HeLa cells were plated 1 day before transfection and were ~60 to 70% confluent at the time of transfection. The indicated amounts of plasmid DNA were added to 100 μl of Opti-MEM (Gibco/BRL), incubated with Lipofectin reagent (6 μl/1 μg of DNA; Invitrogen) for 15 min at room temperature, and then added to the cells. The cells were incubated with the transfection mixture for 5 h before the addition of normal medium. The cells were harvested 48 h posttransfection. Cell lysates were assayed for luciferase activity by a luciferase assay system (Promega). The luciferase activity was normalized by the protein content of the lysates, as determined by a Bradford assay (Bio-Rad) with γ-globulin as a standard.

Nuclear extract preparation. Nuclear extracts were prepared as described previously (49). HUVECs or HeLa cells were harvested by scraping and collected by centrifugation at 400 × *g* for 8 min. Cells were washed once with ice-cold PBS and resuspended in 1.5 volumes of nucleus lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, and 0.2% NP-40) on ice for 3 min. Nuclei were collected by centrifugation for 5 min at 400 × *g*. Nuclei were washed by gentle resuspension in 1.5 volumes of nucleus wash buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, and 3 mM $MgCl_2$) and then collected by centrifugation for 4 min at 400 × *g*. Nuclei were immediately resuspended in an equal volume of low-KCl extract buffer (20 mM HEPES [pH 7.5], 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol), and 1.33 volumes of the same buffer containing 1.2 M KCl was added dropwise. Nuclei were extracted for 45 min at 4°C with constant mixing. The suspension was then centrifuged for 30 min at 150,000 × *g*. Aliquots of the supernatant were frozen on dry ice and stored at −80°C. The protein concentration was measured by the Bradford assay with γ-globulin as a standard. Dithiothreitol (5 mM), phenylmethylsulfonyl fluoride (0.5 mM), and leupeptin (20 μg/ml) were included in all buffers.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSAs) were conducted as described previously (50). Aliquots of HUVEC or HeLa nuclear extracts (3 μg) were incubated in 50 mM HEPES (pH 7.8), 300 mM KCl, 50% glycerol, 5 mM $MgC1_2$, 5 mM dithiothreitol, and 1 μg of poly(dI-dC) with or without 4 nmol of unlabeled double-stranded oligonucleotide or 4 μl of antibody in a final volume of 16 μl for 15 min at 4°C. End-labeled, double-stranded oligonucleotide (40 fmol) was added, and reaction mixtures (20 μl) were incubated for 20 min at 25°C. Samples were resolved on 6.5% nondenaturing polyacrylamide gels in 0.75× Tris-acetate-EDTA running buffer (30 mM Tris-acetate, 0.75 mM EDTA [pH 8.0]) at 180 V for 3 h at 4°C. Gels were preelectrophoresed for at least 10 min at 4°C. DNA binding activity was detected by analyzing dried gels with a PhosphorImager (Molecular Dynamics).

Western blotting. To detect the expression levels of AP-1 subunits, nuclear extracts were prepared from HUVECs or HeLa cells under the indicated conditions. Total protein (20 μg) of nuclear extract was resolved by SDS-polyacrylamide gel electrophoresis on a 10% acrylamide gel. The proteins were transferred to an Immobilon P membrane (Millipore) and detected by immunoblotting with the indicated antibody. Proteins were visualized with ECL-Plus (Amersham).

RNA fluorescence in situ hybridization (FISH) analysis. RNA FISH was performed as described previously (28a). Endogenous *NOTCH4* transcription was detected with an antisense, digoxigenin-labeled, single-stranded DNA probe visualized with fluorescein isothiocyanate-labeled antibodies. The 1,420-bp probe fragment, spanning *NOTCH4* introns 15 to 17, was generated by PCR with

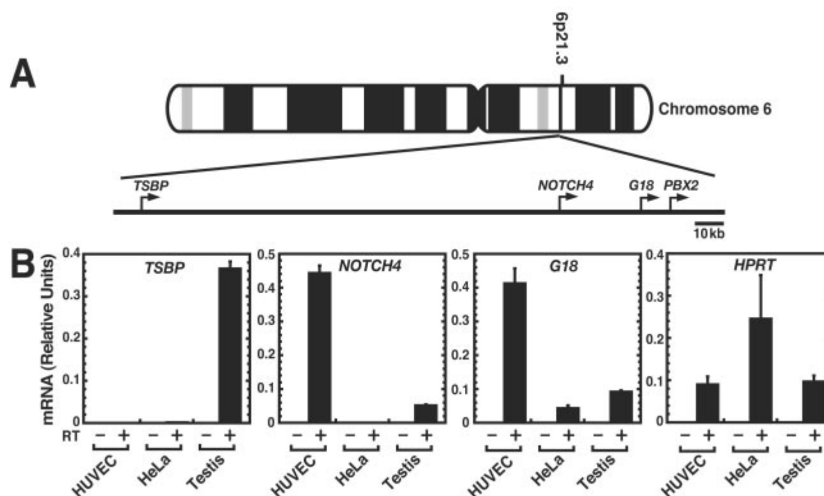


FIG. 1. Distinct cell-type-specific transcription patterns of *NOTCH4* and adjacent genes. (A) Human *NOTCH4* locus on chromosome 6. The nearby genes, testis-specific basic protein gene (*TSBP*), *G18*, and the pre-B-cell leukemia transcription factor-2 gene (*PBX2*) are also shown. (B) Quantitative real-time RT-PCR analysis of *NOTCH4*, *TSBP*, and *G18* mRNA expression in HUVECs, HeLa cells, and testis. *HPRT* mRNA transcripts were measured as a control. Relative expression levels were normalized by total RNA (mean ± standard error of the mean [SEM]; three independent experiments).

the primer pair CAGACAGGTGAGCAGGGCCCAAAGA and ATTCCTGG GTGGAGACTGGTCTGGG.

Analysis of *lacZ* fusion constructs in transgenic mice. DNA constructs for F0 transgenic analysis were linearized, purified with the QIAEX II gel purification kit (QIAGEN), and microinjected into fertilized mouse oocytes. For whole-mount analysis, 5-bromo-4-chloro-3-indolyl β-galactoside (X-Gal) staining was performed with E10.5 embryos as described previously (72). Embryos were fixed with 1% formaldehyde, 0.2% glutaraldehyde, and 0.06% Igepal CA-630 (Sigma) in PBS for 40 min at 4°C. Samples were washed twice with PBS and then incubated for 4 h at 37°C in 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and X-Gal (1 mg/ml) in PBS. After X-Gal staining, embryos were washed twice with PBS and refixed with 10% formaldehyde overnight at 4°C. For tissue sections, the refixed samples were dehydrated with 40 and 80% methanol in PBS and then in 100% methanol. Samples were embedded into paraffin and were dried for 3 days at 4°C before being sectioned. The sectioned samples (each, 4 μm) were counterstained with Kernechtrot staining solution. Genomic DNA was purified from the embryos, and integration of the transgenes was verified by PCR. The following primers were used for the analysis: β-gal-5′ (5′-ACCGAC TACACAAATCAGCG-3′) and β-gal-3′ (5′-CAACCACCGCACGATAGAG A-3′).

RESULTS AND DISCUSSION

Endothelial cell-specific histone modification pattern of the endogenous *NOTCH4* locus. To investigate mechanisms underlying the endothelial cell specificity of *NOTCH4* transcription, we compared the expression of *NOTCH4* and genes flanking *NOTCH4* on chromosome 6 (Fig. 1A) in HUVECs and nonendothelial HeLa cells. *NOTCH4* transcript levels were high in HUVECs, undetectable in HeLa cells, and low in testis (Fig. 1B). The low-level expression likely originates from the endothelium of the vascularized testis. The *TSBP* gene, a testis-specific gene of unknown function (106), resides approximately 100 kb to the 5′ side of *NOTCH4*. *TSBP* transcripts were not detected in HUVECs or HeLa cells (Fig. 1B). Immediately downstream of *NOTCH4* resides *G18*, which is also of unknown function (42). *G18* transcript levels were high in HUVECs and lower in HeLa cells and in testis (Fig. 1B). As expected, the broadly expressed *HPRT* gene was expressed in HUVECs, HeLa cells, and testis.

Given the distinct expression patterns of *NOTCH4* and its flanking genes, we reasoned that *NOTCH4* might assemble a histone modification pattern that segregates regulatory elements associated with these genes. The rationale for this assumption is illustrated by analysis of the chicken β-globin locus. A 15-kb hypoacetylated region lies between the chicken β-globin locus and an upstream folate receptor gene, which has a distinct expression pattern (77). A chromatin insulator, characterized by a sharp peak of histone acetylation, resides at the junction of the 5′ end of the β-globin locus and the 3′-end of the hypoacetylated region (17, 58, 77). Additional examples of broad histone modification patterns that demarcate transcriptional regulatory regions include the high enrichments in histone acetylation and H3-meK4 present at upstream locus control regions of the β-globin (25, 45, 85) and growth hormone (21, 31) loci.

