Endothelial Cells Express a Novel, Tumor Necrosis Factor-α-regulated Variant of HOXA9*

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The expression of the class 1 homebox (HOX) family of "master control" transcription factors has been studied principally in embryogenesis and neoplasia in which HOX genes play a critical role in cell proliferation, migration, and differentiation. We wished to test whether HOX family members were also involved in a differentiation-like process occurring in normal, diploid adult cells, that is, cytokine-induced activation of endothelial cells (EC). Screening of a human EC cDNA library yielded several members of the A and B groups of HOX transcription factors. One clone represented a novel, alternatively spliced variant of the human HOXA9 gene containing a new exon and the expression of which was driven by a novel promoter. This variant termed HOXA9EC appeared restricted to cells of endothelial lineage, i.e. expressed by human EC from multiple sources, but not by fibroblasts, smooth muscle cells, or several transformed cell lines. HOXA9EC mRNA was rapidly down-regulated in EC in response to tumor necrosis factor-α due to an apparent reduction in transcriptional rate. Reporter construct studies showed that the 400 base pairs of genomic DNA directly 5′ to the transcription initiation site of HOXA9EC contained the information required for both up-regulation in response to cotransfection with a HOXA9EC expression vector and tumor necrosis factor-α-dependent down-regulation of this gene. These results provide evidence of a novel HOX family member that may participate in either the suppression or the genesis of EC activation.

Little is known about the molecular mechanisms that regulate and maintain endothelial cells (EC) in their differentiated state or the types of aberrations in the expression of regulatory genes that lead to an activated or pathological state of these cells (1). One important category of nuclear proteins which we hypothesized to be involved in these processes is the homeobox (HOX) class of transcription factors. These "master regulatory" genes specify and govern the body plan of organisms and have been shown to regulate development and differentiation in all metazoa ranging from sponges to vertebrates (for review see Ref. 2). The salient feature of the HOX proteins is a highly conserved 60-amino acid "homeodomain" that allows for sequence-specific DNA binding to regulate target gene expression. Ablation of HOX genes causes homeotic transformations (3–11), and abnormal expression of specific HOX genes can cause both neoplastic transformation in cultured cells and tumors in mammals (12–15). The pattern of HOX gene expression is thought to progress from dictating pattern formation during embryogenesis to a more restricted role in maintaining the differentiated state of cells in an adult organism (2, 16). In addition to the restricted expression of a subset of HOX genes in a tissue, these transcription factors are known to be alternatively spliced (17–22), transcribed from multiple promoters (23–26), post-transcriptionally regulated (27, 28), and post-translationally modified (29, 30).

The pursuit of HOX genes of consequence in vascular cell differentiation and proliferation is a new field. Investigators have begun to identify HOX genes that may play a regulatory role in the adult cardiovascular system (31–33). To date, these studies have focused on the smooth muscle cell with few published reports discussing HOX gene expression in EC (34, 35). The endothelium plays a dynamic role at sites of inflammation and during cell-mediated immune responses. Quiescent EC when activated in response to treatment with cytokines, such as TNF-α or interleukin-1β, exhibit morphological changes and synthesize new proteins not present in resting endothelium (1). The signaling pathways leading to the expression of specific genes involved in the promotion or suppression of EC activation have not yet been defined. Promoter analysis of many EC genes that are induced in response to immune or inflammatory reactions show that ubiquitous transcription factors, such as NF-xB, ATF-2/c-Jun, and HMG-I(Y), are necessary, but not sufficient, to account for EC-specific gene expression (36–41). There is increasing evidence that homeodomain proteins provide control of tissue-specific gene expression by heterodimerizing with other transcription factors (42–47).

As a first step toward determining the possible involvement of HOX gene expression in the biology of EC activation, we have isolated several HOX genes expressed by human EC. Here we report on the characterization of an EC-expressed, novel variant of HOXA9, which is transcribed from a novel promoter that shows both autoregulation as well as modulation during EC activation.

