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# Characterization of the Human $\alpha_1\beta_1$ Soluble Guanylyl Cyclase Promoter

## *KEY ROLE FOR NF-κB(p50) AND CCAAT-BINDING FACTORS IN REGULATING EXPRESSION OF THE NITRIC OXIDE RECEPTOR*[*] [S]

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**Soluble guanylyl cyclase (sGC) is the principal receptor for NO and plays a ubiquitous role in regulating cellular function. This is exemplified in the cardiovascular system where sGC governs smooth muscle tone and growth, vascular permeability, leukocyte flux, and platelet aggregation. As a consequence, aberrant NO-sGC signaling has been linked to diseases including hypertension, atherosclerosis, and stroke. Despite these key (patho)physiological roles, little is known about the expressional regulation of sGC. To address this deficit, we have characterized the promoter activity of human $\alpha_1$ and $\beta_1$ sGC genes in a cell type relevant to cardiovascular (patho)physiology, primary human aortic smooth muscle cells. Luciferase reporter constructs revealed that the 0.3- and 0.5-kb regions upstream of the transcription start sites were optimal for $\alpha_1$ and $\beta_1$ sGC promoter activity, respectively. Deletion of consensus sites for c-Myb, GAGA, NFAT, NF-κB(p50), and CCAAT-binding factor(s) (CCAAT-BF) revealed that these are the principal transcription factors regulating basal sGC expression. In addition, under pro-inflammatory conditions, the effects of the strongest $\alpha_1$ and $\beta_1$ sGC repressors were enhanced, and enzyme expression and activity were reduced; in particular, NF-κB(p50) is pivotal in regulating enzyme expression under such conditions. NO itself also elicited a cGMP-independent negative feedback effect on sGC promoter activity that is mediated, in part, via CCAAT-BF activity. In sum, these data provide a systematic characterization of the promoter activity of human sGC $\alpha_1$ and $\beta_1$ subunits and identify key transcription factors that govern subunit expression under basal and pro-inflammatory (*i.e.* atherogenic) conditions and in the presence of ligand NO.**

Nitric oxide is now well established to play key regulatory roles in numerous, disparate physiological and (patho)physiological processes (1–3). Pivotal to NO-mediated modification of cell function is activation of the hemoprotein soluble guanylyl cyclase (sGC)[3] and consequent production of the second messenger cGMP, which in turn activates specific cyclic nucleotide-dependent protein kinases, ion channels, and phosphodiesterases (4). This fundamental role for sGC as the principal intracellular receptor for NO is exemplified in the cardiovascular system, where the enzyme governs smooth muscle tone (5) and growth (6), vascular permeability (7, 8), platelet reactivity (9, 10), and leukocyte extravasation (11, 12).

Soluble GC functions as an obligate heterodimer composed of $\alpha$ and $\beta$ subunits (13). Within the last decade, the localization and structure of the genes encoding for sGC subunits have been elucidated in several mammalian species, nonvertebrates, and plants (14). In mammals, two isoforms of each subunit, called $\alpha_{1,2}$ and $\beta_{1,2}$, have been cloned and characterized (15). However, to date only $\alpha_1/\beta_1$ and $\alpha_2/\beta_1$ heterodimers have been identified at the protein level. The chromosomal localization of sGC genes has been determined in rodents (16, 17) and humans (18); in both cases the $\alpha_1$ and $\beta_1$ subunits are co-localized on the same locus, whereas $\alpha_2$ and $\beta_2$ lie on separate chromosomes.

Previous reports have provided evidence that expressional regulation of the enzyme is a key means of modulating NO-sGC signaling. For instance, different tissues possess distinct levels of mRNA for sGC isoforms (19), intimating tissue-specific expression of individual sGC subunits. Furthermore, splice variants of the $\alpha_1$ sGC subunit mRNA have been identified (20). In contrast, the human $\beta_1$ subunit is encoded by a single transcript (~3.5kb), suggesting the translation of a single protein. Such observations suggest that the expression of the $\beta_1$ subunit is somewhat invariant, whereas alterations in the expression of the $\alpha$ subunit(s) may be an important physiological control mechanism.

