Expression of endothelial nitric-oxide synthase (eNOS) mRNA is highly restricted to the endothelial cell layer of medium to large sized arterial blood vessels. Here we assessed the chromatin environment of the eNOS gene in expressing and nonexpressing cell types. Within endothelial cells, but not a variety of nonendothelial cells, the nucleosomes that encompassed the eNOS core promoter and proximal downstream coding regions were highly enriched in acetylated histones H3 and H4 and methylated lysine 4 of histone H3. This differentially modified chromatin domain was selectively associated with functionally competent RNA polymerase II complexes. Endothelial cells were particularly enriched in acetylated histone H3 lysine 9, histone H4 lysine 12, and di- and tri-methylated lysine 4 of histone H3 at the core promoter. Histone modifications at this region, which we have previously demonstrated to exhibit cell-specific DNA methylation, were functionally relevant to eNOS expression. Inhibition of histone deacetylase activity by trichostatin A increased acetylation of histones H3 and H4 at the eNOS proximal promoter in nonexpressing cell types and led to increased steady-state eNOS mRNA transcript levels. H3 lysine 4 methylation was also essential for eNOS expression, since treatment of endothelial cells with methylthioadenosine, a known lysine 4 methylation inhibitor, decreased eNOS RNA levels, H3 lysine 4 methylation, and RNA polymerase II loading at the eNOS proximal promoter. Importantly, methylthioadenosine also prevented the trichostatin A-mediated increase in eNOS mRNA transcript levels in nonendothelial cells. Taken together, these findings provide strong evidence that the endothelial cell-specific expression of eNOS is controlled by cell-specific histone modifications.

Endothelial nitric-oxide synthase (eNOS, NOS3) is constitutively expressed in vascular endothelial cells, especially the endothelial layer of medium to large sized arterial blood vessels, where it is known to play a key role in vascular wall homeostasis and regulation of vasomotor tone. Studying the mechanisms regulating the constitutive transcription of eNOS in endothelial cells is essential to understand how these mechanisms may be perturbed in diseases characterized by a decrease in eNOS mRNA in the vascular endothelium. For example, constitutive expression of eNOS is compromised in the endothelial cells overlying advanced human atherosclerotic plaques (1–3).

In general, the basis for endothelial-specific gene expression is not known. Whereas models involving DNA-binding transcription factors (e.g. AP-1, Ets family members, GATA-2, octamer proteins, or Sp1) (4–7) have been invoked to explain the transcriptional control of a variety of endothelial genes, these models cannot fully account for the exquisite specificity of these endothelial-specific promoters, given that these transcription factors are ubiquitously expressed. This can be contrasted with the control of muscle-specific or adipocyte-specific genes, which are controlled by “master regulators,” including MyoD/myocardin/MEF-2 or peroxisome proliferator-activated receptor-γ, respectively (8, 9). Current evidence argues against a prominent role for cell-specific expression of endothelial “master regulators.” This has prompted the search for other paradigms of cell-specific gene expression in endothelial cells. In this regard, the contribution of chromatin-based epigenetic pathways to the regulation of cardiovascular gene expression, especially in vascular endothelial cells, remains poorly understood.

We have recently shown that epigenetics may provide a new paradigm for understanding endothelial cell-specific expression (10). Transient transfection of eNOS promoter/reporter constructs into endothelial and nonendothelial cell types revealed high levels of transcription regardless of whether the native chromatin-based gene was active. However, expression of reporter genes in insertional transgenic eNOS promoter/reporter mice demonstrated endothelial-specific reporter activity when related promoter sequences were utilized (11, 12). These unexpected findings suggested that chromatin-based mechanisms are functionally relevant. By analyzing a large portion of the eNOS/NOS3 locus for cell-specific DNA methylation patterns, we found a differentially methylated region (DMR) that was highly localized to the proximal promoter. Whereas nonendothelial cells were highly methylated at proximal promoter sequences, endothelial cells lacked DNA methylation; VSMC, vascular smooth muscle cell(s); HMVEC, human dermal microvascular endothelial cell(s); ChIP, chromatin immunoprecipitation; DMR, differentially methylated region; CTD, C-terminal domain; CYP-A, cyclophilin A; EMSA, electrophoretic mobility shift assay; BAEC, bovine aortic endothelial cell(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
ylation in this same region. This cell-specific methylation pattern was demonstrated in primary and human transformed cell types as well as in vivo in the vasculature of mice using laser capture microdissection. Methylation of this DMR functionally repressed eNOS transcription. Importantly, although many of these nonendothelial cell types do not basally express appreciable levels of eNOS mRNA transcripts at steady state, we noted that inhibition of DNA methyltransferase activity with 5-azacytidine led to increases in eNOS mRNA levels (10). Complementary studies supporting these findings were subsequently reported by others (13).

We have previously argued that Sp1, Sp3, and Ets-1 trans-factors participate in highly structured nucleoprotein complexes that form on eNOS proximal promoter sequences, termed positive regulatory domains (PRDs) I and II (14, 15), and recently found that their functional activity was impeded by methylation of proximal promoter sequences in cells (10). In the current work, we were unable to demonstrate an effect in electromobility shift assays (EMSA) of methylation of naked double-stranded DNA on the formation of nucleoprotein complexes. Whereas Sp1, Sp3, and Ets-1 are relatively ubiquitously expressed across cell types, curiously they differentially localize to the eNOS promoter in chromatin immunoprecipitation (ChIP) assays of endothelial versus vascular smooth muscle cell chromatin (10).

Chromatin structure is regulated by a plethora of post-translational covalent modifications at the N termini of the H3 and H4 core histones: acetylation, methylation, phosphorylation, deimination, sumoylation, and ubiquitination among others. These modifications are thought to influence the accessibility of DNA to general nuclear factors and serve as target sequences for regulatory proteins and are dynamically regulated (15, 16). Modification patterns are established and maintained by a variety of histone acetyltransferases, HDACs, histone methyltransferases, and the recently discovered arginine-specific histone deiminases and histone H3 lysine 4-specific demethylase (15–19). Recently, the specific sites of acetylation and, more importantly, the combination of sites of acetylation and other covalent modifications, such as methylation and phosphorylation, have been shown to dictate whether a particular region of chromatin is in a transcriptionally active or repressed state (20, 21). The “histone code” model postulates that it is the specific combinations of covalent histone modifications that determine the functional activity of chromatin (20–22). Because the structure of histones in nucleosomal DNA is an important determinant of nucleoprotein complex formation (23) and DNA methylation of promoters is often accompanied by histone modifications that render the chromatin effectively inaccessible to transcription factors (23–26), we considered whether covalent modifications of promoter histones may provide insight into the phenomenon of eNOS cell-specific expression. Here we describe a cell-specific histone code for human eNOS, map a discrete region of uniquely modified nucleosomes, and demonstrate the functional importance of these modifications with respect to transcription.