EXPERIMENTAL PROCEDURES
cDNA and Genomic Cloning—A human umbilical vein EC cDNA library in AgtII (a gift from Vishva M. Dixit, Genentech, San Francisco) was screened using a 128-fold degenerate oligonucleotide probe directed...
toward the highly conserved third helix of the homeodomain and containing two inosine residues. The probe had the following sequence: (where I indicates inosine) 5′-CG/TI/G(T/A)G(T/G/C/T)/G(A/G)-AACC/AAI/C(T/G)/T/TT-3′ (48). We screened 500,000 plaque-forming units and purified 90 cDNA clones. Direct sequencing (Fmoc Sequencing, Genentech) of the cDNA inserts in the phage was performed using 32P-end-labeled oligonucleotide primers specific to the right and left arm of the phage adjacent to the 5′ and 3′ boundary of the cDNA insert. To confirm the presence of a conserved homeodomain, sequencing was performed with the oligonucleotide primer used to screen the library as well as its reverse complement. Identity of the cloned cDNAs was established by homology searches performed against the GeneBank™ nucleotide data base using the Blast algorithm.

The HOXA9 genomic clones were obtained by screening a human placental genomic DNA library in Lambda EMBL3 (CLONTECH) using a uniformly 32P-labeled HOXA9EC cDNA probe. The intervening region between the exons was polymerase chain reaction-amplified and sequenced using appropriate exon-specific oligonucleotide primers. Direct sequencing of the Lambda EMBL3 clone containing the HOXA9 genomic DNA confirmed the authenticity of the sequence. The sequence analysis was performed using MacVector software (Oxford Molecular Group).

**Synthesis of GST-HOXA9EC Fusion Protein in E. coli**—The HOXA9EC cDNA insert in lambda gt11 was excised with EcoRI and cloned into the EcoRI site of pGEXZT, in-frame with the GST-coding sequence. Following transformation in E. coli HB101, the orientation of the insert was determined by sequencing the DNA. Production of the GST-HOXA9EC fusion protein was induced by isopropyl-β-D-thiogalactoside (1 mM), and the cells were lysed by sonication in phosphate-buffered saline containing 0.5% Nonidet P-40 (Sigma). The lysate was incubated with glutathione-Sepharose beads for 6 h at 4 °C. Beads were washed 4 times with 50 volumes of lysis buffer, and protein was eluted with 10 mM glutathione in 25 mM Tris (pH 7.5). Purity of the protein was determined by Coomassie staining following SDS-polyacrylamide gel electrophoresis.

**Electrophoretic Mobility Shift Assay (EMSA)**—The EMSA probes were synthesized by annealing the two overlapping primers and gel-purified on 12% non-denaturing polyacrylamide gel. The double-stranded oligonucleotides were 5′-end-labeled with γ-[32P]ATP and T4 polynucleotide kinase and purified from unincorporated nucleotides by a spin column method. EMSA was performed by incubating 50,000 dpm of probe with 50 ng of GST-HOXA9EC fusion protein in the presence of 10 mM HEPES (pH 7.5), 75 mM KCl, 1 mM dithiothreitol (DTT), 10 µg/ml poly(dI-dC), 5 µg/ml bovine serum albumin, and 15% glycerol. The reaction mixture was incubated at 20 °C for 45 min and then subjected to electrophoresis on a nondenaturing 5% polyacrylamide gel in 0.25× TBE (Tris borate-EDTA) at 20 °C. The EMSA results were visualized using a PhosphorImager (Molecular Dynamics).

**RNase Protection Analysis (RPA) and cRNA Probes**—Total RNA, isolated using Trizol reagent (Life Technologies, Inc.), was obtained from human mesenteric artery EC, human aortic smooth muscle (provided by Dr. Linda Graham, University of Michigan), skeletal muscle (provided by Dr. Linda Graham, University of Michigan, Ann Arbor), and various cultured cells. RNase protection was performed on 10 µg of RNA following the recommended protocol (Ambion). Uniformly 32P-labeled cRNA probes were generated from HOXA9EC (EC exon (E1), 245–644; homeodomain exon, 2114–2465), human E-selectin (49), and human β-actin (50). A genomic DNA fragment containing the 5′-untranslated region (UTR) and upstream sequence of HOXA9EC (−416 to +85) was used to synthesize a cRNA probe to determine the 5′ boundary of the HOXA9EC-exon specific by RNase protection analysis. The size of the 5′-UTR of HOXA9EC transcript was determined by reverse transcriptase-dependent primer extension (51) of human umbilical vein EC poly(A) RNA using a 32P-labeled antisense oligonucleotide to region 125 to 145 of the HOXA9EC sequence.