Quantitative ChIP analysis was used to determine if endogenous *NOTCH4* in HUVECs assembles a broad pattern of acH3, acH4, and H3-meK4 that is distinct from that of HeLa cells. Histone acetylation increases factor access to chromatin (53, 98) and counteracts higher-order chromatin folding (94), which masks *cis* elements. Only a threefold increase in acetylation is required to unfold the higher-order structure of a reconstituted chromatin template (94). H3-meK4 has a distribution similar to that of acH3 and acH4 (45, 58, 70), although establishment and maintenance of these modifications can be differentially regulated (45). These studies utilized chromatin fragments averaging 300 to 400 bp, and real-time PCR was used to quantitate the relative levels of immunoprecipitated DNA fragments under conditions of linearity (data not shown).

The chromosomal region upstream of the *NOTCH4* promoter extending to *TSBP* is saturated with repetitive DNA elements and has only one major region of sequence conservation approximately 4 kb upstream of the *NOTCH4* promoter (Fig. 2A). Little or no enrichment in acH3, acH4, and H3-

FIG. 2. Endothelial cell-specific histone modification pattern of the endogenous *NOTCH4* locus. (A) VISTA plot comparison of the human and murine *NOTCH4* sequences. The amplicons analyzed by ChIP analysis are indicated at the bottom. (B) ChIP analysis of acH3, acH4, and H3-meK4 at the *NOTCH4* locus in HUVECs and HeLa cells. Histone modifications were analyzed at the broadly expressed *cyclophilin* and erythroid cell-specific β-globin promoters as controls (mean ± SEM; at least three independent experiments). pro, promoter; in, intron; ex, exon.

meK4 was detected at this region (N4-1) or at two nonconserved regions at the 3′ side of the conserved region (N4-2 and -3) in either HUVECs or HeLa cells (Fig. 2B). By contrast, amplicons at the promoter (N4-pro1 and N4-pro2), intron 1 (N4-in1), and exon 3 (N4-ex3) revealed enrichments of acH3, acH4, and H3-meK4 in HUVECs but not in HeLa cells. Analysis of amplicons at intron 3, intron 8, exon 11, exon 18, and intron 20 (N4-in3, N4-in8, N4-ex11, N4-ex18, and N4-in20, respectively) revealed little or no enrichment of these histone modifications in HUVECs or HeLa cells. Thus, acH3, acH4, and H3-meK4 were not enriched throughout the open reading frame of *NOTCH4*. Recently, a genomics analysis indicated that multiple human genes lack high enrichments of these modifications downstream of the transcription start site (57). AcH3, acH4, and H3-meK4 were enriched at exon 30 in both HUVECs and HeLa cells. As exon 30 and the *G18* promoter overlap, the enrichments at N4-ex30 likely reflect the transcriptionally active state of the *G18* promoter. Histone modifications at the broadly active *cyclophilin* promoter and the erythroid cell-specific β-globin promoter were analyzed as positive and negative controls, respectively. The enrichments of acH3, acH4, and H3-meK4 at the *cyclophilin* promoter were comparable to enrichments at the *NOTCH4* promoter and at the 5′ portion of the *NOTCH4* open reading frame in HUVECs. Unlike the absolute endothelial cell-specific histone modification pattern of *NOTCH4*, the enrichments of histone modifications at the *cyclophilin* promoter were comparable in HUVECs and HeLa cells. No enrichments were detected at the β-globin promoter, consistent with the erythroid cell-specific histone modification pattern and expression of β-globin (25).

***NOTCH4* histone modification pattern delineates functional sequences.** Based on the nearly undetectable acH3 and acH4 at the *NOTCH4* locus in HeLa cells and the role of acetylation in increasing chromatin accessibility, it is unlikely that RNA Pol II would be able to access the *NOTCH4* promoter in these cells, although there are reports of basal transcription complex and factor recognition of heterochromatin (12, 86). Quantitative ChIP was used to test whether Pol II occupies the locus in a cell-type-specific manner. The greatest enrichments in Pol II occupancy were detected at the promoter amplicon (N4-pro2) closest to the transcription start site and the exon-30 amplicon (N4-ex30), which overlaps the *G18* promoter (Fig. 3A). Little or no enrichment was detected upstream of N4-pro2 or throughout the open reading frame. Little or no enrichment was detected in HeLa cells, with the exception of the N4-ex30 amplicon, in which the level of Pol II occupancy was comparable to the same region in HUVECs (Fig. 3A). Thus, Pol II occupancy of the *NOTCH4* locus was specific to that of HUVECs and was greatest at the *NOTCH4* and *G18* promoters, in which the enrichments of acH3, acH4, and H3-meK4 were also high (Fig. 2B). Pol II occupancy was almost 10-fold higher at the broadly expressed *cyclophilin* promoter versus the *NOTCH4* promoter, and no enrichments were detected at the β-globin promoter.

Although Pol II occupancy was clearly detected at the *NOTCH4* promoter, the enrichment levels were relatively low, which might reflect a low transcription rate or a small percentage of cells expressing *NOTCH4*. To distinguish between these mechanisms, RNA FISH was used to measure primary *NOTCH4* transcripts in single HUVECs. Transcription signals were detected at 23% (72 of 320) of *NOTCH4* alleles (Fig. 3B), and 36% of the cells in the population transcribed one (42 of 160 cells) or both (15 of 160 cells) alleles. Thus, *NOTCH4* is only transcribed in a small fraction of the HUVECs at any given time. These results are consistent with a mechanism in which *NOTCH4* undergoes transcriptional oscillations, which has been shown to be common for mammalian genes in other contexts (45a, 72a). RNA FISH analysis of primary transcripts from the broadly expressed *Rps18* gene approximately 30 Mb from *NOTCH4*, which encodes a riboprotein, revealed that 49% of *Rps18* alleles were active in HUVECs (data not shown). Double-labeling analysis of both *NOTCH4* and *Rps18* expression revealed that 76% of HUVECs had active *NOTCH4* and/or *Rps18* alleles (data not shown), indicating that the RNA FISH assay was capable of detecting expression in at least 76% of the cells in the population.

Strong enrichments of acH3, acH4, and H3-meK4 distal to a promoter are often hallmarks of transcriptional elements such as enhancers, locus control regions, and insulators (9). The lack of such epigenetic marks at the conserved region upstream of the *NOTCH4* promoter (Fig. 2) suggested that the transcriptional determinants of *NOTCH4* reside within the promoter and potentially at sites in the 5′ portion of the *NOTCH4* open reading frame. To assess whether the promoter and/or other regions contain determinants of endothelial cell specificity, transient transfections were conducted with *NOTCH4* promoter-luciferase reporter constructs in HUVECs and HeLa cells. The *NOTCH4* promoter reporter (N4-pro) was 80-fold more active than the pGL3basic reporter in HUVECs, whereas it was only 8-fold more active than pGL3basic in HeLa cells (Fig. 4). Moreover, the *NOTCH4* promoter was twice as active as the simian virus 40 (SV40) promoter (pGL3pro) in HUVECs, whereas it was ~4-fold less active than the SV40 promoter in HeLa cells. Since intron 1 is highly conserved and resides within the restricted region of enriched histone modifications, we tested whether intron 1 regulates promoter activity. A ~800-bp fragment of intron 1 cloned downstream of luciferase (N4-proIN1) induced a small increase in reporter activity in HUVECs and HeLa cells.

Although the region ~4 kb upstream of the promoter was not enriched in acH3, acH4, or H3-meK4, the high conservation of this region suggested that it might be functionally important. Additional constructs containing the *NOTCH4* promoter with ~4 kb of upstream sequence (N4-UPpro) or the *NOTCH4* promoter with a ~1-kb fragment spanning the kb −4 conserved region (N4-CRpro) were tested. These constructs had activities that differed only slightly from the *NOTCH4* promoter alone in HUVECs and HeLa cells (Fig. 4). Thus, a ~650-bp promoter fragment was sufficient to confer cell-type-specific transcriptional activity in HUVECs and HeLa cells in a transient transfection assay. Furthermore, intron 1 and the upstream conserved region lack enhancer activity, as defined by the ability to strongly activate a reporter gene in a transient transfection assay.