MATERIALS AND METHODS

Cell Culture—Human umbilical vein endothelial cells (HUVEC, eNOS(+)) were isolated and maintained as described previously and used at passages 3–4 (27). Single donor human dermal microvascular endothelial cells (HMVEC, eNOS(+)) and human aortic vascular smooth muscle cells (VSMC, eNOS(-)) were obtained from Clonetics (Cambridge, East Rutherford, NJ) and ScienCell (San Diego, CA) and maintained as recommended and used at passages 4–9. HepG2 (human hepatocellular carcinoma cell line, eNOS(-)), HeLa (human cervical carcinoma cell line, eNOS(-)), and JEG-3 (human choriocarcinoma cell line, eNOS(-)) were obtained from ATCC and maintained at 37 °C with 5% CO2. Cell culture reagents were from Invitrogen.

Antibodies Used for ChIP Assays—Antibodies used were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) (anti-acetyl histone H3 (Lys9 and Lys14) (06-599), anti-acetyl histone H3 (Lys9) (06-942), anti-acetyl histone H3 (Lys14) (06-911), anti-acetyl histone H4 (Lys12, Lys14, and Lys16) (06-886), anti-acetyl histone H4 (Lys12) (06-759), anti-acetyl histone H4 (Lys16) (06-760), anti-acetyl histone H4 (Lys16) (06-761), anti-acetyl histone H4 (Lys16) (06-762), anti-acetyl histone H4 (Lys16, ChIP grade) (07-329), anti-dimethyl histone H3 (Lys4) (07-030), anti-MeCP2 (07-013) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (anti-Pol II (N-20; sc-899), anti-HDAC1 (C-19; sc-6298), anti-HDAC2 (C-19; sc-6286), and anti-HDAC3 (H-99; sc-11417), and Abcam (anti-trimethyl histone H3 (Lys39) (ab8590) and anti-dimethyl histone H3 (Lys4) (ab7312), and Covance (against-phosphorylated RNA polymerase II (Ser5) (H14) and anti-phosphorylated RNA polymerase II (Ser2) (H5)). Normal rabbit IgG (sc-2027) was purchased from Santa Cruz Biotechnology.

Treatment of eNOS Promoter/β-Galactosidase Reporter Transgenic Mice with 5-Aza-CdR—As described previously (10), Laser-Trap laser-promoter-reporter eNOSpromLacZ transgenic strain has been primarily reported (12). The −5200/−28 Mu eNOSprom(LacZ) transgene contains 5.2 kb of the native murine eNOS promoter and directs expression of the nuclear localized β-galactosidase reporter. Mice with one copy or five copies of the transgene were used in parallel experiments. Adult and neonatal mice containing the eNOS promoter/β-galactosidase reporter were treated with 5-azacytidine (Sigma) at a dose of 2.5 mg/kg body weight, a dose known not to cause adverse effects in mice and to result in demethylation of Ifg2 (28). Adult mice were injected intraperitoneally with 5-azacytidine or phosphate-buffered saline on days 1 and 4, and organs were harvested on day 16. Neonatal mice were treated on days 12 and 15 of life, and organs were harvested on day 26. Organs (brain, kidney, heart, intestine, liver, lung, and spleen) were harvested, capped, and stained as described previously (10). Laser-trap capture microdissection was used to isolate genomic DNA from aortic endothelial cells, aortic vascular smooth muscle cells, and hepatocytes, as described (10). To assess the DNA methylation status of the inseltransgenic gene, sodium bisulfite sequencing was performed using primers specific for the inseltransgenic gene (10).

Electromobility Shift Assays with Methylated eNOS Promoter Sequences—Nuclear lysates from HeLa cells were obtained from Geneka Biotechnology (Carlsbad, CA). Bovine aortic endothelial cell (BAEC) extracts were prepared as previously described (14). 5 pmol of double-stranded, methylated, or mock-methylated probe was labeled using the Klenow fragment of DNA polymerase I and [α-32P]-dCTP and/or [α-32P]-dATP. For each EMSA reaction, 1.0 × 106 cpm of labeled probe was incubated for 30 min at room temperature with 7 ng of nuclear extract in a total volume of 20 μl of binding buffer (10 mM Tris, pH 8, 1 mM dithiothreitol, 5% glycerol, 20 mM NaCl, 2 mM MgCl2, 0.01 mg/ml bovine serum albumin, and 0.1 mg/ml sonicated salmon sperm DNA). As indicated, 2 μg of antibody was added 20 min after the addition of probe and incubated an additional 15 min at 22 °C. Antibodies used were from Santa Cruz Biotechnology: Sp1 (PEP2, sc-59) and Sp3 (D-20; sc-164). Reacting complexes were analyzed on 4% nondenaturing polyacrylamide gels run at room temperature. The EMSA probes used were CG11, a synthetic 135-bp fragment containing 20 GCCG sites and 7 CCGG sites, and eNOS(−161/+89), a 253-bp fragment spanning PRD I(−104/−95) and PRD II(−144/−115) of the human eNOS promoter, containing seven CpG sites (generated by digestion of the −1193/+109 eNOS promoter/reporter with BamHI and Styl). CG11 is a well studied probe that is known to bind MeCP1 and MeCP2 complexes when methylated (29). The competitor oligonucleotides corresponded to PRD I(−110/−89), PRD II(−151/−110), or both PRD I and II(−151/−89). The CG11 sequence in PUC 19 (a gift from K. Blanchard) and the eNOS linker-anchored complex, PGL2-1193(−164), were in vitro methylated prior to probe purification using SsIl methylase (New England Biolabs) to symmetrically methylate all CG sequences, as described previously (10). Methylation reactions were monitored for completeness with MspI/HpaII isoschizomer restriction enzyme analysis.