**Transient Transfection Assays**—The luciferase reporter constructs were made by subcloning the −416 to +86 region of the HOXA9EC genomic sequence (Fig. 2A) in pGL3 (Promega) in both the forward and reverse orientations. Lipofectin (Life Technologies, Inc.)-mediated transfections of 50% confluent cells at 50% confluence were carried out with endoribonuclease-free DNA for 6 h, and cells were allowed to recover in serum-containing media for 2–4 h before TNF-α treatment. Reporter activity was measured in triplicate and corrected for transfection efficiency by cotransfecting with pRSV β-galactosidase DNA and measuring the β-galactosidase activity with a GalactoLight Plus detection system (Tropix). For cotransfection experiments, HOXA9EC genomic DNA (−416 to +2819) was subcloned into pGEM-T (Promega) and a HOXA-2 (48) cDNA was subcloned into the pcDNA3 expression vector (Invitrogen).

**RESULTS**

Cloning of EC Homeobox cDNAs and Identification of a Novel Variant of HOXA9—A degenerate oligonucleotide probe targeted to the highly conserved third helix of the homeodomain (48) was used to screen a human umbilical vein EC cDNA library. Eighty cDNA clones that hybridized to the probe were isolated and sequenced; 66 were confirmed to contain homeodomains. Identity of the clones was established by sequencing the boundaries of the cDNA inserts as well as the region within and around the homeodomain. We identified cDNAs of HOXA1, HOX2, HOX4, HOX5, HOX7, HOX9, HOXB2, HOXB4, HOXB6, HOXB7, and an unlinked homeobox gene HLX. No members of the other two subgroups of HOX genes, C or D, were isolated among the cloned endothelial cDNAs.

Two partial cDNA clones of 1.6 and 1.4 kb were found to contain 1′ homeodomains and 3′-UTR of the HOXA9 gene as previously reported (13), but their sequences differed from reported sequence upstream of the conserved splice site previously reported for both the murine HOXA-9 cDNA (52) and a partial cDNA of chicken Hoxa-9 (53). These clones therefore represented novel transcripts encoded by the HOXA9 gene (Fig. 1A). The sequence of the 1.6-kb cDNA, which we have
termed HOXA9EC, exhibited a continuous open reading frame with a novel N-terminal 116 amino acids leading into the HOXA9 homeodomain. This sequence when scanned against the data base showed homology to the human HOXA9 cDNAs cloned from CD34+ cells (GenBank™ accession number U82759), from the human fetus (GenBank™ accession number U41813) (13), and to a human PAC sequence spanning 7p-15 to p-21 (GenBank™ accession number AC004080). It was also homologous to the murine (GenBank™ accession numbers M28449, AB008914, and AB005457) (52, 54), alternatively spliced guinea pig HOXA-9 cDNAs (GenBank™ accession numbers X13536 and X13537) (21), chicken HOXA-9 cDNA (GenBank™ accession number X97750) (53), and a Mexican salamander HOxa-9 cDNA (GenBank™ accession number U20941) (55). The novel HOXA9EC cDNA sequence, which differed from the previously described HOxa-9 cDNAs from various species, was apparently the result of differential splicing. The internal splice acceptor site located within the novel exon had been apparently the result of differential splicing. The internal splice acceptor site located within the novel exon had been

The breakpoint for the t(7;11)(p15;p15) translocation that

1A and Hoxa-9

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rhoes confirmed that one of the HOXA9 genomic clones contained the exons encoding the A9 homeodomain, the novel HOXA9EC exon, and the first exon of the previously reported HOXA9 cDNA isolated from fetal tissue (13). The novel HOXA9EC 5’ exon was found to be separated by a 990-bp intron from the common 3’ exon containing the HOXA9 homeodomain. Polymerase chain reaction amplification using exon-specific primers, followed by sequencing, confirmed the presence of a 3.7-kb intron separating the 5’ exon of the fetal HOXA9 cDNA sequence that splices to the HOXA9 homeodomain-containing exon (13) and HOXA9EC exon 1. The colocalization of the previously mapped fetal HOXA9 exon upstream of the EC exon 1 and the HOXA9 homeodomain exon in the human genomic clones confirmed that HOXA9EC cDNA was derived from the HOXA9 locus (Fig. 2D).

