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Three Repeats of CCCCTCC on the Pyrimidine-rich Sequence in the Proximal 5′ Flanking Region are Required for Efficient Transcriptional Activity of the Human Endothelial Nitric Oxide Synthase Gene

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The endothelial nitric-oxide synthase (eNOS) gene is constitutively expressed in endothelial cells, but numerous regulatory elements in the promoter region should contribute to the regulation for cell specific expression and the response to exogenous stimuli. A Sp1-binding consensus motif (−104 to −96) is essential for a core promoter activity of the human eNOS gene. In this study, we show that three repeats of CCCCTCC element (−74, −61, and −47), which located periodically at 13 and 14 nucleotide intervals on a pyrimidine-rich string in the proximal 5′-flanking region, were required for efficient transcriptional activity of the eNOS gene. In electrophoretic mobility shift assays, a specific DNA-protein complex was formed with a binding ability depending on the number of the CCCCTCC element while only one element did not retain any binding ability. Dinucleotide-substitution mutants at the repeat sequences reduced their transcriptional activities of the eNOS gene in transient transfection assays as diminishing their abilities to form the complex. Further, DNase I footprinting analyses indicated that nuclear extracts continuously protected a proximal region from −108 to −16, which includes pyrimidine-rich and purine-rich strings containing three CCCCTCC repeats and the Sp1-binding motif. UV-crosslink assay revealed the CCCCTCC repeat probe bound to a 97 kDa protein in the complex. A huge protein complex including Sp1-related factors and a 97 kDa protein might be formed along the proximal promoter of the eNOS gene for efficient transcriptional activity.

Keywords: NO; NOS; Endothelium; Promoter; Transcription; Sp1

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase

INTRODUCTION

Nitric oxide (NO) was demonstrated to be a regulatory substance in a number of diverse physiological processes, including smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission, immune regulation and penile erection (for review, see Refs. [1–3]). NO is produced from L-arginine by nitric oxide synthase (NOS, EC 1. 14. 13. 39.) with a concomitant production of L-citrulline. There are three distinct isoforms of NOS in animal bodies. Neuronal (nNOS) and endothelial nitric-oxide synthase (eNOS) are constitutive but dormant until temporal activation by Ca^{2+} transient elevation which sustains the binding of calmodulin. Thus, their catalytic activities are rapidly stimulated by increases in intracellular calcium ion through specific signal induction pathways. The third NO synthase (iNOS) is expressed in several tissues including arterial endothelium only after transcriptional induction. Once synthesized, this iNOS is active for prolonged periods without a requirement for elevation of Ca^{2+} above the physiological levels in cells.

Recent studies show that regulation of eNOS expression has important physiological and pathological implications. Indeed, lipopolysaccharide,[4] tumor necrosis factor-α,[5] and hypoxia[6] decrease the eNOS expression post-transcriptionally by specific destabilization of the eNOS mRNA.

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Shear stress,[7,8] estrogen,[9] lysophosphatidylcholine,[10] transforming growth factor-β,[11] and platelet-derived growth factor[12] upregulate eNOS expression by increasing the transcriptional activity of the eNOS gene. Further, eNOS expression is relatively restricted to endothelial cells in blood vessels, except *in situ* immunohistochemical studies demonstrated the location of eNOS to hippocampal pyramidal cells.[13] Few transcriptional factors, however, have been detected on the regulation of the eNOS gene in such conditions.

The eNOS gene is constitutively expressed in endothelial cells, but numerous regulatory elements in the promoter region should contribute to the regulation for cell specific expression and the response to exogenous stimuli. We[14] and others[15,16] reported that the gene for the human endothelial NOS is composed of 26 exons and spans 21 kb. Sequence analysis of the 5′-flanking region of the eNOS gene shows the promoter lacks a typical TATA element but contains a number of Sp1 binding consensus sequences in GC-rich regions. These characters are often found on many so called "housekeeping" genes. We previously demonstrated that the 5′-flanking region up to −116 of the human eNOS gene exhibited a sufficient transcriptional activity in endothelial cells.[17] Further, a Sp1 binding motif located between −104 and −96 in the GC-rich region was essential for a core promoter activity of the eNOS gene.[17–19] In the present study, we report the importance for transcriptional regulation by three repeats of CCCCTCC, which located periodically at 13 and 14 nucleotide intervals on a pyrimidine-rich string in the proximal 5′-flanking region of the eNOS gene.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from Amersham Corp. (Amersham, England). Klenow fragment, T4 polynucleotide kinase, exonuclease III, and T4 DNA ligase were from New England Biolab (Beverly, MA). Restriction enzymes were Takara (Kyoto, Japan) or NEB. The host cell for molecular cloning was DH5αF′IQ of *E. coli* (Life Technologies, Gaithersburg).