Cell-type-specific AP-1 complexes occupy the *NOTCH4* promoter and confer high-level transcription in endothelial cells. Analysis of conserved sequence motifs within the *NOTCH4* promoter revealed several prospective *cis* elements, including an AP-1 motif. EMSA was performed with overlapping oligo-

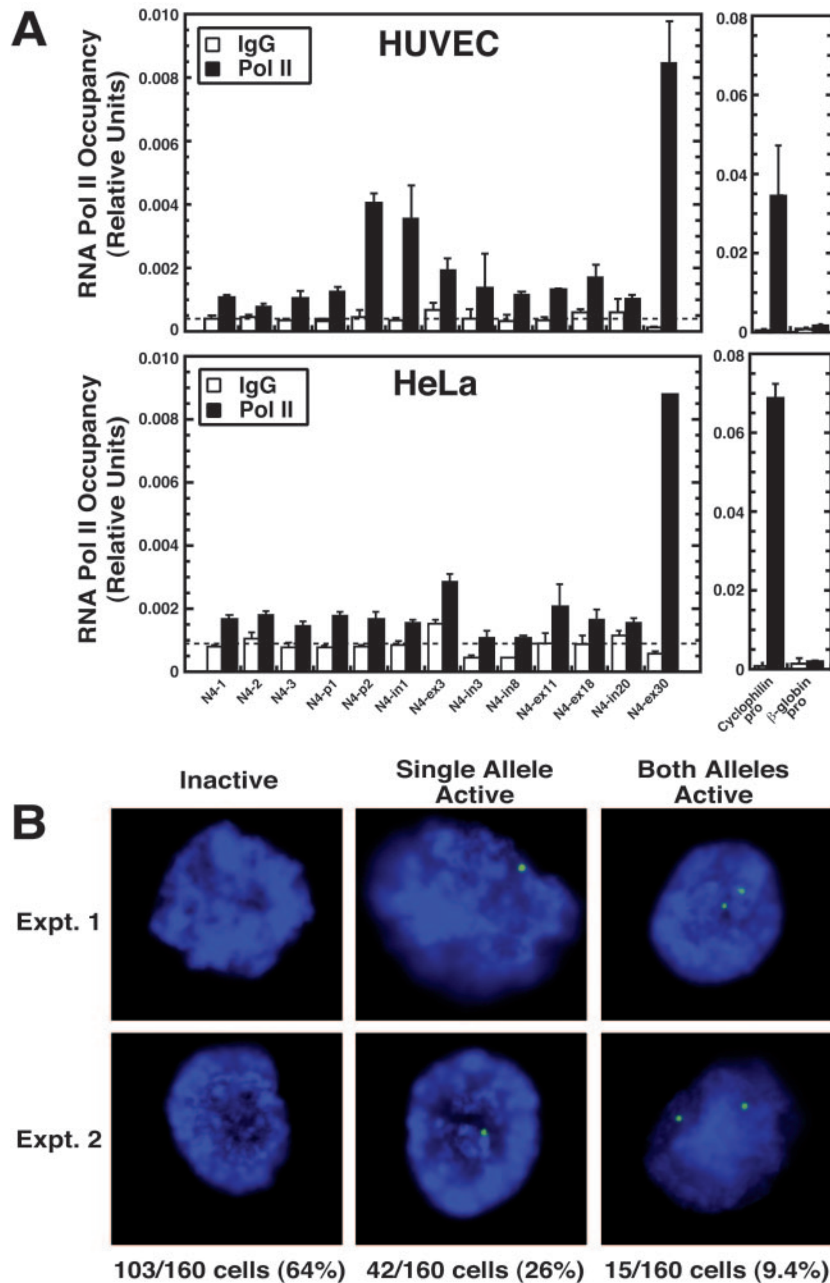


FIG. 3. RNA Pol II selectively occupies the *NOTCH4* locus in endothelial cells, but only a small percentage of the *NOTCH4* alleles are transcriptionally active. (A) ChIP analysis of Pol II occupancy at the *NOTCH4* locus in HUVECs (top) and HeLa cells (bottom). Pol II binding was analyzed at the broadly expressed *cyclophilin* and erythroid cell-specific β-globin promoters as controls (mean ± SEM; at least three independent experiments). N4-p1 and N4-p2, N4-pro1 and N4-pro2. (B) In situ RNA FISH analysis of endogenous *NOTCH4* primary transcript levels in HUVECs. *NOTCH4* transcription was detected by RNA FISH with an antisense, digoxigenin-labeled, single-stranded DNA probe and visualized with fluorescein isothiocyanate-labeled antibodies. Representative pictures (two independent experiments) of a transcriptionally inactive cell and cells expressing either one allele or both alleles of *NOTCH4* are shown. The numbers and percentages of transcriptionally inactive cells and cells expressing either one or two alleles of *NOTCH4* are shown at the bottom.

nucleotides spanning ~300 bp of *NOTCH4* promoter sequence to test whether these sequences mediate assembly of unique protein-DNA complexes with HUVEC versus HeLa nuclear extracts. Whereas no complexes were uniquely formed with HUVEC extracts (data not shown), the HUVEC complex that assembled on the AP-1(wt) oligonucleotide (Fig. 5A) had a slightly faster mobility in the gel than the corresponding HeLa complex (Fig. 5B). The complexes were abrogated by inclusion of a 100-fold excess of unlabeled AP-1(wt) probe, whereas an unrelated oligonucleotide containing two GATA motifs (GATA) had no effect.

To determine if the AP-1 motif mediates complex formation, EMSA analysis was conducted with the labeled AP-1(wt) oligonucleotide, an AP-1 motif-mutated oligonucleotide [AP-

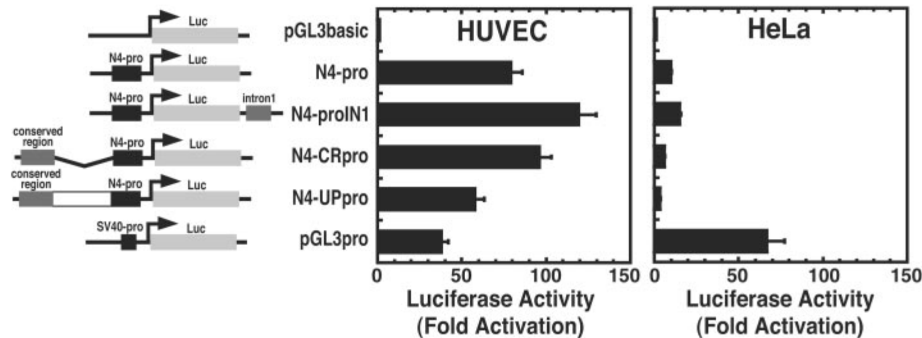


FIG. 4. Endothelial cell-specific *NOTCH4* promoter activity. HUVECs and HeLa cells were transiently transfected with the following luciferase reporter constructs: pGL3basic that lacks a promoter, pGL3basic containing the *NOTCH4* promoter (N4-pro), pGL3basic containing the *NOTCH4* promoter and intron 1 (N4-proIN1), pGL3basic containing the *NOTCH4* promoter and an upstream conserved region (N4-CRpro), pGL3basic containing the entire upstream region including the conserved region and the *NOTCH4* promoter (N4-UPpro), and pGL3basic containing the SV40 promoter (pGL3pro). Luciferase activity was normalized by the protein content of the lysates (mean ± SEM; five independent experiments). Relative luciferase activities are shown as fold activation, with pGL3basic activity designated as 1.0.

1(mut-1)], and an oligonucleotide in which a sequence of no known function downstream of the AP-1 motif was mutated [AP-1(mut-2)]. Mutation of the AP-1 motif [AP-1(mut-1) probe], but not the downstream sequence [AP-1(mut-2) probe], abolished complex formation (Fig. 5C). Competitive binding assays were conducted with a 100-fold excess of the unlabeled AP-1(wt) oligonucleotide or the GATA oligonucleotide. The AP-1(wt) oligonucleotide abrogated binding of the HUVEC factors to the AP-1(wt) and AP-1(mut-2) probes, whereas the GATA oligonucleotide had no effect (Fig. 5C). These results strongly indicate that AP-1 or a highly related factor binds the conserved AP-1 motif of the *NOTCH4* promoter in vitro.