Specificity of HOXA9EC Expression—Northern analysis of human umbilical vein EC mRNA revealed a single 2.1-kb transcript hybridizing to either the full-length HOXA9EC cDNA probe (Fig. 3A) or the HOXA9EC exon L-specific probe (not shown) suggesting that HOXA9 transcribes a single major transcript of low abundance in EC. This was in contrast to the expression of the murine Hoxa-9 gene that was shown to encode a major transcript of 2.5 kb (52). The RPA using a HOXA9EC exon 1-specific probe revealed that HOXA9EC was expressed in freshly isolated EC from human mesenteric artery but not in smooth muscle cells from the same artery (Fig. 3B). HOXA9EC expression was also examined in a variety of cultured cells by RPA using the exon 1-specific probe. HOXA9EC mRNA was detected in primary cultures of human aortic, as well as microvascular, EC, but not in fibroblasts, smooth muscle cells, or a variety of transformed cell lines (Fig. 3C).

A multiple tissue Northern blot probed with full-length HOXA9EC cDNA revealed a major 2.5-kb transcript in skeletal muscle and a weak signal for a similar transcript in kidney (Fig. 3D). No such hybridization was detected with a HOXA9EC exon 1-specific probe (not shown) suggesting that the HOXA9EC-specific exon may not be expressed in skeletal muscle and kidney. We then confirmed by RPA that the A9 homeodomain-coding exon, but not the EC exon, was expressed in skeletal muscle (Fig. 3E). Our failure to detect HOXA9EC message in vascularized tissues may be due to the low abundance of this message in EC and the fact that EC comprise only a small fraction of the cellular content of these tissues.

Rapid Down-regulation of HOXA9EC Transcription by TNF-α—Activation of the endothelium is a differentiation-like process, believed to be critical in the initiation of a variety of inflammatory processes in the vessel wall (59, 60). To determine whether HOXA9EC expression was involved in the process of EC activation, we measured the steady state levels of HOXA9EC mRNA in resting and TNF-α-activated human umbilical vein EC. We observed a rapid and nearly complete (≥8 fold) down-regulation in the steady state level of HOXA9EC mRNA in response to TNF-α treatment for 4 h (Fig. 4A). E-selectin as a marker of EC activation served to verify the efficacy of TNF-α in stimulating these cells. Similar down-regulation of HOXA9EC mRNA was observed in EC treated with either interleukin-1β or lipopolysaccharide but not phorbol 12-myristate 13-acetate (not shown).

Pretreatment of human umbilical vein EC with cycloheximide did not block the effect of TNF-α (Fig. 4B), indicating that new protein synthesis was not required for TNF-α-dependent down-regulation of HOXA9EC mRNA. To determine whether the down-regulation was due to de-stabilization of the existing HOXA9EC mRNA in human umbilical vein EC or whether it directly affected transcription of the HOXA9EC gene, we measured the stability of the mRNA of the gene in the presence and


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FIG. 2. A, composite DNA sequence of cDNAs and genomic clones of HOXA9EC. The sequence in lowercase from 2429 to 21 and 647 to 1563 denote the intervening sequences separating the HOXA9EC exon 1 from the exons encoding fetal HOXA9 (13) and the HOXA9 homeodomain-encoding exon (HD), respectively; the triangle marks the internal splice acceptor site present in HOXA9EC; the putative TATA box, CAAT box, and polyadenylation sequence are in bold. The single letter amino acid code is used for the HOXA9EC protein. The HOXA9 homeodomain is shown in italics; the polyhistidine tract and the Pro-Tyr dipeptide are underlined.

B, the 5′9 boundary of the HOXA9EC exon E1 was determined by RNase protection analysis of a cRNA probe generated from the HOXA9 genomic DNA sequence (2420 to 155) and hybridized to 20 μg of total RNA from human umbilical vein EC.