Site-directed Mutagenesis

To generate pGLeNOS-116 mutants, single-stranded DNAs of Bluescript II(+)SK containing the −116/+8 fragment of the eNOS gene were prepared using helper phage R408. Complementary strands were synthesized by T7 DNA polymerase using dinucleotide-substituted oligonucleotides: −88/−62M10U(5′-CAGCACTGctGAGCCCCCTCCCACTGC-3′), M20U(5′-CAGCACTGGAGAGCCggCT CCCACTGC-3′), M30U(5′-CAGCACTGGAGAGCCCCCTggCACTGC-3′), M40U(5′-CAGCtgTGGAGAGCCCCCTCCCACTGC-3′), −60/−31M02L(5′-TTAGGAAGAGGGAGccGACCGAGAGGAGGG-3′), and −60/−31M03L(5′-TTAGGAAGAGccAGGGGACCGAGAGGAGGG-3′) as primers and ligated by T4 DNA ligase according to the manufacturer's manual (United State Biochemical, Cleveland). The double stranded DNAs were transfected into SDM *E. coli* cells (mcr−). The resulting mutant plasmids were sequenced to validate the mutagenesis at proper sites. The 5′-flanking regions containing the mutations were ligated to the luciferase expression vector pGL3-basic again.

Cell Culture, Trasfections and Luciferase Assays

Human umbilical venous endothelial cells were purchased from Clonetics (USA) and were cultured in EGM-2 medium according to the manufacture's protocol. Plasmid DNAs for transfection were prepared according to alkaline lysis method[20] and purified by two successive centrifugations through CsCl gradient. tHUE-2 cells[21] were cultured at 37°C in serum-free ASF301 medium (Ajinomoto, Tokyo, Japan). The tHUE-2 cells on a 35-mm culture plate were transfected with 1 μg of a luciferase reporter construct together with 0.2 μg of a β-galactosidase expression vector, pCH110, using 6 μl of Lipofectin reagent (Life Technologies) according to the manufacturer's instructions. The cells were treated for 24 h and then incubated in fresh medium for 24 h. The transfected cells were harvested and cell extracts were prepared in 200 μl of PicaGene lysis buffer (Wako, Tokyo). Luciferase activities were measured using PicaGene assay kit (Wako) for 10 s on a luminometer, Lumat LB9501 (EG&G Berthold, Germany). Variations in transfection efficiencies were normalized by determining the β-galactosidase activities of pCH110.

Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from culture cells on a 10-cm plate according to a small-scale preparation method.[22] Oligonucleotides, −88/−62U(5′-CAGCACTGGAGAGCCCCCTCCCACTGC-3′), −76/−51 U(5′-GCCCCCTCCCACTGCCCCCTCCTCTC-3′), −60/−16U(5′-CCCTCCTCTCGGTCCCCTCCCTCTTCCTAAGGAAAAGGCCAGGGC-3′), and their anti-sense oligonucleotides, −88/−62L, −76/−51L, −60/−16L, were synthesized and purified through an OPC column. To be used as a probe, an oligonucleotide was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. It was annealed with

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its complementary oligonucleotide in 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA after heating to 75°C for 5 min. Longer DNA fragments were synthesized by polymerase-chain-reaction (PCR) using pairs of proper oligonucleotides, and purified through polyacrylamide gel electrophoresis. To make a fragment −76/−51 flanked with multi-cloning site sequence of Bluescript II(+)SK, fragment −76/−51 was cloned into the Eco RV site. One molecule of fragment −76/−51 inserted into the Eco RV site was digested with Sac I and Kpn I, then was isolated through polyacrylamide gel after electrophoresis. Two molecules of fragment −76/−51 in tandem into the Eco RV site was digested with Eco RI and Hind III, then was isolated as above. For multiple copies of fragment −76/−51, fragment −76/−51 was phosphorylated with ATP by T4 polynucleotide kinase and ligated together by T4 DNA ligase. To be used as probes, these isolated DNA fragments were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. On binding reactions, nuclear extracts (2.1 μg protein) were incubated for 20 min at 25°C with a radiolabeled probe in a 10-μl reaction buffer containing 10 mM HEPES–KOH, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 1 μg of poly(dI–dC)–poly(dI–dC). The reaction mixtures were resolved at 4°C by a nondenaturing electrophoresis in a 5% polyacrylamide gel for 1 h at 100 V in 1 × Tris–glycine buffer (50 mM Tris, pH 8.5, 380 mM glycine, and 2.1 mM EDTA). The gel was dried and exposed to a piece of X-ray film (Fuji XR, Fuji). For competition experiments, a 200-fold molar excess of unlabelled oligonucleotides was added to the reaction buffer before binding reaction. Monoclonal antibodies against Sp1 (PEP2), Sp3 (D-20), and GATA-2 (C-20) were from Santa Cruz Biotechnology Inc. A monoclonal antibody against MAZ and a polyclonal antibody against ZBP-89 were kindly gifted by Dr K.B. Marcu and Dr J.L. Merchant, respectively. In supershift experiments, antibodies were added to the reaction buffer before binding reaction.

DNase I Footprinting Analysis

Probe DNAs were labeled at the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP or labeled at the 3′ end with DNA polymerase Klenow fragment (New England Biolab) and [α-^{32}P]dCTP. A 100 μl-binding reactions contained 28 μg or 84 μg of tHUE-2 nuclear extracts, 10 mM Hepes(pH7.9), 60 mM KCl, 0.1 mM EDTA, 10 μg of poly(dI–dC)–poly(dI–dC), 0.5 mM DTT, 5% glycerol, and an appropriate amount of radiolabeled probe DNA. The nuclear extracts were dialyzed against buffer D (20 mM Hepes at pH 7.9, 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT).[23] After reactions were incubated at 25°C for 20 min, DNase I (0.5 or 1.4 units, Takara) was added to the reactions and incubated for 2 min at 25°C. The reactions was stopped with 100 μl of stop buffer containing 200 mM NaCl, 20 mM EDTA, and 1% SDS. The reaction products were purified by phenol/chloroform extraction and ethanol precipitation, and were analyzed on DNA sequence gel with a chemical sequencing reaction of the probe DNA as a marker.