Chromatin Immunoprecipitation—ChIP was performed essentially as previously described (10, 30). Briefly, −3 × 106 cells were used per ChIP. Formaldehyde was added directly to the medium to a final concentration of 1% and incubated at 37 °C for 10 min. Sonication was performed on ice using a Misonix Sonicator with a 3-mm tip set at 30% maximum power using 10-s pulses with a 20-s interval between sonications to completely solubilize chromatin and achieve chromatin fragments ranging between 200 and 400 bp in size. For histone ChIPs, chromatin was precleared using 80 μl of salmon-sperm DNA/protein A-agarose for 30 min. Immunoprecipitation was performed overnight using 5 μg of antibody or a background control (no antibody or 5 μg of normal rabbit IgG). A 20-μl aliquot of a 2000-μl

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IgM (Serotec, Star86) for phosphorylated RNA Pol II ChIPs.

10 mM KCl, 0.5 mM dithiothreitol, and protease inhibitor tablet (Roche). Determinations were performed in triplicate on 2 μl of DNA from 5'-GGG AGG ACC AGG AAC ACC-3' and 5'-TCA CTC TCT TCG CCA TCA C-3'; probe, 5'-FAM CAG CCG TGA AGT CCT CCG C Blackhole Quencher-3'.

Copy number of transcripts was determined by comparison with a standard curve using known amounts of amplicon-containing plasmid.

RESULTS

DNA Methylation of Proximal Promoter Sequences of eNOS Promoter|β-Galactosidase Reporter Transgenic Mice Is Not Essential for Endothelium-specific Expression

We previously demonstrated that DNA methylation participates in the endothelium-specific expression of eNOS (10). Proximal promoter regions were heavily methylated in eNOS-nonexpressing cell types, such as VSMC, and devoid of methylation in endothelial cell types. This correlation was true both in vitro and in vivo and was functionally relevant. Treatment of cell types with a DNA methyltransferase inhibitor, 5-azacytidine, led to eNOS expression. Expression of reporter genes in eNOS promoter/reporter transgenic mice is highly restricted to vascular endothelial cells. To determine whether DNA methylation contributed to transgene cell-specific expression, we treated either adult or neonatal eNOS promoter/reporter mice with 5-azacytidine and assayed reporter β-galactosidase expression in a wide variety of organs: aorta, brain, kidney, heart, intestine, liver, lung, and spleen. We used maximally tolerated 5-azacytidine treatment regimes and concentrations known to activate the methylated Igf2 gene (28). In contrast to in vitro studies, there were no major alterations in reporter expression profiles. Robust expression of β-galactosidase was only detected in endothelial cells. Shown in Fig. 1A are representative sections from the renal arteries or livers of control and 5-azacytidine-treated neonatal mice. Across all tissues studied, baseline expression of the reporter in endothelial cells of medium and large sized arteries was unaltered, the absence of important expression in microvascular endothelial cells (such as the sinusoidal bed of the liver) continued, and de novo expression in nonendothelial cells was not observed. To understand why 5-azacytidine failed to induce eNOS reporter expression in nonendothelial cells, we analyzed the DNA methylation status of the insertional transgene in endothelial and smooth muscle cells of the aorta and hepatocytes. These studies used laser capture microdissection combined with sodium bisulfite genomic sequencing of the hemizygous insertional −5200/+28 Mu eNOS<sup>−/−</sup> LacZ transgenic mice. As shown in Fig. 1B, methylation of the proximal promoter of the transgene was not cell-specific, with very low levels of methylation detected in all cell types examined. Comparable findings were evident in different founder transgenic lines with either single or multiple copies (data not shown). These studies indicated that a cell type-specific methylation pattern was not observed for these cell types.
random integration events. Although it is not our preferred model, we cannot exclude the possibility that the insertional transgenes exhibit a cell-specific DNA methylation pattern during a brief period of developmental patterning or exclude the possibility that DNA methylation is cell-specific in other regions of the transgene. Because eNOS promoter/reporter expression in transgenic founders was specific to the endothelium, we take these data to indicate that additional chromosome-based mechanisms might be operative in controlling the cell-specific expression.

**Absence of a Methylation Effect of eNOS Promoter Sequences on Nucleoprotein Complex Formation in EMSA**—Using deletion analysis and linker-scanner mutagenesis, two cis-regulatory regions were previously identified in the proximal region of the eNOS promoter: PRD I (residues −104/−95 relative to transcription initiation) and PRD II (−144/−115). Analysis of trans-factor binding and functional expression studies revealed that the nucleoprotein complexes that form upon these regions in endothelial cells contained Sp1, variants of Sp3, YY1, Ets-1, Elf-1, and MAZ (14). We have shown that methylation of eNOS promoter/reporter constructs inhibits transactivation by threshold amounts of Sp1, Sp3, and Ets-1 (10). We have also shown using ChIP assays that in vivo binding of Sp1, Sp3, and Ets-1 to the eNOS proximal promoter is enriched in endothelial cells compared with VSMC, despite comparable amounts of these factors in both cell types. To determine whether DNA methylation alone can block access of these factors to eNOS promoter sequences, we used SsII-methylated or mock-methylated −161/+92 regions (seven CpG sites) of the eNOS promoter as probes in EMSAs. Representative EMSAs using protein extracts from BAEC (Fig. 2A) and HeLa cells (Fig. 2B) are indicated. Complexes that formed on the −161/+92 eNOS promoter were specific. For instance, smaller probes spanning PRD I or PRD I and II were effective competitors, whereas mutant PRD I/II probes were not (Fig. 2A, lanes 4–7). Sp1 and Sp3 are among the factors present in these nucleoprotein complexes, since shift abrogation and supershifts were demonstrated upon antibody addition (Fig. 2A, lanes 8 and 9). Importantly, the formation of nucleoprotein complexes using BAEC (Fig. 2A) or HeLa (Fig. 2B) extracts and the pattern of shifts with Sp1 and Sp3 antibodies (data not shown) were not altered when the probe was methylated (Fig. 2, A and B). Methylated cold probes competed as effectively as mock-methylated cold probes (data not shown). In contrast to the absence of an effect of methylation in EMSAs using an eNOS probe, methylation of the control CG11 probe (27 CG sites) had a major effect on complex formation. As demonstrated in EMSA experiments using protein extracts from BAEC (Fig. 2A) or HeLa (Fig. 2B) cells, methylation of the CG11 synthetic probe abrogated the formation of prior complexes and was associated with the de
**novo** formation of others. A detailed analysis of these CG11 nucleoprotein complexes has been reported for other cell types (29). These data indicate that DNA methylation alone does not affect the formation of nucleoprotein complexes on proximal regions of the eNOS promoter, at least on naked double-stranded DNA templates.