C, identification of the transcription initiation site of HOXA9EC. Primer extension was performed on 5 μg of poly(A)1 RNA from human umbilical vein EC using reverse transcriptase and a 32P-end-labeled oligonucleotide primer complementary to bases 120–145 (GCTCAGCT-CATCCCGCCGCGCTCAGG). The extension products were separated on a denaturing polyacrylamide gel.

D, localization of the novel HOXA9EC-specific exon. The lambda EMBL3 clone containing human HOXA9 was sequenced to determine the location of the HOXA9 homeodomain-containing exon (HD), the novel exon of HOXA9EC (E1), and the exon specific to the HOXA9 cDNA isolated from a human fetal cDNA library (F1, Ref. 13).
HOXA9 Expression in Endothelial Cells

Multiple examples exist of HOX gene autoregulation in systems where these genes must be continuously expressed to maintain the differentiated state of a cell (2, 16, 63). We tested whether HOXA9EC exhibited autoregulation in EC. In cotransfection experiments a 4-fold increase in HOXA9EC promoter activity was observed in response to overexpression of HOXA9EC (Fig. 6A). This up-regulation of the HOXA9EC promoter was TNF-α-sensitive, and it was specific since neither the vector alone nor the expression of another HOX gene expressed by EC Hox-2 had any effect on HOXA9EC promoter activity. Under identical conditions, ectopic expression of HOXA9EC in HeLa cells not only up-regulated promoter activity but also conferred TNF-α sensitivity to the HOXA9EC promoter (Fig. 6B).

DISCUSSION

The HOX family of transcription factors regulate proliferation and differentiation in the developing embryo and play a critical role in the early events of embryonic pattern formation. HOX gene expression is often developmental stage-specific and tissue-specific, and the developmental potential of a particular tissue may be governed by the different types of HOX genes expressed in that tissue (64). It is also possible that the maintenance of the differentiated state of a cell type depends on the regulated expression of HOX gene family members. The dearth of information on HOX gene function in adult and differentiated tissue is striking when compared with our knowledge of the role of HOX genes during embryogenesis (2). The possibility that this gene family may also play a role in defining either the quiescent, non-activated state or the cytokine-stimulated state of cells has also received little attention, and this formed the rationale for the current study.

We have isolated and identified several HOX cDNAs from EC. Results of extensive screening revealed that out of the four groups of clustered HOX genes, only two groups, namely the A and B classes, were present in our cultured EC library. Two novel cDNAs containing the HOXA9 homeodomain sequence, termed HOXA9NT and HOXA9EC, differed at the N terminus from the previously reported alternatively spliced partial cDNAs of HOXa-9 from guinea pig (53), as well as the HOXA9 cDNA isolated from a fetal human library (13). The HOXA9NT cDNA represented a non-translatable transcript arising from the HOXA9 locus. This mRNA may play a post-transcriptional regulatory role as has been suggested for several HOX gene transcripts in Xenopus embryos (65) and for one of the transcripts from the HOXa-10 locus (17) which has been shown to be incapable of translation. HOXA9EC on the other hand has an open reading frame capable of generating a 29-kDa homeodomain-containing protein, the N-terminal 170 amino acids of which are contributed by a novel exon.

HOXA9EC Is Transcribed from an Auto-regulated TNF-α-responsive Novel Promoter—Based on primer extension assays the transcription initiation site of HOXA9EC was localized to the novel EC exon. This finding together with the transcript size of 2.1 kb suggested that the EC-specific exon and the HOXA9 homeodomain-coding exon were sufficient to generate the HOXA9EC transcript and that HOXA9EC may be transcribed from a novel promoter element immediately upstream of the EC-specific novel exon. Transient transfections of human umbilical vein EC, human microvascular EC, bovine aortic EC, as well as HeLa and fibrosarcoma HT-1080 cell lines, with a plasmid containing a genomic DNA fragment (420 to +16, Fig. 1A) consisting of the putative HOXA9EC promoter driving a luciferase reporter yielded 50-fold higher activity than the identical genomic fragment cloned in the reverse orientation (Fig. 5). TNF-α treatment of the transfected cells resulted in a 40–60% reduction in HOXA9EC promoter activity only in cells of endothelial origin. No such effect was observed in either HeLa or HT-1080 cells, which are TNF-α-responsive cell lines (61, 62). Thus, the EC-specific, TNF-α sensitivity of HOXA9EC transcription was shown to reside within 0.42 kb of the novel promoter.

absence of TNF-α after blocking new transcription with actinomycin D (Fig. 4C). The apparent half-life of the HOXA9EC mRNA, approximately 90 min, was the same in actinomycin D-treated human umbilical vein EC in the presence or absence of TNF-α, suggesting that the TNF-α-dependent down-regulation occurred at the level of transcription.