AP-1 exists in multiple heterodimeric configurations, which can have different biochemical and biological activities (2, 7, 14, 18–20, 26, 30, 43, 44, 54, 55, 68, 78, 80, 90, 110). For example, tethering AP-1 subunits via a flexible peptide linker revealed that c-Jun–Fra-2, but not c-Jun–Fra-1 or c-Jun–c-Fos, inhibited growth arrest of immortalized fibroblasts (4). The slightly different mobility of the HUVEC and HeLa AP-1 complexes suggested that they contain distinct AP-1 components or that the components differ in posttranslational modifications. To define the subunit composition of HUVEC versus HeLa AP-1 complexes that form on the *NOTCH4* promoter, extracts were preincubated with antibodies against multiple Fos and Jun family members, and DNA binding was measured by EMSA. HUVEC complexes were supershifted or inhibited by antibodies reacting with multiple Fos species (All-Fos), Fra-1, multiple Jun species (All-Jun), c-Jun [H-79 and c-Jun(N)], and JunD (Fig. 5D, top). HeLa complexes were supershifted or inhibited by antibodies reacting with All-Fos, All-Jun, JunD, and JunB (Fig. 5D, bottom). The greatest difference in the AP-1 complexes is that the HUVEC AP-1 complex preferentially contained the Fos family member Fra-1. A second major difference between HUVEC and HeLa AP-1 complexes is that the HUVEC complexes contained c-Jun almost exclusively, whereas the HeLa complexes contained JunB predominantly.

As HUVEC and HeLa AP-1 complexes that assemble on the *NOTCH4* promoter in vitro differ in composition, it is attractive to propose that the differential composition constitutes a mechanism that confers or contributes to endothelial cell specificity of *NOTCH4* transcription. AP-1 integrates information via diverse cellular signals (20, 88), including signals initiated by FGF-2 (66, 67), a vascular angiogenic remodeling factor. FGF-2 signaling activates mitogen-activated protein kinase and extracellular signal-regulated kinase, which stimulate AP-1 activity via regulation of the synthesis and activity of AP-1 components (66, 67).

The different compositions of the HUVEC and HeLa AP-1 complexes might result from the cell-type-specific expression or activation of AP-1 components. To determine the basis of the distinct AP-1 complexes, the relative levels of AP-1 components in HUVEC and HeLa nuclear extracts were measured by Western blotting (Fig. 5E). Multiple Fos species were detected with the All-Fos antibody in both extracts, consistent with the ability of this antibody to supershift AP-1 complexes formed with both extracts. By contrast, Fra-1 levels were high in HUVEC extracts but were nearly undetectable in HeLa extracts. The inability of the anti-Fra-1 antibody to supershift or inhibit the HeLa AP-1 complex (Fig. 5D) can therefore be explained by low levels of Fra-1 in HeLa cells. The All-Jun antibody detected a major species in HUVEC extracts and multiple low-level components in HeLa extracts. HUVEC extracts contained very little JunB compared to HeLa extracts, consistent with the supershift results. JunD and tubulin were detected in the two extracts at nearly identical levels. These results indicate that the differential expression of AP-1 subunits in HUVECs versus HeLa cells gives rise to cell-type-specific complexes that assemble on the conserved AP-1 motif of the *NOTCH4* promoter in vitro.

Despite the utility of EMSA in identifying prospective *trans*-acting factors, DNA recognition motifs in cells are often inaccessible. Quantitative ChIP analysis was used to determine whether AP-1 complexes occupy the *NOTCH4* promoter in HUVECs and HeLa cells. Multiple AP-1 antibodies were used to test for AP-1 occupancy in HUVECs at the promoter of the established AP-1 target gene *IL-8* (Fig. 6A). The only antibodies that yielded specific signals were the All-Fos and Fra-1 antibodies (Fig. 6B). Under conditions in which Fos and Fra-1

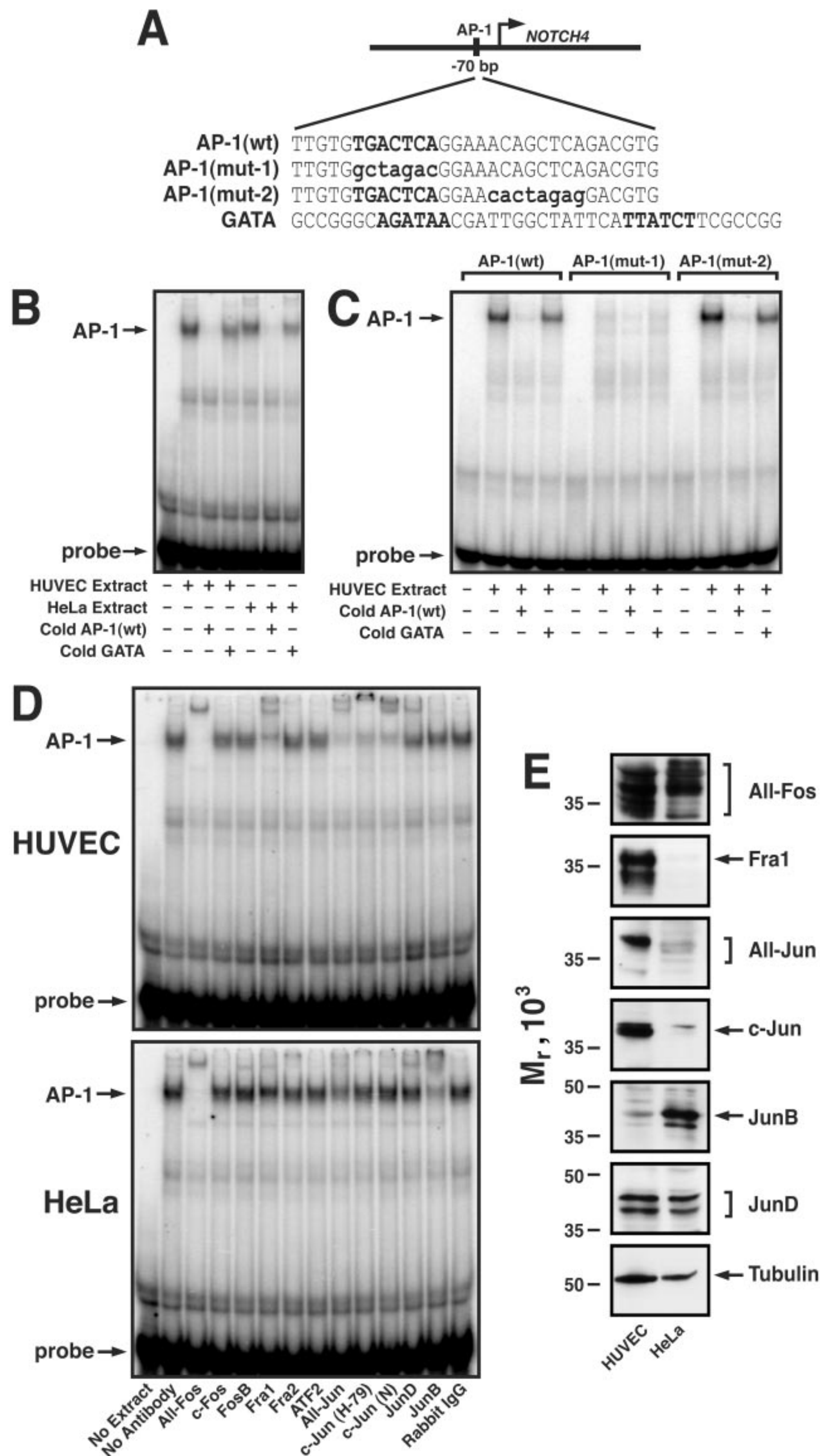


FIG. 5. Assembly of AP-1 complexes on a conserved AP-1 motif of the *NOTCH4* promoter in vitro. (A) Sequences of oligonucleotides used in the EMSA: AP-1(wt), an oligonucleotide consisting of a *NOTCH4* promoter sequence with a conserved AP-1 motif; AP-1(mut-1), an oligonucleotide with a mutated AP-1 motif; AP-1(mut-2), an oligonucleotide with a sequence downstream of the AP-1 motif mutated; GATA, an oligonucleotide with two GATA binding motifs. The conserved AP-1 motif and the GATA motifs are indicated in uppercase boldface type. The specific bases mutated are indicated in lowercase boldface type. The GATA oligonucleotide was used as a nonspecific competitor. (B) EMSA analysis of nucleoprotein complex assembly on the AP-1(wt) oligonucleotide with nuclear extracts from HUVECs or HeLa cells. Nuclear extracts (5 μg) were preincubated with or without a 100-fold excess of unlabeled AP-1(wt) or GATA oligonucleotides and then incubated with ^{32}P-labeled AP-1(wt) oligonucleotide (40 fmol). (C) AP-1 motif-specific mutation abolishes its complex formation with HUVEC extract. Nuclear extract (5 μg) from HUVECs was preincubated with or without 100-fold excess of unlabeled AP-1(mut-1) or AP-1(mut-2) and then incubated with ^{32}P-labeled oligonucleotide (40 fmol). (D) Unique AP-1 complexes assemble on the *NOTCH4* promoter in HUVECs versus HeLa cells. Nuclear extract (5 μg) from HUVECs (top) or HeLa cells (bottom) was preincubated with the indicated antibodies (reading from left to right, lanes 3 to 14) and then incubated with ^{32}P-labeled AP-1(wt) oligonucleotide (40 fmol). Lane 1, reaction mixture lacking nuclear extract; lane 2, reaction mixture lacking antibody. (E) Nuclear extract (20 μg) from HUVECs or HeLa cells was analyzed by Western blotting with antibodies against specific AP-1 components or tubulin as a control.