**MeCP2 Binds to the eNOS Proximal Promoter in VSMC—** MeCP2 was a potent inhibitor of transcription from methylated versus mock-methylated eNOS promoter/reporter templates *in vitro* (10). However, MeCP2 can influence chromatin structure independently of DNA methylation (33). Although methylation of naked DNA templates failed to affect formation of nucleoprotein complexes in EMSA, we used ChIP combined with real time PCR to assess the binding of endogenous MeCP2 at the eNOS proximal promoter (166 to 26). As shown in Fig. 3, MeCP2 was highly enriched at the proximal promoter of eNOS in human aortic VSMC compared with HUVEC. The differences in ChIP results for MeCP2 were not due to the absence of this methyl cytosine binding protein in endothelial cells, as demonstrated by Western blotting (data not shown). Importantly, MeCP2 is known to play an important role in modulating chromatin structure by recruiting HDACs and thereby mediating changes in nucleosomal histone acetylation (34).

**FIG. 2.** Methylation of eNOS promoter sequences does not affect the binding of nucleoprotein complexes. EMSA was used to determine the effect of methylation on the nucleoprotein complexes forming on the eNOS promoter. Probes were subjected to mock methylation or M.SssI methylation prior to 32P labeling. A, EMSA of BAEC nuclear protein binding to the −161/+89 eNOS probe. BAEC cell nuclear extract was added in lanes 2–9. The probe was methylated in lane 3 and was mock-methylated in all other lanes. In lanes 4–7, 250-fold molar excesses of PRD I, II, I/II, and mutant PRD I/II were added as cold competitors. In lanes 8 and 9, the addition of Sp1 and Sp3 antibodies to the binding reaction resulted in supershifted and/or abrogated complexes (indicated by an arrow at the right). Complexes that are specific for PRD I and II are indicated by arrows at the left. B, EMSA of HeLa nuclear protein binding to the −161/+89 eNOS probe. Extract was added in lanes 3–6. Lanes 3 and 5 are from one set of extracts; lanes 4 and 6 are from a different extract. C, EMSA of BAEC nuclear protein binding to the synthetic CG11 probe. Extract was added in lanes 3 and 4. Loss and gain of complexes are indicated by the arrows at the left and right, respectively. D, HeLa nuclear protein binding to the synthetic CG11 probe. Extract was added in lanes 3–6. Loss and gain of complexes are indicated by the arrows at the left and right, respectively. Lanes 3 and 5 are from one set of extracts; lanes 4 and 6 are from a different extract.

**FIG. 3.** MeCP2 is differentially recruited to the eNOS proximal promoter in endothelial versus smooth muscle cells. ChIP was used to assess the binding of MeCP2 to the proximal promoter of eNOS (166 to 26) in HUVEC and VSMC. Using real time PCR analysis, immunoprecipitated DNA was calculated by determining the number of copies of the indicated genomic region in the immunoprecipitated samples, subtracting the number of copies in a no antibody control, and dividing by the number of copies in a diluted input sample. *, a statistical difference between endothelial and nonendothelial cell types ($p < 0.05$).
The eNOS Proximal Promoter Is Hyperacetylated at Histones H3 and H4 in eNOS-expressing endothelial cells. Using a quantitative ChIP technique, we assessed post-translational histone modifications of nucleosomes across the eNOS locus in chromatin isolated from human primary cell types that express eNOS (HUVEC and HMVEC) and human primary and tumor cell types that do not express eNOS (VMSC, HeLa, HepG2, and JEG3 cells). A systematic analysis of the locations of specific chromatin modifications was performed across the eNOS locus: the enhancer (−4718 to −4596), an upstream region of the eNOS promoter (−891 to −796), the proximal promoter (−166 to −26), exon 2 ( +1463 to +1514), and exon 14/intron 14 (+12,652 to +12,733). CpG sites are indicated by vertical lines, and repetitive DNA elements are indicated above. The DMR is also indicated. ChIP was performed on eNOS-expressing endothelial cell types (HUVEC and HMVEC; white bars) and eNOS-nonexpressing cell types (VMSC, HeLa, HepG2, and JEG3; black bars) using anti-acetylated histone H3 (Lys9 and Lys14) or anti-acetylated histone H4 (Lys5, Lys8, Lys12, and Lys16) antibodies. *, a statistical difference between endothelial and nonendothelial cell types for the genomic region studied (p < 0.05). C, histone Western blot of acid-extracted histones from HUVEC and VSMC using antibodies to detect histone H3 and H4 acetylation. D, real time reverse transcription-PCR was used to determine the number of copies of eNOS and CYPA per microgram of RNA extracted from HUVEC and VSMC. E, ChIP assay using anti-acetylated histone H3 and anti-acetylated histone H4 antibodies and primers that amplify the CYPA promoter (−161 to −19).
increases in histone acetylation can be functionally relevant in the unfolding of reconstituted chromatin templates (35). Neither an upstream region (−891 to −796) nor 3′ distal regions (+12652 to +12733) were highly acetylated in HUVEC or VSMC when compared with the proximal promoter. Whereas acetylation of histone H4 at upstream regions (−891 to −796) and histone H3 and H4 at 3′ distal regions (+12,652 to +12,733) was slightly enriched in HUVEC compared with VSMC, the level of acetylation was modest compared with the marked hyperacetylation evident at the proximal promoter (−166 to −26) and exon 2 (+1463 to +1514) regions. Importantly, and in contrast to the enhancer regions of other genes that are expressed in a cell type-specific fashion (36, 37), only low levels of histone acetylation were present in an upstream DNase I-hypersensitive site/enhancer (38) in either HUVEC or VSMC.