UV-crosslink Assay

A fragment −97 to +8 of the eNOS gene cloned into pBluescript II (+) was amplified by Taq DNA polymerase with SK and KS primers using bromodeoxyuridine(BrdU) and [α-^{32}P]dCTP. After digested with appropriate restriction enzymes, the BrdU-fragment DNA was isolated and purified on 5% polyacrylamide gel. Using this BrdU-DNA as a probe, electrophoretic mobility shift assays were performed as mentioned above, and the whole gel was irradiated for 20 min with 320 nm UV light at 2.5-cm distance on a UV illuminator.[24] Shifted complexes corresponding to appropriate bands detected on X-ray films were isolated and eluted by incubation in 2 × SDS-loading buffer. Eluted complexes were analyzed on SDS-polyacrylamide gel and detected by autoradiograph on an X-ray film.

RESULTS

Transcriptional Regulation by the Proximal Region of the Human eNOS Gene

We previously analyzed the promoter activities of a series of various lengths of the 5′ flanking region of the human eNOS gene using transient transfection with CAT reporter constructs.[17] The region up to −116 of the eNOS gene still retained a sufficient promoter activity. We also showed that the distal Sp1-binding motif (−104 to −96), but not the proximal one (−99 to −91), was essential for a basal promoter of the eNOS gene.[17] Figure 1A shows the nucleotide sequences of the eNOS gene promoter region and indicates DNA fragments and mutant constructs used in the present report. To analyze whether any other elements located in the proximal region up to -116 participated in the regulation of eNOS gene transcription, we constructed deleted mutants of the internal regions in the 5′ flanking region ligated to the luciferase reporter gene. We transfected tHUE-2 (a human umbilical venous endothelial cell line)[21] cells with the deletion mutants to measure their transcriptional activities (Fig. 1B). A deletion of nucleotides from −98 to −34 (pGLeNOS−116Δ−98/−34), which disrupted the Sp1-binding motif (−104 to −96), reduced its transcriptional activity to only 8%

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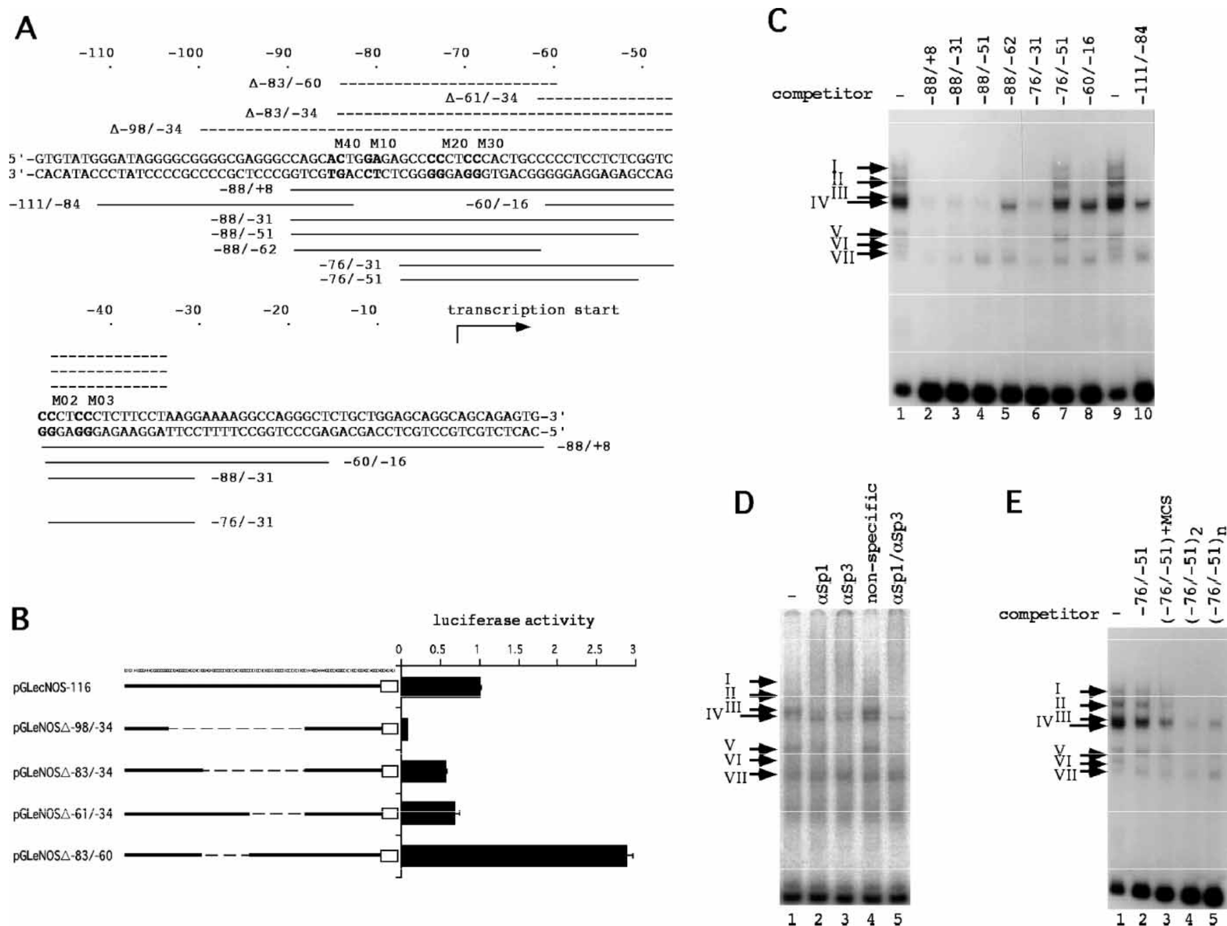