Fig. 3. A, expression of HOXA9EC mRNA. Northern blot analysis of poly(A)+ RNA (4 μg) from cultured human umbilical vein EC revealed a single 2.1-kb transcript identified by the HOXA9EC cDNA probe. B, in vivo expression of HOXA9EC. Total RNA (5 μg each) from freshly isolated endothelium from human mesenteric artery (MA), smooth muscle cells from human mesenteric artery (MASMC), and cultured human umbilical vein EC (HUVEC) were subjected to RNase protection analysis with a cRNA probe generated from exon E1 (303–641, Fig. 2A). A γ-actin cRNA probe was included as a control. C, HOXA9EC expression was detected in cultured EC but not cells of other lineages. RNase protection analysis was performed using total RNA (10 μg) isolated from various cultured cells hybridized to the cRNA probe generated from exon E1 of HOXA9EC. A γ-actin cRNA probe was included as a control. D, Northern blot analysis of HOXA9EC expression. A multi-tissue Northern blot (CLONTECH) containing poly(A)+ RNA isolated from human tissue was hybridized to uniformly 32P-labeled HD or HOXA9EC cDNA. E, HOXA9EC exon E1 was not expressed in skeletal muscle. RNase protection analysis of total RNA (10 μg) isolated from human skeletal muscle and probed with either HOXA9EC exon E1-specific cRNA or homeodomain containing exon (HD)-specific RNA. A γ-actin cRNA probe was included as a control.

Our Northern blot analysis demonstrated that HOXA9EC
existed as a single transcript of 2.1 kb in EC. Exon mapping of genomic clones, as well as primer extension experiments, indicated that the complete open reading frame and 5' UTR of HOXA9EC were contained within 2 exons separated by a 990-bp intron. This finding raised the possibility of an alternative promoter that directed transcription of the HOXA9EC gene in EC. A number of HOX genes are transcribed from alternative promoters resulting in transcripts differing in size, stability, and tissue distribution (23–26). The duplication and reduplication of HOX genes on separate chromosomes and sub-

**Fig. 4.** A, TNF-α-dependent down-regulation of HOXA9EC mRNA. Human umbilical vein EC were treated with TNF-α (10 ng/ml for 4 h), and total RNA (10 μg) was subjected to RNase protection analysis with a mixture of cRNA probes for the following genes: E-selectin, γ-actin, HOXA9EC novel exon (E1), and the HOXA9 homeodomain-containing exon (HD). B, the down-regulation of HOXA9EC mRNA in response to TNF-α is independent of new protein synthesis. Umbilical vein EC were pretreated for 1 h with cycloheximide (10 μg/ml, lane 1) and then treated with TNF-α (10 ng/ml) for an additional 2 or 4 h (lanes 2 and 3, respectively) in the continued presence of cycloheximide. Total RNA was isolated from control and TNF-α-treated EC and subjected to RNase protection analysis using a cRNA probe for the HOXA9EC exon 1 (E1). C, TNF-α has no effect on the half-life of HOXA9EC mRNA. RNase protection analysis was performed to detect HOXA9EC exon 1 mRNA. Total RNA was isolated at various time points from human umbilical vein EC treated with either (i) actinomycin D (5 μg/ml) alone or (ii) TNF-α (10 μg/ml) added 30 min after actinomycin D. The intensities of the protected bands were compared by PhosphorImager analysis.
DNA inserts, and 0.2 mg, TNF- control EC, respectively. The construct in TNF- HOXA9EC vector DNA either alone or containing either the HeLa cells (B) using 1 mg HOXA9EC transcriptional script. In response to cytokines transcription of this transcription factor gene is suppressed leading to a rapid and dramatic reduction of HOXA9EC mRNA, and ultimately protein, allowing EC to progress to an activated state. An alternative hypothesis, albeit less simple, is that HOXA9EC acts in a positive manner on activation genes in coordination with cytokine-induced, transcription factors but that HOXA9EC mRNA down-regulation represents an "off switch" for cytokine-induced genes in activated EC. The latter hypothesis would help to explain the highly, transient nature of transcriptional up-regulation of many of the cytokine-induced genes in EC. Future studies will determine if either of these two possible mechanisms of action of HOXA9EC is correct.