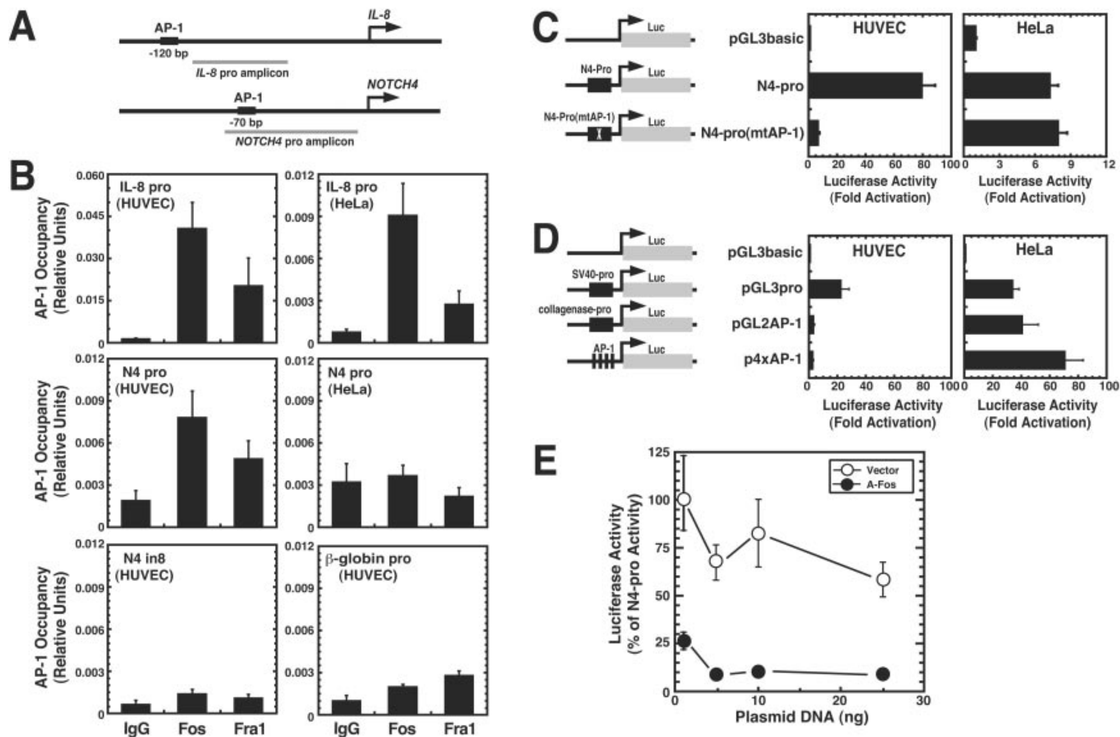


FIG. 6. Endothelial cell-specific occupancy of the *NOTCH4* promoter by AP-1 and preferential requirement of the AP-1 motif for promoter activity in endothelial cells. (A) Diagram of the *IL-8* promoter and *NOTCH4* promoter amplicons used in the ChIP assay. The amplicon either includes or is very close to the conserved AP-1 motif. (B) Quantitative ChIP analysis of AP-1 occupancy at the *IL-8* promoter and *NOTCH4* promoter in HUVECs and HeLa cells (mean ± SEM; at least three independent experiments). Anti-Fos antibody against multiple Fos components and anti-Fra-1 antibody were used in the ChIP assay. Rabbit IgG was used as a control. AP-1 occupancy was also analyzed at intron 8 of *NOTCH4* and the β-globin promoter as negative controls. pro, promoter; in, intron. (C) The conserved AP-1 motif on the *NOTCH4* promoter is required for endothelial cell-specific transcription. HUVECs and HeLa cells were transiently transfected with the following luciferase reporter constructs: pGL3basic, pGL3basic containing *NOTCH4* promoter (N4-pro), or pGL3basic containing the *NOTCH4* promoter with a mutated AP-1 motif [N4-pro(mtAP-1)] (mean ± SEM; at least three independent experiments). (D) HUVECs and HeLa cells were transiently transfected with the following luciferase reporter constructs: pGL3basic, pGL3basic containing the SV40 promoter (pGL3pro), and pGL2basic containing either the collagenase promoter (pGL2AP-1) or four synthetic AP-1 motifs (p4xAP-1). Luciferase activity was normalized by the protein content of the lysates. Relative luciferase activities are shown as fold activation, with the activity of pGL3basic designated as 1.0 (mean ± SEM; at least three independent experiments). (E) HUVECs were transiently cotransfected with the N4-pro reporter construct and increasing amounts of the blank vector pCMV500 or an expression vector encoding the dominant-negative AP-1 molecule A-Fos. Relative luciferase activities, normalized by the protein content of the lysates, are shown as the percentage of the activity obtained with the N4-pro construct (mean ± SEM; at least three independent experiments).

occupied the *IL-8* promoter, occupancy was also detected at the *NOTCH4* promoter in HUVECs but not in HeLa cells. By contrast, no occupancy was detected at *NOTCH4* intron 8 (N4-in8) or at the β-globin promoter. As expected from the results of Fig. 3B, which show that only a small percentage of the HUVECs express *NOTCH4* primary transcripts, the All-Fos and Fra-1 enrichment levels were relatively low.

We tested whether the preferential activity of the *NOTCH4* promoter in transfection assays in HUVECs versus HeLa cells requires the AP-1 motif. Mutation of the AP-1 motif nearly abolished the strong reporter activity conferred by the *NOTCH4* promoter in HUVECs (Fig. 6C). As mutation of the AP-1 motif did not affect the low activity of the promoter in HeLa cells (Fig. 6C), the HUVEC AP-1 complexes are uniquely able to activate the *NOTCH4* promoter. Taken together with the selective AP-1 occupancy of endogenous *NOTCH4* chromatin in HUVECs (Fig. 6B), these results indicate that the unique ability of a cell-type-specific AP-1 complex to access or to form a stable complex with the *NOTCH4* chromatin template contributes to or confers endothelial cell-specific *NOTCH4* transcription. Since antibodies against other AP-1 components were not efficacious in the ChIP assay, one cannot rule out the possibility that other AP-1 components also occupy the *NOTCH4* locus.

Since the HUVEC and HeLa AP-1 complexes have distinct compositions, HUVEC AP-1 might have an intrinsically greater transactivation capacity, which could explain the results shown in Fig. 6C. We tested this by measuring the activity of two AP-1 reporter constructs in HUVECs and HeLa cells. These constructs, containing either the collagenase promoter with a single AP-1 motif or four synthetic AP-1 motifs, are known to be activated only by AP-1. The AP-1 reporters had considerably higher activities in HeLa cells, whereas pGL3pro had high activities both HeLa cells and HUVECs (Fig. 6D). This result indicates that AP-1 complexes are not more efficacious in HUVECs than HeLa cells in activating transcription

through AP-1 motifs. A model in which AP-1 functionally interacts with other components of the *NOTCH4* promoter can therefore explain the preferential requirement of the *NOTCH4* promoter AP-1 motif, and this interaction occurs in HUVECs but not HeLa cells.

As endogenous Fos species occupy the endogenous *NOTCH4* promoter in HUVECs but not HeLa cells and as the AP-1 motif is selectively required for promoter activity in HUVECs but not HeLa cells, it is highly likely that endogenous AP-1 confers high-level *NOTCH4* promoter activity in HUVECs. To further test this, we asked whether expression of a dominant-negative molecule that antagonizes endogenous AP-1, A-Fos (71), affects *NOTCH4* promoter activity in HUVEC cells. Transfecting increasing amounts of A-Fos expression vector strongly reduced promoter activity, whereas the blank vector pCMV500 had little effect (Fig. 6E). This result solidifies a role for endogenous AP-1 in conferring activation through the AP-1 motif of the *NOTCH4* promoter.