Selective enrichment of histone modifications at cell-specific promoters is a newer area of study, and studies in vascular endothelial cells are beginning to address broad or specific histone modification patterns (13, 39–41). Yet it is not known whether histone acetylation is broadly enriched in endothelial cells compared with nonendothelial cells. Therefore, histone proteins were extracted from HUVEC and VSMC and Western blots were used to assess global steady-state H3 and H4 acetylation levels (Fig. 4C). Levels of acetylated histones did not differ significantly between cell types, suggesting that bulk histone acetylation differences could not account for the endothelium-specific acetylation of eNOS promoter histones. CYPA is a widely expressed housekeeping gene (Fig. 4D). Assessment of histone acetylation at the CYPA promoter revealed no significant differences between HUVEC and VSMC (Fig. 4E). Therefore, histone acetylation differences between cell types at the eNOS proximal promoter are a specific rather than a global phenomenon.

Endothelial Cells Are Highly Enriched in Acetylated H3 (Lysine 9) and Acetylated H4 (Lysine 12) at the eNOS Promoter—The histone code model suggests that it is not simply the presence or absence of acetylation of histones H3 and H4 that is important in transcriptional regulation but that the combination of acetylation, methylation, phosphorylation, and other covalently bound residues determines whether chromatin is transcriptionally active or silent (20, 21). Since the antibodies directed against acetylated histones used in Fig. 4 recognize different acetyl residues of histones, the acetylation of specific lysine residues of histones H3 and H4 at the eNOS proximal promoter was assessed using monospecific antibodies directed against unique lysine modifications. The acetylated histone H3 lysine 9 (AcH3(K9)) modification was found to be highly enriched in endothelial cells, whereas AcH3(K14) was not significantly different between cell types (Fig. 5, A and B, respectively). Examination of lysines 5, 8, and 16 of histone H4 revealed no significant differences between expressing and nonexpressing cell types (Fig. 5, C, D, and F, respectively). AcH4(K5) and AcH4(K16) levels were low in all cell types tested, and AcH4(K8) levels were moderately high in all cell types. The low levels of H3 or H4 monospecific changes, such as AcH3(K14) and AcH4(K16), have been confirmed using multiple antibodies (data not shown), each of which has been demonstrated to immunoprecipitate chromatin in ChIP assays for other genes (42). Importantly, we found that lysine 12 acetylation of H4 was significantly higher in endothelial cells than nonendothelial cells (Fig. 5E). Thus, an endothelium-specific histone code consisting of lysine 9 acetylation of H3 and lysine 12 acetylation of H4 exists at the eNOS proximal promoter.

**FIG. 5.** Endothelial cells are enriched in acetylated histone H3 (lysine 9) and acetylated histone H4 (lysine 12) compared with nonendothelial cell types. ChIP assay using (A) anti-acetyl histone H3 (Lys9), (B) anti-acetyl histone H3 (Lys14), (C) anti-acetyl histone H4 (Lys5), (D) anti-acetyl histone H4 (Lys8), (E) anti-acetyl histone H4 (Lys16), and (F) anti-acetyl histone H4 (Lys8) in eNOS-expressing (HUVEC and HMVEC; white bars) and nonexpressing (VSMC, HeLa, HepG2, and JEG3; black bars) cell types. Shown in all panels is ChIP analysis of the proximal promoter of eNOS. *, a significant difference between endothelial and nonendothelial cell types (p < 0.05).
endothelial cell types had very low levels of dimethylated lysine 9 of histone H3 at the eNOS promoter. Although lacking important levels of H3 lysine 9 acetylation, we did not find high levels of H3 lysine 9 dimethylation at the eNOS core promoter in nonexpressing cell types (Fig. 6).

Serine 5-modified RNA Polymerase II Is Preferentially Loaded at the Proximal Promoter of eNOS in Endothelial Cells—We next sought to correlate histone modification status with transcriptional activity. Previously, we found that Pol II is differentially localized at the eNOS proximal promoter in HUVEC versus VSMC (10). This was the same region that had contrasting DNA methylation patterns across cell types and, as shown above, also represents a discrete region of differentially modified histones. Using quantitative ChIP assays, we directly compared Pol II density, distinguishing between total (N terminus) and differentially modified CTD regions of Pol II (serine 2 and serine 5), and measured transcriptional activity by nuclear run-on assays across broader regions of NOS3 in a variety of expressing and nonexpressing cell types (Fig. 7).

Pol II was highly enriched at the proximal promoter in endothelial cells compared with other cell types (Fig. 7A). Although Pol II is selectively recruited to upstream enhancer genomic regions in certain cell-specific genes (45), we failed to detect Pol II at upstream enhancer regions (−4718 to −4596) of eNOS (Fig. 7A). Compared with the proximal promoter, we also failed to detect a robust Pol II signal at downstream genomic regions in endothelial cells. HUVEC demonstrated ~10-fold less Pol II ChIP signal in exon 2 (+1463 to +1514) or exon 14/intron 14 (+12,652 to +12,735) genomic regions compared with the promoter (~166 to ~26). Despite this decrease in Pol II compared with the proximal promoter, Pol II levels were reproducibly higher than 5′-flanking regions and well above background signals obtained with nonspecific antibody controls in these downstream genomic regions in both endothelial and nonendothelial cells. Therefore, although regions 1500 bp downstream of the core promoter are markedly enriched in marks of transcriptionally active chromatin (acetylated histones H3 and H4 and methylated lysine 4 of histone H3) in endothelial cells, they are not highly enriched in Pol II. This was surprising because of the dramatic differences in steady-state levels of eNOS mRNA between cell types (Fig. 4D) and since it has been argued that the association of Pol II with downstream coding regions correlates well with transcriptional signal obtained with nuclear run-on studies, especially for stable mRNA transcripts like eNOS (46).