FIGURE 1 Transcriptional active elements exist in the proximal sequence of the human eNOS gene. (**A**) Nucleotide sequences of both strands of the 5′ flanking region from −116 to +8 relative to the cap site (+1) of the human eNOS gene are shown. An arrow indicates the transcription start site. Dotted lines represent internal deleted sequences in a series of deletion mutants of pGLeNOS−116. Bold lines represent DNA fragments used for electrophoretic mobility shift assays. Bold letters shows the substituted dinucleotides of GA to CT (M10), CC to GG (M20), CC to GG (M30), AC to TG (M40), CC to GG (M02), and CC to GG (M03) at respective sites. (**B**) The construct pGLeNOS−116 contains the 5′ flanking region up to −116 relative to the cap site (+1) of the human eNOS gene fused to the luciferase gene of pGL3-basic plasmid. Deleted sequences of the eNOS promoter region are shown by dotted lines. For example, the construct pGLeNOS-116Δ−98/−34 lacks the 65 nucleotides from −98 to −34. These reporter constructs were transfected into tHUE-2 cells and assayed for luciferase activities. The luciferase activities were normalized using β-galactosidase expression from co-transfected pCH110 vector as an internal control. Relative activities of reporter constructs were calculated when the normalized luciferase activity of pGLeNOS−116 was arbitrarily set to 1. The data are mean ± SD of three experiments. (**C**) Electrophoretic mobility shift assays of the eNOS gene promoter region. The ^{32}P-end-labeled −88/+8 fragment probe was incubated with nuclear extracts from tHUE-2 cells in the absence (lanes 1 and 9) or the presence of a 200-fold molar excess of unlabeled fragment DNA, −88/+8 fragment (lane 2), −88/−31 fragment (lane 3), −88/−51 fragment (lane 4), −88/−62 fragment (lane 5), −76/−31 fragment (lane 6), −76/−51 fragment (lane 7), −60/−16 fragment (lane 8), or −111/−84 fragment (lane 10). (**D**) The ^{32}P-end-labeled −88/+8 fragment probe was incubated with nuclear extracts from HUVEC in the absence (lane 1) or the presence of an anti-body for Sp1 (lane 2), Sp3 (lane 3), GATA-2 (lane 4), or both anti-Sp1 and anti-Sp3 (lane 5). (**E**) The ^{32}P-end-labeled −88/+8 fragment probe was incubated with nuclear extracts from tHUE-2 cells in the absence (lane 1) or the presence of a 200-fold amount of unlabeled DNA, −76/−51 fragment (lane 2), −76/−51 fragment flanked by multicloning site region of Bluescript II SK(+) (lane 3), a dimer of −76/−51 fragment (lane 4), or a multimer of −76/−51 fragment (lane 5).

of the intact construct pGLeNOS−116. This reconfirmed the previous conclusion that the Sp1-binding motif was necessary for the basal activity.[17] Further, a deletion of nucleotides from −83 to −34 containing a long pyrimidine-rich tract (pGLeNOS−116Δ−83/−34) reduced its activity to 56%. A proximal shorter deletion of nucleotides from −61 to −34 (pGLeNOS−116Δ−61/−34) also slightly reduced its activity to 69%. The pyrimidine-rich sequence in the deletion should contain regulatory elements for a full transcriptional activity. In contrast, a deletion of the distal nucleotides from −83 to −60 (pGLeNOS−116Δ−83/−60) led to a 2.9-fold increase in the activity. This increase was an unexpected result. It is possible that some transcriptional factors bind to the distal region (from −83 to−60) to exhibit an inhibitory effect whereas other factors for enhancing effects bind to the proximal region (from −61 to −34).

Three Repeats of CCCCTCC are Required for Efficient Binding to Nuclear Factors

We performed electrophoretic mobility shift assays with nuclear extracts using fragment probes from