Endothelial cell growth supplement reprograms the *NOTCH4* gene in HeLa cells from a repressed to a transcriptionally active state. HUVECs are cultured in media containing a low-serum growth supplement with FGF-2, epidermal growth factor, hydrocortisone, and heparin. FGF-2, an important regulator of vascular angiogenic remodeling, activates AP-1-responsive transcriptional elements and AP-1 DNA binding activity in cultured murine corneal cells and a calvarial osteoblast cell line (66, 67). We reasoned that the signaling molecules in the supplement might induce components of the HUVEC-specific AP-1 complexes both in endothelial cells and in nonendothelial cells. We tested this by measuring *NOTCH4* promoter activity and the expression of AP-1 components in HeLa cells cultured for 24 h with the supplement. The supplement increased the activity of the N4-pro reporter construct ~3-fold without affecting the activity of the N4-pro(mtAP-1) reporter containing a mutated AP-1 motif or the SV40 promoter-containing reporter pGL3pro (Fig. 7A). Fra-1, c-Jun, and JunB expression were strongly induced by the supplement, whereas low-level JunD expression was unchanged (Fig. 7B). Thus, signal-dependent reconfiguration of the expression profile of AP-1 components, yielding a profile that resembles the HUVEC profile, is associated with elevated *NOTCH4* promoter activity.

We tested whether the supplement-mediated induction of AP-1 components is associated with changes in expression of the endogenous, inactive *NOTCH4* gene in HeLa cells. HeLa cells were derived from an epidermoid carcinoma of the cervix and have epithelial cell properties (34). HeLa cells might lack multiple factors required for *NOTCH4* transcription, or transcriptional repression might result from a deficiency of specific AP-1 components, such as those expressed in HUVECs. Quantitative RT-PCR revealed that culturing HeLa cells in medium containing the growth supplement for 24 h under conditions that induce AP-1 components and increase *NOTCH4* promoter activity in transient transfections (Fig. 7B) was sufficient to reprogram the endogenous *NOTCH4* gene from a repressed state to a transcriptionally active state (Fig. 7C). However, the *NOTCH4* expression level was considerably lower than in HUVECs. The supplement-mediated induction of *NOTCH4* expression was confirmed by quantitative RT-PCR analysis with an independent primer set (NOTCH4-2), was maximal after 24 h of treatment, and persisted for at least 6 days in the continued presence of the supplement (data not shown). *NOTCH1* was expressed in HeLa cells and was not induced by the endothelial cell supplement (Fig. 7C). To determine whether the endothelial cell supplement induces expression of other endothelial cell-specific genes, we measured the expression of genes encoding endothelial cell nitric oxide synthase (*eNOS*), von Willebrand factor (*vWF*), and the type 2 receptor for vascular endothelial cell growth factor (*Flk-1*) (Fig. 7D). The supplement had little if any effect on the expression of these genes. This result indicates that signaling molecules in the supplement have the capacity to induce AP-1 components that are expressed in HUVECs and to selectively activate endogenous *NOTCH4* transcription in HeLa cells.

AP-1-dependent high-level activity of *NOTCH4* promoter in vascular endothelium in vivo. To test whether the *NOTCH4* promoter AP-1 motif is important for endothelial cell-specific transcription in vivo, *NOTCH4* promoter-*lacZ* fusion constructs were assayed in F_0 E10.5 transgenic mouse embryos (Fig. 8). A construct containing the endothelial cell-specific *Tie-2* enhancer and promoter (Tie-2–LacZ) (81) was used as a control. As expected, analysis of whole mount embryos revealed that Tie-2–LacZ was expressed in the vascular endothelium (Fig. 8A). By contrast, the *NOTCH4* promoter alone (N4-pro-LacZ) was almost completely inactive (Fig. 8A), although 3 of 15 transgene-positive embryos revealed ectopic staining (Fig. 8B). Inclusion of intron 1 in the N4-pro-LacZ construct (N4-proIN1-LacZ) resulted in endothelial cell expression in 7 of 17 embryos with a similar frequency of ectopic expression (Fig. 8). Mutation of the *NOTCH4* promoter AP-1 motif in the presence of intron 1 [N4-pro(mtAP-1)IN1-LacZ] strongly reduced LacZ expression, although careful analysis revealed very low level expression in subregions of the vascular endothelium and at ectopic sites in 10 of 19 transgene-positive embryos (Fig. 8). Inclusion of an additional ~4 kb of sequence upstream of the *NOTCH4* promoter (N4-UPpro-LacZ) conferred high-level expression in vascular endothelium but only at a low frequency (2 of 14 transgene-positive embryos) (Fig. 8). Extensive attempts (analysis of >150 embryos) to analyze an additional construct containing both the upstream sequences and intron 1 were unsuccessful, as the stable integration of this construct in chromosomal DNA could not be reliably established (data not shown).

To investigate the vascular expression pattern of *NOTCH4* promoter-*lacZ* constructs in detail, sections of E10.5 embryos were analyzed for LacZ expression in endothelial cells of the dorsal aorta (Fig. 9A), yolk sac (Fig. 9B), neural tube (data not shown), and the endocardium (data not shown). The Tie-2–LacZ and N4-UPpro-LacZ constructs were expressed in endothelial cells of all four anatomical regions (Fig. 9C). The N4-proIN1-LacZ construct was expressed in endothelial cells of the dorsal aorta and the neural tube (Fig. 9C). By contrast, mutation of the AP-1 motif [N4-pro(mtAP-1)IN1-LacZ] abolished expression in endothelial cells of the dorsal aorta and reduced expression in endothelial cells of the neural tube (Fig. 9C).

The transgenic analysis indicated that a transgene containing the *NOTCH4* promoter alone is insufficient to confer expression in vascular endothelium in E10.5 transgenic mouse embryos. However, either intron 1 or sequences upstream of the