Assessments of nuclear run-on signal revealed that competent transcriptional complexes were differentially localized over the NOS3 locus (see representative result in Fig. 7B). In general, run-on signal was stronger in endothelial cells compared with nonendothelial cells. Signal was also enriched in both endothelial cells and nonendothelial cells at the core promoter/exon 1/intron 1 relative to exon 8-exon 16 downstream...
regions. Across the cell types that were examined, we found comparable transcription rates at the GAPDH locus, as a positive control, and the absence of signal for the pBluescript prokaryotic vector, as a negative control. We observed a comparable signal at genomic regions that correspond to the transcriptional overlap of the \textit{NOS3} and \textit{NOS3AS} genes. Studies with single-stranded probes in nuclear run-on assays have indicated that the double-stranded probe used in these run-on assays detects transcription in both directions at these overlapping genomic regions (30). \textit{NOS3AS} encodes the antisense sONE mRNA transcript, sits in an overlapping tail-to-tail configuration with \textit{NOS3}, and participates in the post-transcriptional control of eNOS expression through sense/antisense RNA interactions (30).

As shown in Fig. 7A, at exon 2 and exon 14/intron 14 regions, levels of Pol II were not dramatically higher in endothelial cells compared with nonendothelial cell types. These studies used a ChIP antibody that recognizes the N terminus of Pol II. In general, Pol II is loaded onto promoters in an unphosphorylated form and is phosphorylated by TFIIH at serine 5 of the CTD heptad repeats when transcription is initiated (47). The presence of this modification has been correlated with H3 lysine 4 methylation in mammalian cells (48) and serves as a signal for the binding of other proteins needed for RNA processing, such as the 5' capping complex (49). Therefore, we considered whether modifications of Pol II are cell type-specific.

As shown in Fig. 7C, we found no important difference between HUVEC and VSMC at exon 2 (+1463 to +1514) using an anti-Pol II (N terminus) ChIP analysis to assess the distribution of Pol II along the eNOS gene in expressing (HUVEC; white bars) and nonexpressing (VSMC, HeLa, HepG2, and JEG3; black bars) cell types. Since the antibody used recognizes the N terminus of Pol II, signals represent total Pol II, independent of CTD phosphorylation status. B, nuclear run-on was used to assess transcription of the eNOS gene in HUVEC, VSMC, HeLa, HepG2, and JEG3. GAPDH and pBluescript were used as positive and negative controls for transcription, respectively. The eNOS exon 1-intron 1 and eNOS exon 8–16 double-stranded probes recognize only eNOS transcriptional events, whereas the eNOS exon 17–26 probe also measures transcription of the overlapping transcriptional unit, sONE/NOS3AS (intron 9-exon 12). Shown is a representative blot of three identical experiments. C, ChIP using a protocol optimized for use with antibodies directed toward phosphorylation of the CTD of Pol II. HUVEC (white bars) and VSMC (black bars) were analyzed for the presence of total Pol II levels (N terminus) and serine 2- and serine 5-phosphorylated Pol II (CTD) for the proximal promoter and exon 2. All values are expressed relative to the immunoprecipitated (IP) DNA signal using the N-terminal Pol II antibody in HUVEC at the proximal promoter. *, a significant difference between endothelial and nonendothelial cell types ($p < 0.05$).
antibody that recognizes the N terminus of Pol II (HUVEC/ VSMC Pol II ChIP signal ratios of 8.1 and 1.7 at promoter and exon 2, respectively). Qualitatively, however, we observed an important difference between cell types in the amount of Pol II that was phosphorylated at serine 5 of the CTD at both the proximal promoter and in exon 2 (HUVEC/VSMC serine 5 Pol II ChIP signal ratios of 49.1 and 7.6 at promoter and exon 2, respectively). In contrast, we found no appreciable levels of serine 2 phosphorylated Pol II at either site in HUVEC or respectively). In contrast, we found no appreciable levels of serine 2 phosphorylated Pol II at either site in HUVEC or respectively). Therefore, ChIP assessments of the serine 5-phosphorylated CTD form of Pol II correlated closely with nuclear run-on assays. This suggests that serine 5-phosphorylated Pol II is a better measure of transcriptional activity than total levels of Pol II with respect to cell-specific expression patterns, at least for eNOS. Similar findings have recently been described for active genes on human chromosome 21 and 22 (48).

**Functional Relevance of Differentially Acetylated Histones at the eNOS Promoter**—Treatment of cell types that do not express appreciable amounts of eNOS with 5-azacytidine, a DNA methyltransferase inhibitor, results in increased steady state levels of the eNOS transcript (10, 13). HDACs are known to be tethered to DNA via methyl DNA-binding domain proteins such as MeCP2 (34), and HDACs can also be recruited to cis-elements through trans-factor interactions (17). Since DNA methylation alone did not prevent trans-factor binding in EMSA experiments, we sought to determine whether repressive histone post-translational modifications might play a functional role in repressing eNOS expression. We treated endothelial and nonendothelial cells with TSA, a potent class I and II HDAC inhibitor. Treatment of the nonexpressing cell types HeLa and VSMC with TSA resulted in increased eNOS steady state levels as detected by real-time PCR (Fig. 8A). Results were comparable regardless of whether eNOS mRNA levels were normalized to the housekeeping genes GAPDH or CYP A. In contrast to the effect of TSA on nonendothelial cell types, treatment of HUVEC with equivalent concentrations of TSA led to a decrease in eNOS steady-state mRNA levels, as published previously by us and others (30, 50). To dissect the mechanism by which steady-state eNOS mRNA increased following TSA treatment of nonendothelial cells, we performed ChIP assays. The levels of acetylated histones H3 and H4 increased at the eNOS proximal promoter in VSMC and HeLa following 24 h of TSA treatment (Fig. 8B). Similar treatment of HUVEC resulted in no change in histone acetylation at the eNOS promoter. The potent effect of TSA on eNOS expression in nonexpressing cell types implies that HDAC functional activity is mechanistically relevant to eNOS repression in these cell types. We used ChIP combined with real-time PCR to assess the binding of endogenous HDAC1, -2, and -3 at the eNOS proximal promoter (~166 to ~26). As shown in Fig. 8C, HDAC1 was highly enriched at the proximal promoter of eNOS in VSMC compared with HUVEC. A similar enrichment was not evident for HDAC2 or HDAC3. The differences in ChIP results for HDAC1 were not due to the absence of this class I HDAC in endothelial cells, as demonstrated by Western blotting (data not shown).