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the eNOS promoter region. The −88/+8 fragment probe made a major complex (denoted as IV) and six minor complexes (Fig. 1C). The mobilities of complex III and IV were very similar but the complexes were distinct as mentioned below. The presence of a 200-fold molar excess unlabeled −88/+8 fragment specifically competed to diminish the shifted bands of complexes I–VI but not complex VII (lane 2). Complex VII appeared not to be specifically formed on the probe. Relatively long fragments −88/−31 (lane 3) and −88/−51 (lane 4) also competed for formation of all the specific complexes I to VI. With a shorter fragment −88/−62 only complex IV was still detectable while the other specific complexes were competed well (lane 5). These results indicate that the sequence from −62 to −51 should include a binding site for complex IV formation. On the other hand, another long fragment −76/−31 competed for complex IV formation to be faintly detectable, and it competed completely for formation of all the other complexes (lane 6). However, a shorter fragment −76/−51 abolished complex VI formation, but it did not compete at all for formation of all the others (lane 7). Similarly, these results indicate that the sequence from −51 to −31 should also include a binding site for IV. Both sequences of −62 to −51 and −51 to −31 include a partial sequence of the CCCCTCC element. It should be noted that three CCCCTCC elements (−74, −61, and −47) locate periodically at 13- or 14-nucleotide intervals on a pyrimidine-rich string in the proximal 5′-flanking region of the eNOS gene (Fig. 1A). Although a proximal fragment −60/−16 contains one CCCCTCC element, it did not compete for complex IV formation at all (lane 8). Not less than two CCCCTCC elements should be necessary for formation of complex IV. We doubted whether these specific complexes included some Sp1- related factors as the fragment probe contained sequences that were similar to the consensus motif for Sp1. Fragment −111/−84 containing the Sp1-binding motif has a high binding ability to Sp1 factor.[17] In competition with fragment −111/−84, only complex IV still remained to be detectable (lane 10). Furthermore, an addition of anti-Sp1 antibody resulted in supershift of complex III to higher molecule complexes and anti-Sp3 antibody supershifted complex V as well as III (Fig. 1D). However, complex IV still remained to be detectable in the presence of both anti-Sp1 and anti-Sp3 antibodies. These results indicate that the CCCCTCC repeat formed complex IV which did not contain such Sp1-related factors although it possessed a low affinity binding site for Sp1- related factors.

It should be noted that although both fragments −88/−51 and −76/−51 contained two repeats of the three CCCCTCC elements, only −76/−51 fragment did not compete at all for formation of complex IV and others (Fig. 1C, lanes 4 and 7). Further, the fragment −76/−51 itself as a probe did not form any detectable complex (data not shown). To examine whether complex IV required for its formation any more sequence as well as the CCCCTCC repeat, we constructed three DNA fragments as competitors. One was a fragment −76/−51 flanked with an extra sequence of multi-cloning site from BlueScript II SK(+) vector, and the two others were self-ligated fragments containing two and multiple copies of fragment −76/−51 in tandem, respectively. In competition assays, the original fragment −76/−51 did not compete for complex IV formation while it competed only for complex VI formation (Fig. 1E, lane 2). The longer fragment −76/−51 with an extra sequence specifically competed for complex IV formation whereas complex III and other minor complexes I, II, and V remained to be detectable (lane 3). Further both DNA fragments with two and multiple copies of fragment −76/−51 also competed for complex IV formation while complex III was still slightly detectable (lanes 4 and 5). These results demonstrate that the formation of complex IV requires at least two CCCCTCC elements together with some unrecognized sequences. Such requirement of unrecognized sequences for binding was generally observed for restriction enzymes as well as other factors. Further, the binding ability for complex IV becomes stronger as the number of CCCCTCC element increases.

Mutations at the CCCCTCC Repeats Reduce the Binding Capacity and the Transcriptional Activity

We introduced dinucleotide-substitutions into the CCCCTCC repeat elements and the adjacent sequences (see Fig. 1A). Using the substitution mutant fragments as competitors, we performed electrophoretic mobility shift assays (Fig. 2A). An intact fragment −88/−31 competed for complex IV formation to the probe −88/+8 (lane 3) at the same level as fragment −88/+8 with a full length (lane 2). Mutant fragments −88/−31M10 and −88/−31M40, with substitutions at −80/−79 and −84/−83, respectively, also competed for complex IV formation (lanes 4 and 7) as the control fragment −88/−31 did. Whereas, fragment −88/−31M20 (CCCCTCC) with a substitution on the distal element did not affect complex IV formation so much (lane 5) and fragment −88/−31M30 (CCCCTCC) only slightly competed for complex IV formation (lane 6). Similarly, fragments −88/−31M02 (CCCCTCC) and −88/−31M03 (CCCCTCC) with a substitution on the proximal element did not compete for complex IV formation so much (lanes 9 and 10). Thus, the substitution mutants on the distal or the proximal CCCCTCC element impaired their binding ability