Changes in the expression of sGC have also been linked with disease states, intimating that expressional regulation of the

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[3] The abbreviations used are: sGC, soluble guanylyl cyclase; TF, transcription factor; LPS, lipopolysaccharide; HASMC, human aortic smooth muscle cell; DETA-NO, DETA-NONOate; EMSA, electrophoretic mobility shift assay; NFY, nuclear factor Y; A, $\alpha$ fragment; B, $\beta$ fragment.

enzyme is likely to have (patho)physiological significance. For instance, in aged and spontaneously hypertensive rats, expression of the $\beta_1$ subunit is diminished and correlates with elevations in systemic blood pressure (21, 22). Moreover, salt-sensitive hypertension in Dahl rats is associated with decreased and increased expression of the $\beta_1$ and $\beta_2$ subunits, respectively, suggesting that exchange of $\beta$ subunits may be critical to blood pressure regulation (16). In animal models of pulmonary hypertension, sGC expression is reduced (23, 24), and pulmonary artery smooth muscle cells exposed to hypoxia lose their ability to express sGC (25). Reduced $\beta_1$ sGC expression also correlates with intimal thickening following balloon injury (26). Exposure of cells and/or tissues to pro-inflammatory cytokines, or NO itself, also induces changes in sGC expression. For example, lipopolysaccharide (LPS), interleukin 1$\beta$, and NO donors cause reduced sGC $\beta_1$ mRNA expression in pulmonary artery smooth muscle cells (27), and in atherogenic lesions reduced sGC expression is linked to diminished cGMP-dependent signaling and neointimal proliferation (28). In contrast, augmented $\beta_1$ subunit expression has been linked to increased nitrovasodilator potency in animals with endothelial dysfunction (29, 30) and increased vasodilator response in aortic rings from rats after myocardial infarction (31).

These observations intimate that expressional regulation of sGC plays a key role in the cardiovascular system, both in terms of physiological homeostasis and pathogenesis. Despite these reports, however, there is a paucity of information regarding the regulation of sGC promoter activity, particularly in the human vasculature where the enzyme performs such an important physiological function. Previous studies have examined the promoter activity of individual sGC subunits in different species (32, 33), yet only a single study has focused on the human $\beta_1$ gene (34). Here, a key role for a CCAAT-binding factor (CCAAT-BF) was revealed, although this analysis was conducted in a neuronal, rather than cardiovascular, cell line. Thus, there is a key requirement to understand the factors regulating human $\alpha_1$ and $\beta_1$ sGC promoter activity in the human vasculature to more fully appreciate the physiological roles and regulation of the NO receptor and how aberrant expression may underlie cardiovascular pathogenesis. To address this need, herein we investigated the transcriptional regulation of human $\alpha_1$ and $\beta_1$ sGC genes in human aortic smooth muscle cells (HASMCs).

## MATERIALS AND METHODS

*Cell Culture*—HASMCs (PromoCell) were cultured in smooth muscle cell growth medium 2 supplemented with 5% fetal bovine serum, 0.5 ng/ml recombinant human epidermal growth factor, 2 ng/ml recombinant human basic fibroblast growth factor, 5 mg/ml recombinant human insulin, and 0.62 ng/ml phenol red (PromoCell). The cells were maintained at 37 °C and 5% $CO_2$, and cells in passages 2–10 were used.

*Isolation of Genomic Clones for Human $\alpha_1$ and $\beta_1$ sGC Subunits*—Bacterial artificial chromosome clones containing $\alpha_1$ or $\beta_1$ sGC subunits were purchased from BacPac Resources (Children's Hospital Oakland Research Institute). Bacterial artificial chromosome DNA was isolated according to the manufacturer's protocol. The full promoters of human $\alpha_1$ and $\beta_1$ sGC were isolated from the bacterial artificial chromosome clones by PCR using Accuprime™ Taq high fidelity DNA polymerase, using primers based on the 5′-flanking regions of $\alpha_1$ (AY034777) and $\beta_1$ (AY034778) sGC genes including the first exon. The identity of both promoter regions was verified by direct sequencing.

*Construction of Luciferase Deletion Plasmids*—Different 5′-flanking regions of the $\alpha_1$ and $\beta_1$ sGC genes upstream of the transcription start site (+1) and extending to the noncoding part of the first exon (+9) were cloned into NheI-XhoI restriction sites of the pGL3-basic luciferase reporter vector (Promega). Fragments were obtained by PCR as previously described for the full promoter. Insertions in the pGL3 reporter vector expressing firefly luciferase were identified by restriction digest, and the integrity of the constructs was confirmed by direct sequencing. Primer sequences used to generate the full promoter, and deletion constructs are shown in supplemental Fig. S1.