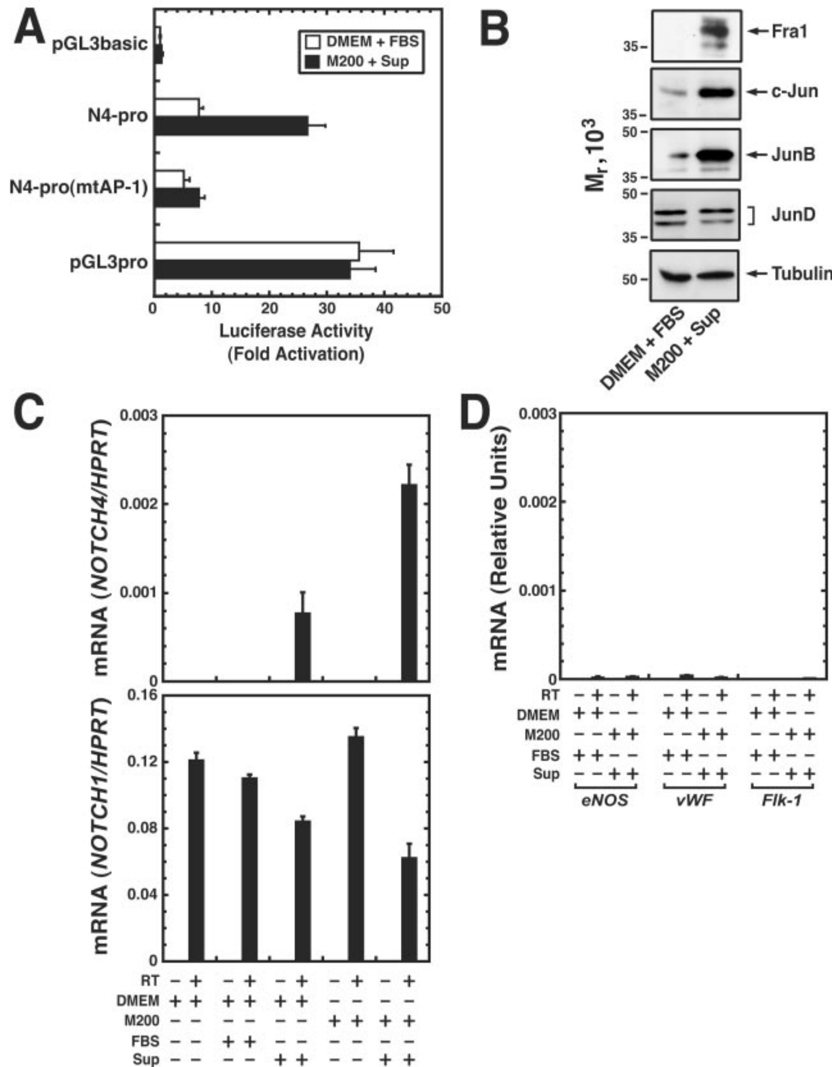
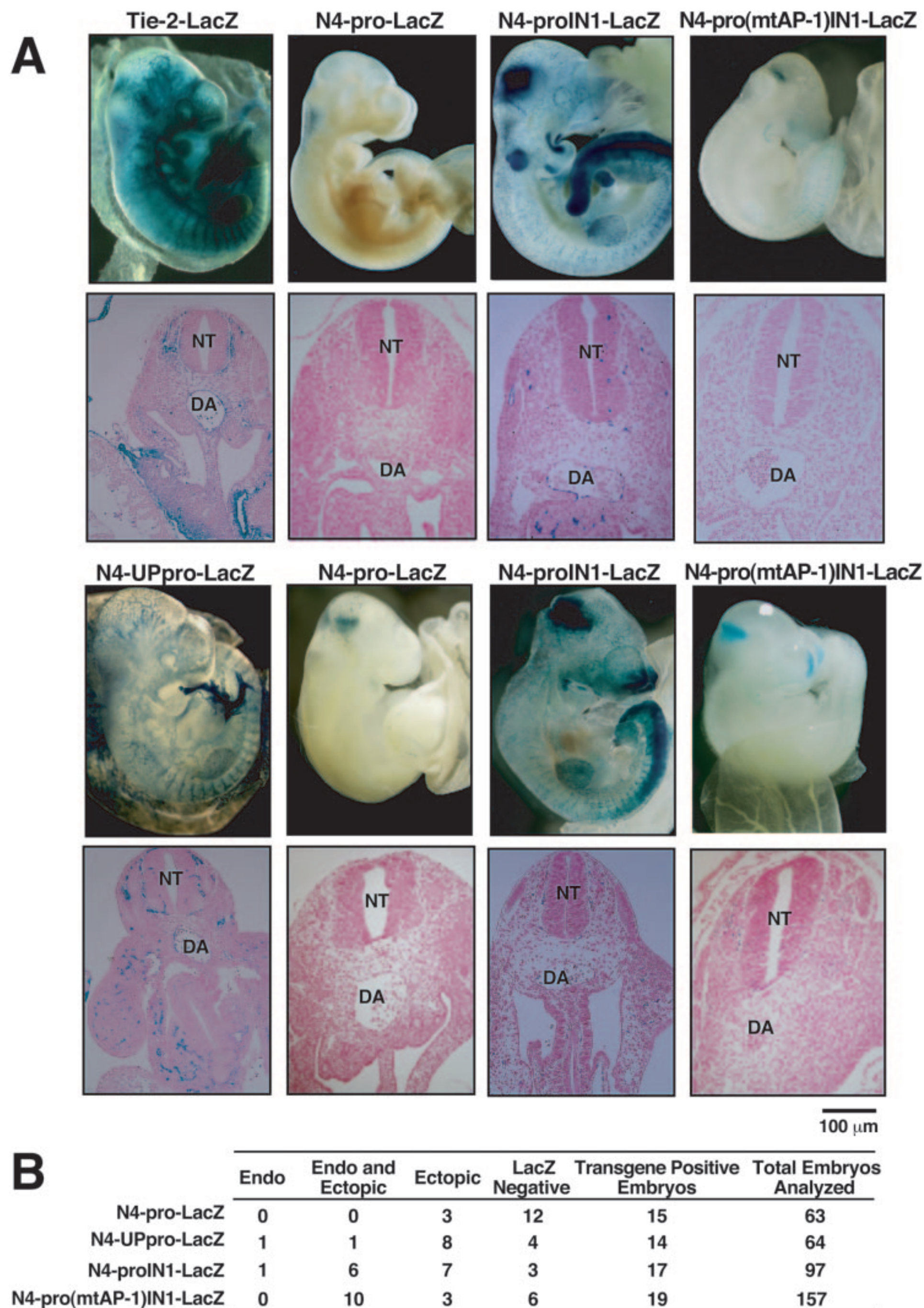


FIG. 7. Endothelial cell growth supplement induces AP-1 components and reprograms endogenous *NOTCH4* from a repressed to a transcriptionally active state in HeLa cells. (A) HeLa cells were transiently transfected with the following luciferase reporter constructs: pGL3basic, pGL3basic containing *NOTCH4* promoter (N4-pro), pGL3basic containing the *NOTCH4* promoter with a mutated AP-1 motif [N4-pro(mtAP-1)], or pGL3basic containing the SV40 promoter (pGL3pro). Transfected HeLa cells were cultured in normal medium (DMEM plus 10% FBS) or in the endothelial medium (Medium 200 plus endothelial cell supplement) for 24 h before harvest. Luciferase activity was normalized by the protein content of the lysates. Relative luciferase activities are shown as fold activation, with the activity of pGL3basic designated as 1.0 (mean ± SEM; three independent experiments). (B) Nuclear extract (20 μg) from HeLa cells cultured in normal medium or endothelial medium was analyzed by Western blotting with antibodies against specific AP-1 components or tubulin as a control. (C) Quantitative real-time RT-PCR analysis of *NOTCH4* and *NOTCH1* mRNA expression in HeLa cells cultured in DMEM or Medium 200 with or without 10% FBS or the endothelial cell supplement. *NOTCH4* and *NOTCH1* transcripts were also measured in HUVECs cultured in endothelial cell medium. The levels of *NOTCH4* and *NOTCH1* mRNA were normalized by *HPRT* mRNA. The *NOTCH4*/*HPRT* or *NOTCH1*/*HPRT* mRNA ratios in HUVEC cells were designated as 1.0. The graph shows the expression pattern of *NOTCH4* (top) and *NOTCH1* (bottom) in HeLa cells (mean ± SEM; three independent experiments). (D) HeLa cells and HUVECs were cultured as described in panel C. *eNOS*, *vWF*, and *Flk-1* mRNA levels were normalized by *HPRT* mRNA levels, and the transcript ratios in HUVECs were designated as 1.0. The graph shows the expression patterns of *eNOS*, *vWF*, and *Flk-1* in HeLa cells (mean ± SEM; three independent experiments).

promoter conferred promoter activity in vascular endothelium. Intron 1 and the upstream sequences therefore have intrinsic enhancer activity in vivo, which was not apparent in transient transfections in HUVECs and HeLa cells (Fig. 4). The strongest expression in vascular endothelium was achieved with the N4-UPpro-LacZ construct. However, based on the low frequency (14%) of N4-UPpro-LacZ expression in transgenic embryos, this construct could not be used for additional mechanistic analysis. By contrast, N4-proIN1-LacZ had a considerably higher frequency of expression (41%), making additional mechanistic analysis tractable. Mutation of the AP-1 motif in the N4-proIN1-LacZ construct strongly reduced expression in vascular endothelium, even though intron 1 was present, indicating that AP-1 cooperates with intron 1 to confer expression in vivo.

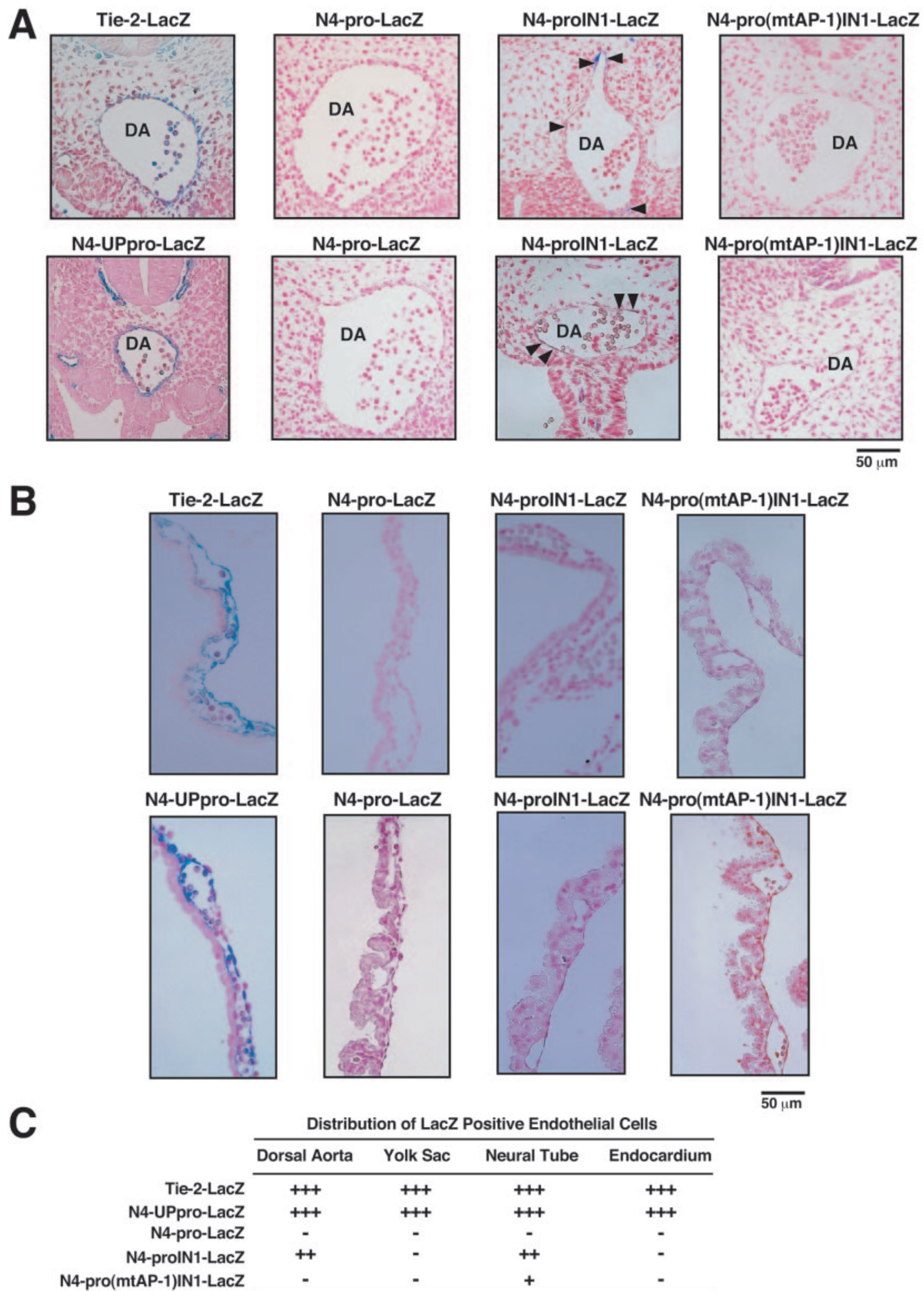
Our results, showing that the *NOTCH4* promoter has cell-