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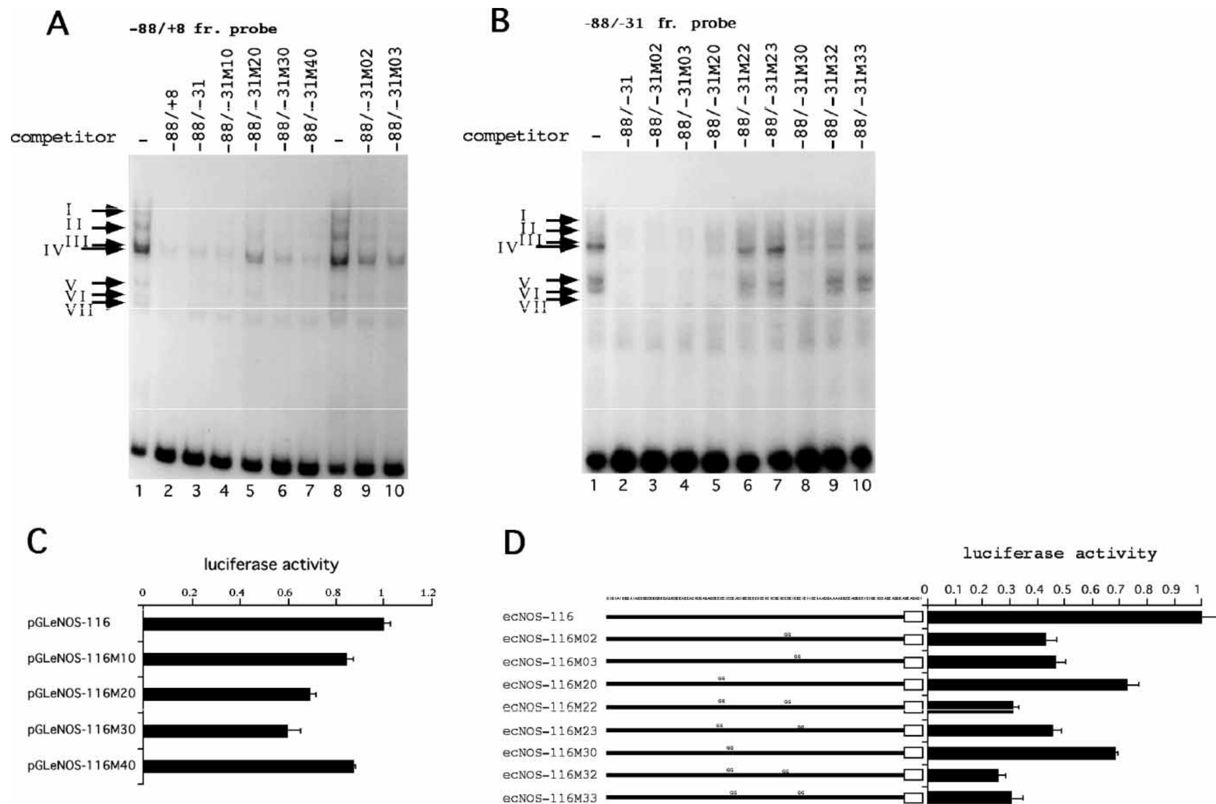

FIGURE 2 Three repeats of CCCCTCC element are efficient to the transcriptional activity of the eNOS promoter. (**A**) Dinucleotide-substituted mutant fragments of the eNOS gene promoter region were used as competitors. The ^{32}P-end-labeled −88/+8 fragment probe was incubated with nuclear extracts from tHUE-2 cells in the absence (lanes 1 and 8) or presence of a 200-fold molar excess of unlabeled fragment DNA, −88/+8 fragment (lane 2), −88/−31 fragment (lane 3), −88/−31M10 fragment (lane 4), −88/−31M20 fragment (lane 5), −88/−31M30 fragment (lane 6), −88/−31M40 fragment (lane 7), −88/−31M02 fragment (lane 9), or −88/−31M03 fragment (lane 10). **B**, The ^{32}P-end-labeled -88/-31 fragment probe was incubated with nuclear extracts from tHUE-2 cells in the absence (1ane 1) or presence of a 200-fold molar excess of unlabelled fragment DNA, −88/−31 fragment (lane 2), −88/−31M02 fragment (lane 3), −88/−31M03 fragment (lane 4), −88/−31M20 fragment (lane 5), −88/−31M22 fragment (lane 6), −88/−31M23 fragment (lane 7), −88/−31M30 fragment (lane 8), −88/−31M32 fragment (lane 9), or −88/−31M33 fragment (lane 10). (**C**) Transcriptional activities was assayed for the dinucleotide- substitutions on the promoter region of the human eNOS gene. The substitutions of GA to CT (pGLeNOS−116M10), CC to GG (pGLeNOS−116M20), CC to GG (pGLeNOS−116M30), AC to TG (pGLeNOS−116M40), CC to GG (pGLeNOS−116M02), or CC to GG (pGLeNOS−116M03) was introduced at respective sites on the promoter region of the human eNOS gene. Relative activities of reporter constructs were calculated when the normalized luciferase activity of pGLeNOS−116 was arbitrarily set to 1. The data are mean ± SD of three experiments. (**D**) Dual substitution constructs, pGLeNOS−116M22, M23, M32 and M33, were produced by combining two respective substitutions together. These reporter constructs were transfected into tHUE-2 cells and assayed for luciferase activities.

to form complex IV. Substitutions on the middle CCCCTCC element showed similar effects (data not shown). These substitution mutants also decreased their competition for formation of other complexes. It means that Sp1- related factors recognize some similar sequence in the CCCCTCC element. Furthermore, on the combination of the two substitution mutations in the distal and proximal CCCCTCC elements, these dual mutants resulted in no competition for formation of any complexes (Fig. 2B, lanes 6, 7, 9 and 10). The dual mutants reduced their binding capacities to greater extents than the corresponding single mutations. A triple mutant at all the three repeats was not competitive at all either (data not shown). These results indicate that the intact fragment containing three repeat elements formed complex IV at a higher binding affinity than two intact repeat elements. Fragments containing one intact element did not retain any or little affinity for complex IV. Thus, its binding affinity for complex IV seemed to depend on the number of intact CCCCTCC element.