*Site-directed Mutagenesis*—The A0.3- and B0.5-kb fragments, respectively, were subjected to site-directed mutagenesis by using the QuikChange II site-directed kit (Stratagene) and high fidelity *Pfu* Turbo DNA polymerase (Stratagene). This kit was also used to repair errors during the construction of luciferase deletion constructs. All of the constructs were verified by direct sequencing.

*Transient Transfections*—3,500 HASMCs/well and 15,000 COS-7 cells/well were seeded in 96-well clear plates 1 day before transfection. The cells were transfected with 50 ng of plasmid DNA/well using FuGENE 6 reagent (Roche Applied Science) with a 3:1 ratio ($\mu$l reagent:$\mu$g DNA). pGL3 control vector (Promega) was used as a transfection control. In each case, the cells were co-transfected with a pRL-TK vector expressing *Renilla* luciferase (Promega) at a 10:1 ratio (test vector:pRL-TK vector). Twenty-four hours later, where indicated cells were treated with either (*a*) "pro-inflammatory mixture" (100 mg/ml LPS (*Salmonella typhimurium*; Sigma), 200 units/ml IFN-$\gamma$, 400 units/ml interleukin-1$\beta$, and 2000 units/ml tumor necrosis factor-$\alpha$ (all Preprotech) in cell culture medium) or (*b*) the NO donor DETA-NONOate (DETA-NO; 0–200 $\mu$M).

*Luciferase Assays*—Forty-eight hours after transfection, the cells were washed in phosphate-buffered saline and lysed for 30 min at room temperature using the passive lysis buffer (Promega). Luciferase activity was determined using the dual luciferase reporter assay system (Promega) on the Wallac Victor 2 Multilabel Counter (PerkinElmer Life Sciences). Firefly luciferase activity was normalized to *Renilla* luciferase derived from pRL-TK vector, and the data are expressed as relative luciferase light units.

*Electrophoretic Mobility Shift Assays (EMSA)*—HASMC plated in 6-cm tissue culture dishes were stimulated for 2 h with the pro-inflammatory mixture or DETA-NO (100 or 200 $\mu$M), nuclear extracts were prepared, and samples were subjected to EMSA, as we have previously described (35). The primers used to generate the double-stranded probes are shown in supplemental Fig. S2.

*Western Blot Analysis*—Protein concentrations were determined by BCA protein assay (Pierce, distributed by Perbio Sciences Ltd., Northumberland, UK). Equal concentrations of protein were subjected to 15% SDS-PAGE under reducing

**TABLE 1**
**The positions are according to the transcription start site (+1)**

| Repeat | No. of elements | Begin | End | Length | Percentage of sequence |
|---|---|---|---|---|---|
| | | | | *bp* | |
| **Human $\alpha_1$ sGC promoter repeats according to RepeatMasker[a]** | | | | | |
| LTR (MaLRs) | 1 | −3038 | −2853 | 185 | 6.1 |
| DNA element (MER1_type) | 1 | −2188 | −2031 | 158 | 5.2 |
| Total interspersed repeats | | | | 343 | 11.2 |
| Low complexity | 1 | −80 | −42 | 39 | 1.3 |
| **Human $\beta_1$ sGC promoter repeats according to RepeatMasker[b]** | | | | | |
| LINE1 | 1 | −2429 | −1671 | 759 | 22.7 |
| Total interspersed repeats | | | | 759 | 22.7 |

[a] Total length, 3048 bp; GC level, 36.5%; based masked, 382 bp (12.5%).
[b] Total length, 3344 bp; GC level, 40.0%; based masked, 759 bp (22.7%).

conditions. The proteins were transferred to a nitrocellulose membrane (Amersham Biosciences) using the mini Trans-blot electrophoresis transfer cell (Bio-Rad). The membranes were stained with Ponceau S stain to confirm equal transfer and incubated in 5% milk in wash buffer (phosphate-buffered saline, 0.1% Tween 20) overnight at 4 °C with gentle shaking. The membranes were then incubated with primary antibody (anti-sGC $\beta_1$) or anti- glyceraldehyde-3-phosphate dehydrogenase (Abcam, Coventry, UK) diluted 1:2000 ($\beta_1$) or 1:1000 (glyceraldehyde-3-phosphate dehydrogenase) in 5% milk in wash buffer for 3 h at room temperature with constant agitation followed by five washes (5 min/wash). The membranes were then incubated with shaking for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako, Cambridgeshire, UK) diluted 1:1000 in 5% milk in wash buffer. The membranes were washed as described above, and the proteins were visualized using enhanced chemiluminescence (Amersham Biosciences). The bands were quantified by densitometry using AlphaImager (Alpha Innotech, San Leandro, CA).