	Endo	Endo and Ectopic	Ectopic	LacZ Negative	Transgene Positive Embryos	Total Embryos Analyzed
N4-pro-LacZ	0	0	3	12	15	63
N4-UPpro-LacZ	1	1	8	4	14	64
N4-proIN1-LacZ	1	6	7	3	17	97
N4-pro(mtAP-1)IN1-LacZ	0	10	3	6	19	157

FIG. 8. Requirement of *NOTCH4* promoter AP-1 motif for high-level promoter activity in E10.5 transgenic mouse embryos. (A) Two representative pictures of E10.5 transgenic mouse embryos (top) and transverse embryo sections (bottom) containing the N4-pro-LacZ, N4-proIN1-LacZ, and N4-pro(mtAP-1)IN1-LacZ transgenes are shown. One representative picture of Tie-2–LacZ and N4-UPpro-LacZ transgenic embryos is shown. DA, dorsal aorta; NT, neural tube. (B) The table summarizes the total numbers of embryos analyzed, the number of embryos that genotyped positive for the *lacZ* transgenes, and the number of embryos that stained positive or negative for LacZ.

type-specific activity in cultured cells but is insufficient to recapitulate this activity in transgenic mice, are reminiscent of results obtained from analyses of other tissue-specific genes, such as the β-globin genes. Extensive studies with β-globin locus transgenes in mice have revealed transcriptional silencing and ectopic expression with constructs containing only the β-globin promoters (48a). Subsequent efforts identified the β-globin locus control region, which overcomes chromosome position effects and confers copy number-dependent expression in transgenic mice (24a, 29a). Importantly, the β-globin promoters, analogous to the *NOTCH4* promoter, bind factors that confer cell-type-specific transcription in cultured cells.



	Dorsal Aorta	Yolk Sac	Neural Tube	Endocardium
Tie-2-LacZ	+++	+++	+++	+++
N4-UPpro-LacZ	+++	+++	+++	+++
N4-pro-LacZ	-	-	-	-
N4-proIN1-LacZ	++	-	++	-
N4-pro(mtAP-1)IN1-LacZ	-	-	+	-

FIG. 9. *NOTCH4* transgene expression in the vasculature of E10.5 mouse embryos. (A) Expression of *NOTCH4* promoter-*lacZ* constructs in vascular endothelium. The photomicrographs show embryo sections, which reveal the dorsal aorta (DA) of E10.5 transgenic mouse embryos with the indicated transgenes. The arrowheads indicate endothelial cells with LacZ positivity. (B) Expression of *NOTCH4* promoter-*lacZ* constructs in sections of E10.5 transgenic mouse embryo yolk sac. (C) The table summarizes a quantitative analysis of the distribution of LacZ-positive endothelial cells in the dorsal aorta, neural tube, endocardium, and the yolk sac. +++, all endothelial cells were LacZ positive; ++, intermediate number of LacZ-positive endothelial cells; +, rare LacZ-positive endothelial cells; −, LacZ-negative endothelial cells.

The locus control region establishes an additional level of regulation, which allows promoter activity to be manifested at ectopic chromosomal sites in mice. Mechanistically, these results showing that isolated promoters with intrinsic cell type specificity determinants need additional regulatory sequences in vivo can be explained by a requirement for chromatin modifying or remodeling activities conferred by the additional sequences, e.g., by intron 1 and upstream sequences of the *NOTCH4* locus. Such activities can be crucial for counteracting chromatin-mediated repression of promoter-only constructs integrated at ectopic chromosomal sites.

An AP-1–Notch4 axis for angiogenic vascular remodeling and oncogenesis? Targeted deletions in mice have established an important role of Fra-1 (83), JunB (82), and Notch4 (47) in vascular development. *fra-1*-null mice die at E10 to E10.5 due to severe reduction in vascular endothelial cells and defective placental vascularization (83). Large vessels develop in the chorionic plate but fail to undergo vascular remodeling. Similarly, *junB*-null mice die at E8.5 to E10 due to defective vascularization of extraembryonic tissues, including defective vascularization of the placental labyrinth (82). Given the sequence homology of AP-1 subunits, the scope of these vascular phenotypes might be restricted due to functional redundancies in specific cell types. Functional redundancies among AP-1 family members have been revealed by gene targeting experiments in mice, including the knock in of *junB* into c-*jun*-null mice, which rescues defects in liver and cardiac development (74). Moreover, the knock in of *fra-1* into c-*fos*-null mice rescues bone development and light-induced photoreceptor apoptosis (24). In the case of Fra-1 and c-Fos, this is not an absolute redundancy, however, as Fra-1 does not rescue defective transcriptional responses in c-*fos*-null fibroblasts (24).

Our analysis has clearly shown that AP-1 preferentially activates the *NOTCH4* promoter in HUVECs versus HeLa cells (Fig. 6); the conserved AP-1 motif of the *NOTCH4* promoter, in the context of the N4-proIN1-LacZ construct, is crucial for promoter activity in transgenic mouse embryos (Fig. 8). Thus, it is attractive to consider the possibility that vascular phenotypes associated with *fra-1* and *junB* knockouts might result, in part, from defective regulation of *NOTCH4* transcription. It is unlikely, however, that such phenotypes in the *Fra-1*- and *JunB*-null mice can be explained solely by defective *Notch4* transcription, since *Notch4*-null mice do not have detectable vascular phenotypes (29, 47). Almost certainly, AP-1 regulates additional genes besides *Notch4* that are dysregulated in the *Fra-1* and *JunB* knockout mice and that mediate angiogenic remodeling. Given the collective requirement of Notch1 and Notch4 for angiogenic remodeling during mouse embryogenesis (29, 47), we propose that AP-1-dependent *Notch4* transcription is required to establish an AP-1–Notch4 vascular angiogenic pathway.

Based on our finding that the endothelial growth supplement reprograms the *NOTCH4* gene in HeLa cells from a repressed to a transcriptionally active state, it is attractive to propose that aberrant signaling in nonendothelial cells, for example in breast cancer cells, ectopically activates *NOTCH4* expression. This mode of establishing ectopic Notch4 signaling would have important consequences for the control of cellular proliferation and differentiation, since many examples exist in which experimental strategies that deregulate Notch signaling are oncogenic (3, 6, 10, 23, 76, 101). Intriguingly, NIC-1 represses expression of the endogenous AP-1 target genes *IL-8* and *MMP-1* and represses AP-1-mediated transactivation in transfection assays (15, 16). Thus, activation of Notch1 signaling might suppress AP-1-mediated induction of *NOTCH4* expression, establishing a crucial molecular link between Notch1 and Notch4 signaling. The studies described herein, dissecting the mechanism underlying endothelial cell-specificity of *NOTCH4* transcription, have laid the groundwork for identifying the factors and signals required to reprogram the repressed *NOTCH4* gene and for testing the validity of our model that such factors contribute to oncogenesis via *NOTCH4* deregulation.

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