Genes that are silenced by epigenetic mechanisms can respond differentially to inhibition of repressive chromatin-based transcriptional pathways. Some genes can be activated by 5-azacytidine and some by TSA, whereas others require the combined addition of both 5-azacytidine and TSA (51, 52). For other genes, the addition of both inhibitors leads to a synergistic activation compared with either agent alone (53, 54). To determine whether there was a synergistic interaction between these two inhibitors, we treated VSMC with 5-azacytidine for 7 days and TSA for the last 24 h. The combined treatment did not result in a synergistic effect (Fig. 8D). These studies, which used maximally tolerated concentrations of pharmacological agents, indicated that the effect of TSA was clearly more potent than 5-azacytidine. This suggests that class I and II HDACs play an important role in repressing eNOS expression in nonendothelial cells.

**Inhibiting Histone Deacetylase Activity in Nonendothelial Cells Does Not Alter Steady-state Levels of sONE**—The finding that inhibiting HDACs led to increased expression of eNOS in nonexpressing cell types suggested a transcriptional mechanism. However, TSA is known to decrease eNOS RNA levels in endothelial cells by post-transcriptional mechanisms (50). We have suggested previously that this reduction in eNOS steady-state RNA abundance may be mediated by sONE, an overlapping antisense transcript to eNOS (30). sONE has been demonstrated to negatively regulate eNOS at the post-transcriptional level. Importantly, levels of sONE increase prior to the decrease in eNOS RNA levels following TSA treatment of endothelial cell types (30). In TSA-treated nonendothelial cells, the increase in eNOS could be similarly due to post-transcriptional mechanisms, namely a decrease in sONE levels and hence loss of negative regulation of eNOS. However, no significant changes in sONE steady-state levels were noted in VSMC or HeLa following TSA treatment, whereas sONE levels were clearly elevated in parallel experiments in HUVEC (data not shown).

**eNOS Expression Is Controlled by Both Histone Acetylation and Lysine 4 Methylation of Histone H3 at eNOS Proximal Promoter Regions**—The dramatic difference in lysine 4 methylation at the eNOS proximal promoter between cell types was intriguing. To determine the functional importance of lysine 4 methylation to eNOS expression, eNOS steady-state levels (Fig. 9A) and histone modifications (Fig. 9B) were assessed in VSMC treated with TSA for 4 or 24 h. While eNOS mRNA was markedly increased following 24-h treatment of VSMC with TSA, at 4 h steady-state levels were unchanged (Fig. 9A). Since histone acetylation is known to rapidly increase following inhibition of HDACs, it was curious that steady-state levels did not also rapidly increase. ChIP revealed that whereas histone acetylation rapidly increased by 4 h of TSA treatment, lysine 4 methylation of H3 did not increase until 24 h (Fig. 9B), a time point at which eNOS RNA levels are greatly elevated. This same pattern was also evident in TSA-treated HeLa cells (data not shown). These findings may suggest that lysine 4 methylation of histone H3 is a modification that is mechanistically downstream of histone acetylation and that this modification, together with histone acetylation, is necessary for eNOS mRNA expression. To determine the mechanism involved, we treated VSMC with MTA a potent inhibitor of lysine 4 methylation (55, 56) for 2 h followed by the addition of TSA for 22 h. Importantly, treatment of VSMC with MTA blocked TSA-induced increases in eNOS mRNA (Fig. 9C). This was not due to MTA preventing TSA-mediated increases in histone acetylation, since histone acetylation was still increased in MTA-treated cells that were treated with TSA for 4 or 22 h (Fig. 9D). Importantly, MTA prevented the increase in lysine 4 methylation following 22 h of TSA treatment.

To assess the importance of lysine 4 methylation to the constitutive expression of eNOS in endothelial cells, HUVEC were treated with MTA. Treatment of HUVEC with MTA for 48 h resulted in dramatically lower levels of eNOS RNA (Fig. 9E). We also noted a decrease in H3 lysine 4 dimethylation and Pol II recruitment at the eNOS promoter (Fig. 9F). These results imply that lysine 4 methylation of H3 is important not
only for the TSA-induced expression of eNOS in nonendothelial cells but also the basal expression of eNOS in endothelial cells.

**DISCUSSION**

The current work provides strong evidence that epigenetics, or chromatin-based mechanisms, play a key role in the constitutive expression of an endothelium-restricted gene. The eNOS gene serves as an excellent model to dissect the mechanisms of endothelium-specific gene expression. The eNOS proximal promoter has been mapped in detail (14, 57); distal regulatory elements have been identified (38) and transgenic reporter mice have been developed using eNOS promoter sequences (12, 58). Expression of the eNOS mRNA is abnormal in a number of

**FIG. 8.** Histone deacetylases negatively regulate eNOS expression in nonendothelial cell types. A, HUVEC, VSMC, and HeLa were treated with 1000 nM TSA for 24 h, and relative eNOS levels were determined by real time reverse transcription-PCR. eNOS RNA levels were normalized to GAPDH and CYPA levels. B, ChIP analysis was performed using anti-AcH3 and anti-AcH4 antibodies in control and 24-h TSA-treated HUVEC, VSMC, and HeLa, and real time PCR was used to assess the eNOS proximal promoter (−166 to −26). C, ChIP was also used to assess the presence of HDAC1, -2, or -3 in HUVEC and VSMC. Shown is a representative experiment. D, to determine the interrelationship between DNA methylation and histone acetylation, VSMC were treated with 1000 nM TSA for 24 h or a combination of 7 days with the DNA methyltransferase inhibitor 5-azacytidine and 24 h of TSA, followed by quantification of eNOS RNA by real time PCR. Shown is a representative result of five comparable experiments. *, a statistically significant difference (p < 0.05).
vascular diseases (59). Therefore, the finding that the expression of eNOS is very tightly regulated by epigenetic mechanisms raises the possibility that perturbations in these pathways in disease may have profound effects on eNOS steady-state RNA levels in the endothelium. This has important implications for the control of local vascular tone and systemic blood pressure. It is of interest that epigenetic pathways have also been recently implicated in other facets of vascular gene expression, such as the pathophysiology of atherosclerosis (60, 61), the microvasculature environment of tumors (62), cytokine-inducible gene expression in vascular endothelium (41), and developmental regulation of vascular remodeling (39). As an example of the disease relevance of epigenetics and hypertension, epigenetic pathways have recently been implicated in the renal epithelial cell-specific expression of 11-β-hydroxysteroid dehydrogenase type II, an enzyme that controls blood pressure by regulating mineralocorticoid bioactivity and total body sodium content (63).