We asked whether the effects of these substitution mutations to the binding affinity contributed to their transcriptional activities. We examined transient expressions of luciferase reporter genes with the same substitutions in the eNOS promoter (Fig. 2C and D). Mutant constructs with substitutions unrelated to the CCCCTCC elements, pGLeNOS−116M10 and pGLeNOS−116M40, still retained relatively high activities, 85 and 88%. Mutant constructs with substitutions on the distal element, pGLeNOS−116-M20 and pGLeNOS-116M30, however, possessed their activities reduced to 69 and 60%, respectively (Fig. 2C). Furthermore, dual mutations on both the distal and proximal CCCCTCC elements, M22, M23, M32, and M33, resulted in much severe reduction of transcriptional activities (Fig. 2D). These results

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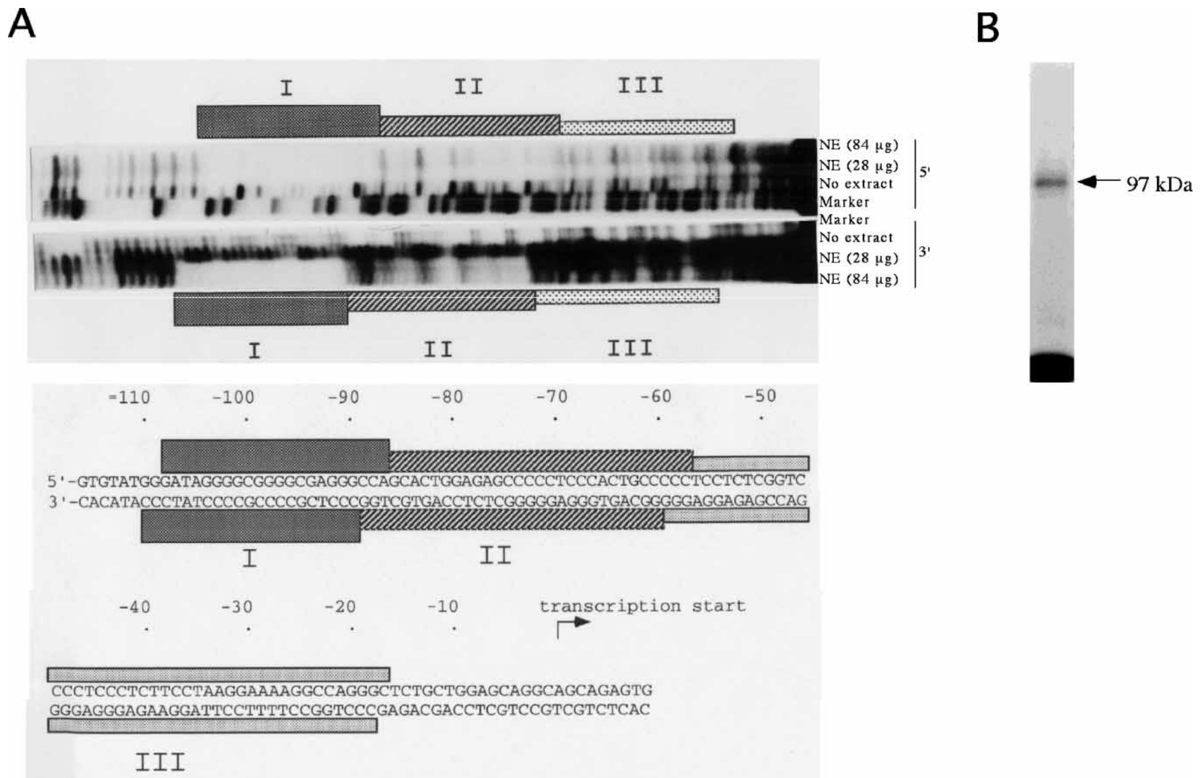

FIGURE 3 (**A**) DNase I footprinting analyses were performed on the upper (coding) and the lower (non-coding) DNA strands of the eNOS fragment from −142 to +8. ^{32}P-labeled DNA fragment probes were incubated with 28 μg or 84 μg of nuclear extracts from tHUE-2 cells and followed by an appropriate digestion with DNase I. The reaction products were subjected to electrophoresis on 8% sequencing gels with DNA maker ladders. Three regions protected strongly, middle, and slightly in DNase I footprinting analyses are indicated by shaded boxes (I), hatched boxes (II) and dotted boxes (III), respectively. The human eNOS promoter sequence from −116 to +8 is shown with an arrow at a transcription start site. (**B**) After electrophoretic mobility shift assays were performed with a BrdU-DNA fragment −97 to +8 of the eNOS gene as a probe, an DNA-protein complex corresponding to complex IV band was cross-linked covalently by UV irradiation and subjected to SDS-polyacrylamide gel. A cross-linked protein was migrated at approximate 97 kDa on the gel.

demonstrate that the transcriptional activity of the promoter of the eNOS gene corresponded to the binding affinity for the CCCCTCC elements. Complex IV should have a stimulative effect to the transcriptional regulation of the eNOS gene.

A Continuous Broad Region is Protected in DNase I Footprinting Analysis

To define sequences for binding to nuclear factors we performed DNase I footprinting analyses on both strands of the proximal promoter region of the eNOS gene (Fig. 3A). A broad region was continuously protected on both strands with a stagger alignment of a few nucleotides. The continuous region could be divided into three regions according to different strengths for the protection from DNase I attack. A GC-rich sequence from −108 to −87 (region I) was clearly protected corresponding to the Sp1-binding motif (−104 to −96). Further, a middle region from −86 to −58 (region II) was protected significantly and a proximal region from −57 to −16 (region III) was also protected slightly. The protected regions II and III contained the three repeats of CCCCTCC as well as a long pyrimidine-rich string and a short purine-rich string. It suggests that a huge complex of binding proteins can form along the proximal promoter region of the eNOS gene.