Fig. 5. HOXA9EC promoter activity is repressed by TNF-α in EC. HOXA9EC promoter DNA (−420 to +85) was subcloned into a luciferase reporter vector pGL3 basic (Promega) in both the forward and reverse orientation. Transient transfections were carried out in triplicate using 1 μg of reporter plasmid DNA. TNF-α treatment (16 h) was initiated 2–4 h after the transfections in human microvascular EC (HMVEC), human umbilical vein EC (HUVEC), bovine aortic EC (BAEC), Hela and HT-1080 cells. The reporter luciferase activity was normalized by cotransfecting pSV β-galactosidase DNA (0.2 μg). The open and striped bars represent the reporter activities of the HOXA9EC promoter constructs in reverse and forward orientations in untreated control EC, respectively. The solid bar shows the activity of the forward construct in TNF-α-treated EC.

Fig. 6. HOXA9EC coexpression confers TNF-α sensitivity on the HOXA9EC promoter in a cell-independent manner. Cotransfections were carried out in triplicate in bovine aortic EC (A, BAEC) and HeLa cells (B) using 1 μg of HOXA9EC/pGL3 reporter DNA, 0.5 μg of vector DNA either alone or containing either the HOXA9EC or Hoxa-2 DNA inserts, and 0.2 μg of pBS+V β-galactosidase (control untreated [ ], TNF-α-treated [ ■ ]). The consequent provision of their individual regulatory elements ensures a diversity of expression unique to homologous genes of the HOX complex. We have shown that a novel promoter for HOXA9EC is contained in the genomic sequence immediately upstream of the transcription initiation site. This promoter may provide the cis-acting, control elements that ensure the proper timing, level, and tissue specificity of HOXA9EC gene expression.

Activation of the endothelium is a differentiation-like process, believed to be critical in the initiation of a variety of inflammatory processes in the vessel wall and in adjacent tissue (1, 59). The activation process includes the induction of leukocyte adhesion molecules on the EC surface leading to hyperadhesivity between leukocytes and the endothelium, changes in the expression of growth regulatory and vasoreactive molecules, and a shifting from an anti-coagulant to a procoagulant phenotype. There is a growing literature demonstrating the involvement of multiple transcription factors in cytokine-mediated induction of activation-related genes. Potential players include the Rel family member NF-κB (36, 37, 68), a cyclic AMP-independent ATF family member ATF2 (39), HMGI-Y (38), and Egr-1 (69). Other unidentified factors specifically expressed in endothelium may also play a role in this induction. Our results suggest the possible involvement of HOXA9EC, since the novel promoter we have identified is sensitive to TNF-α in an EC-specific manner.

Our studies suggest two distinct hypotheses for the role of HOXA9EC in EC gene expression. Since this novel gene within the HOX9 locus is transcriptionally suppressed by the inflammatory cytokine TNF-α, we might speculate that this gene is constitutively expressed and helps to maintain a quiescent, non-activated EC state. Autoregulation may help to preserve a sufficient, steady state level of the short-lived HOXA9EC transcript. Both cytokines transcription of this transcription factor gene is suppressed leading to a rapid and dramatic reduction of HOXA9EC mRNA, and ultimately protein, allowing EC to progress to an activated state. An alternative hypothesis, albeit less simple, is that HOXA9EC acts in a positive manner on activation genes in coordination with cytokine-induced, transcription factors but that HOXA9EC mRNA down-regulation represents an “off switch” for cytokine-induced genes in activated EC. The latter hypothesis would help to explain the highly, transient nature of transcriptional up-regulation of many of the cytokine-induced genes in EC. Future studies will determine if either of these two possible mechanisms of action of HOXA9EC is correct.

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