We previously established that DNA methylation is important in controlling transcription of the eNOS gene. However, several observations suggested to us that additional mechanisms must also be operative. First, DNA methylation did not have an effect on nucleoprotein complex formation on naked eNOS probes in EMSA studies. Second, whereas eNOS transgenes were active in an endothelial cell-specific fashion in vivo, DNA methylation patterns of promoter sequences of the transgene did not differ in a cell-specific manner, and an inhibitor of DNA methyltransferase activity did not affect in vivo expression. Because the transgene expressed with exquisite endothelial cell specificity, and expression was independent of the insertional site in genomic DNA, we assessed the chromatin environment of the native eNOS gene in expressing and non-expressing human cell types. Within endothelial cells, the nucleosomes that encompassed the eNOS core promoter and proximal downstream coding regions were highly enriched in acetylated histones H3 and H4 and methylated lysine 4 of histone H3. A unique combination of specific histone modifications was found at the differentially modified chromatin region. These modifications were not evident in a wide variety of non-expressing primary and tumor cell types. Furthermore, in this
paper, we have provided evidence that histone modifications at these regions are functionally relevant to eNOS expression.

Endothelial cells were particularly enriched in acetylated histone H3 lysine 9 and histone H4 lysine 12 at proximal regions of the NOS3 gene. These findings could not have been predicted empirically. Indeed, recent genome mapping studies of H3 lysine 9 and lysine 14 acetylation modifications have not distinguished between these two modifications (48). However, it is becoming increasingly important to distinguish one modification from another. For example, recent work has indicated that the combined acetylation of lysines 9 and 14 of histone H3 is key to the recruitment of TFIIID to the interferon-β promoter (15), whereas H3 lysine 9 acetylation was more relevant than lysine 14 to the induction of the COX-2 or E-selectin promoters (41, 42).

As with H3, the acetylation of lysine residues located on the N terminus of histone H4 are important for transcriptional processes (15, 64). Lysine 5 acetylation is associated with the derepression of some genes (65), and lysine 8 acetylation was shown to dramatically increase at the interferon-β promoter following viral infection (15). Lysine 16 acetylation is important in the inappropriate expression of genes that are normally repressed by MeCP2 in humans with Rett syndrome (66). At the eNOS promoter, H4 lysine 8 acetylation was moderately high in all cell types. In contrast, levels of acetylated H4 lysine 5 and 16 were low in all cell types. Importantly, it was H4 lysine 12 that was preferentially acetylated at the eNOS promoter in endothelial cells. Again, these findings could not have been predicted empirically. Several recent studies have demonstrated that lysine 12 acetylation is increased during the activation of select genes, some of which were uniquely associated with H3 lysine 9 acetylation (15, 41, 42). The combination of specific acetyl modifications may be especially relevant, such as H3 lysine 9 with H4 lysine 12, or H4 lysine 8 with H4 lysine 12. Attention has recently focused on double bromodomain-containing proteins, such as Brd2. This particular protein participates in transcriptional activation through transcriptional scaffolding mechanisms and preferentially binds acetylated H4 lysine 12 (67, 68). The identification of proteins that can read the acetylation histone code is an important future question. Since histone H3 is deposited onto newly forming chromatin with acetylated lysine 9 and 14 residues and histone H4 is acetylated at lysines 5 and 12 in newly synthesized chromatin (69), histone acetyltransferases and HDACs must remodel the acetylation pattern to set up the cell-specific histone code at the eNOS promoter. Therefore, determining which proteins are implicated in establishing and maintaining these specific modifications represent the next key series of questions.

Because inhibition of HDACs can alter the repressive histone code in nonendothelial cells, we speculated that HDACs were specifically recruited to the eNOS proximal promoter in nonendothelial cells. We found that HDAC1, but not HDAC2 or HDAC3, was selectively bound to the eNOS promoter in VSMC versus HUVEC. The mechanisms regulating HDAC1 recruitment are not known, but the patch of heavily methylated cytosine residues found in the proximal promoter DMR region, which does not extend to upstream regions, is probably important (10). DNA methylation is known to recruit HDACs via MBDs such as MeCP2 (34). Moreover, we report in the present studies that MeCP2 is present at the eNOS proximal promoter in VSMC but not HUVEC. However, whereas MeCP2 engagement at the eNOS promoter may be important in localizing HDAC1 to the eNOS promoter in VSMC, the presence of a DMR may not be essential for cell-specific expression. Inhibit-
events, including promoter clearance. The current work indicates that the differentially modified chromatin domain at the proximal region of the eNOS gene was selectively associated with functionally competent RNA Pol II complexes, as assessed by nuclear run-on studies, and the enrichment of these regions for serine 5-modified RNA Pol II in endothelial cells. Serine 5-phosphorylated Pol II can interact with a variety of chromatin-associate proteins (78, 79), and attention has recently focused on the relationship between H3 lysine 4 methylation and this RNA Pol II modification (48). Models of transcriptional activation and promoter clearance have suggested that both phosphorylation and dephosphorylation of serine 5 can be controlled in a cell-specific fashion. Of interest, recent evidence indicates that small class-C CTD phosphatases function in silencing neuron-specific genes in nonneuronal cell types (80). These phosphatases exhibit specificity for serine 5-modified Pol II and are active in silencing gene expression in nonexpressing cell types. The involvement of such phosphatases in eNOS cell specificity is not known.

In summary, we have demonstrated here that the cell-specific expression of eNOS is controlled by a unique cell-specific histone code. Decreases in eNOS mRNA expression in cardiovascular disease have been well documented. However, the mechanism controlling this decrease in mRNA levels is not understood. We posit that epigenetic mechanisms, especially covalent histone modifications of the eNOS promoter, may be important in some of these disease processes.

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Histone Code of the Human eNOS Gene