To determine the molecular mass of proteins in the DNA-protein complex IV, a bromodeoxyuridine-substituted −88/+8 fragment was incubated as a probe with nuclear extracts from tHUE-2 cells. After Tris–glycine gel electophoresis, DNA-protein complexes corresponding to complex IV band were cross-linked covalently by UV irradiation and subjected to SDS-polyacrylamide gel. A major cross-linked protein was detected at the molecular mass of 97 kDa (Fig. 3B).

DISCUSSION

In this study, we characterized a proximal promoter region of the human eNOS gene that could direct a significant promoter activity. We noted three repeats of CCCCTCC element (−74, −61, and −47), which located periodically at 13 and 14 nucleotide intervals on the pyrimidine-rich string in the proximal region. We indicated that a specific DNA-protein complex was formed with a binding affinity

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according to the number of the CCCCTCC element while only one element did not retain any binding affinity. The binding protein could attach to the one face of DNA duplex on more than two sites. In transfection expression experiments, curiously, an internal deletion of the distal sequence from −83 to −60 (pGLeNOS−116Δ−83/−60) led to a 2.9-fold increase in the transcriptional activity (Fig. 1B). The deleted sequence of −83/−60 might contain a negative regulatory element for repression of expression. However, substitution mutants on the sequence, −88/−31M10 and M40, did not affect their binding capacities or their transcriptional activities (Fig. 2A and C). Substitutions in the distal CCCCTCC element at the −83/−60 sequence, −88/−31M20 and M30, also never stimulated their transcriptional activities, but reduced them (Fig. 2C). Further, −88/+8 fragment with the same deletion −83/−60 did not abolish any complexes or did not form any novel complex *in vitro* (data not shown). The upstream Sp1-binding motif (−104 to −96) is essential for a basal transcription activity. Alternatively, we presumed that the deletion from −83 to −60 might more strongly stimulate the interaction of Sp1 factor and complex IV at a shorter distance between their binding sites in the deletion −83/−60.

In the human eNOS promoter, three repeats of CCCCTCC element (−74, −61, and −47) located periodically at 13 and 14 nucleotide intervals on a pyrimidine-rich string in the proximal 5′-flanking region. We compared the sequence of the human eNOS promoter to those of other species, i.e. bovine and mouse, which have been reported.[25,26] The bovine and mouse sequences conserve two repeats of CCCCTCC element at 13 and 14 nucleotide-intervals, respectively, which similarly located in the proximal promoters. They do not fully contain three repeat elements, but two elements were enough to retain complex IV formation and transcriptional activity though the efficiencies were somewhat lower. These suggest that the CCCCTCC repeats have a conserved role in transcriptional regulation of the eNOS gene among these animals.

We found that the sequence in the upstream region of the c-myc gene was similar to a pyrimidine-rich string in the proximal promoter region of the eNOS gene. Based on the ability to bind to a probe with such a pyrimidine-rich string, several groups have identified cDNA clones, eg. MAZ and CTCF, which were different types of zinc finger proteins.[27,28] We examined whether MAZ or CTCF factor was a component of complex IV formed on the CCCCTCC repeats of the eNOS promoter. Incubation of nuclear extracts with antibodies against MAZ or CTCF did not cause any change in the amount or mobility of complex IV in our conditions (unpublished data). Furthermore, several sequence-specific, single-stranded DNA binding proteins were suggested to regulate c-myc expression through the homopyrimidine–homopurine sequence.[29,30] However, it is not possible that the homopyrimidine sequence region of the eNOS gene promoter binds to single strand-specific binding proteins because both strands of the eNOS promoter were similarly protected in the DNase I footprinting experiments (Fig. 3A). We further noted that the CCCCTCC sequence is similar to the consensus sequence for the ZBP-89 binding site, GCCCCTxCxCC (x represents untested residues).[31,32] In fact, we got a cDNA clone for ZBP-89, which binds to the CCCCTCC repeats on the eNOS promoter. However, an anti-ZBP-89 antibody did not recognize complex IV on the CCCCTCC repeats (unpublished data). We are now investigating to identify a protein factor in complex IV.

The eNOS protein was detected mainly in endothelial cells constitutively expressed under most physiological conditions. Several studies demonstrated modest but potentially important degrees of eNOS expression. It has been reported that several binding sites for transcription factors located in the TATA-less promoter of the eNOS gene. GATA-2, Ets-1, MAZ and Elf-1 factors engaged in the transcriptional activity of the eNOS gene.[18,33,34] Further, a *cis*-acting sequence located 4.9 kb upstream of the eNOS gene acts as an enhancer.[35] The enhancer sequence can form multiple protein complexes involving Erg, other Ets-related factors, AP-2, Sp1-related factors, and MZF- related factors. These transcription factors may be necessary for a restricted pattern of expression of the eNOS gene in endothelial cells. It should be noted that a factor in complex IV locates at the most proximal site to the initiation site of the eNOS gene. Furthermore, a huge protein complex including Sp1- related factors and a 97 kDa protein might be formed along the proximal promoter of the eNOS gene for efficient transcriptional activity. Therefore, the huge complex could function in cooperation with these other factors in the regulation of the eNOS gene.

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