*Cyclic GMP Measurements*—HASMC ($10^6$ cells) were plated in 10-cm dishes and 24 h later exposed to the pro-inflammatory mixture for a further 24 h. At this time, the cells were harvested by trypsinization and centrifuged at 200 × g for 10 min. The cell pellet was resuspended in assay buffer (2.36 mg/100 ml Tris-HCl, 760 mg/100 ml NaCl, 22.2 mg/100 ml KCl, 29.6 mg/100 ml $MgSO_4$, 16.6 mg/100 ml $Na_2HPO_4$, 0.01 mg/100 ml $CaCl_2$, pH 7.4) and treated with 1 mM 3-isobutyl-1-methylxanthine (Sigma). The cells were then exposed to either 1 or 10 μM spermine-NONOate (Axxora, Nottingham, UK) for 5 min ("basal" samples did not receive spermine-NONOate), and the reaction was terminated by heating at 90 °C for 10 min. The cyclic GMP content was then measured using a commercially available assay (Amersham Biosciences).

*Bioinformatics and Statistical Analysis*—BLAST was used to identify and retrieve orthologous $\alpha_1$ sGC promoters from mouse, rat, and human genomes from project *Ensembl* at The Wellcome Trust Sanger Institute. The $\alpha_1$ sGC upstream sequence was analyzed for the presence of repeat regions and transposable elements, using Repeat Masker version open-3.1.5. Confirmation of Pol II promoter was conducted with Promoter Scan II and Novel Neural Network Promoter Prediction. Identification of CpG islands was performed using CpGProD (CpG Island Promoter Detection) software. In silico analysis for transcription factor-binding sites was performed with MatInspector Suite, release 7.4 (36). The results are expressed as the means ± S.E. A Student's *t* test was used to determine differences between data groups, where $p < 0.05$ was considered significant.

## RESULTS

*Characterization of the 5′-Flanking Region of Human $\alpha_1$ and $\beta_1$ sGC Genes*—The transcriptional start sites were determined by pair-wise blasting of $\alpha_1$ and $\beta_1$ sGC 5′-flanking regions with the complete mRNA sequences (NM_000856 and NM_000857, respectively). Scanning both $\alpha_1$ and $\beta_1$ sGC sequences with the Novel Neural Network Algorithms for Improved Eukaryotic Promoter Site Recognition did not reveal any TATA box in close proximity to the transcription start site of both $\alpha_1$ and $\beta_1$ sGC promoters. Analysis of promoter core elements (37) showed the presence of consensus Inr (initiator) and DPE (downstream core promoter) elements but not TFIIB recognition (BRE) or the newly discovered motif 10 (MTE) elements in the $\alpha_1$ sGC promoter (supplemental Fig. S3). For the $\beta_1$ sGC promoter, only DPE elements were found (supplemental Fig. S3). GC and CCAAT-BF boxes were detected in both proximal promoters. Furthermore, using Repeat Masker, we were able to identify three repeats in the $\alpha_1$ sGC promoter but only one in the $\beta_1$ promoter region (Table 1). Finally, CpGProD software did not reveal the presence of CpG islands in either the $\alpha_1$ or $\beta_1$ sGC promoters.

*Analysis of Promoter Activity within the 5′-Flanking Region*—Transiently transfected luciferase reporter constructs containing 0.3–3.0 kb of human $\alpha_1$ or 0.3–3.2 kb of human $\beta_1$ sGC upstream sequence were analyzed for promoter activity in HASMCs. The A0.3-kb fragment showed the highest level of activity among the $\alpha_1$ sGC promoter constructs (Fig. 1). Reduced activity in the remaining (longer) constructs suggested the presence of repressor element(s) further upstream. The B0.5- and B0.8-kb fragments showed the highest level of activity among the $\beta_1$ sGC promoter constructs (Fig. 1). The B1.4-kb fragment showed a decreased promoter activity, suggesting the presence of a repressor element(s) between −830 and −1392 bp. Extension of the promoter fragment up to 3.2 kb in length did not significantly alter the transcriptional activity of the B1.4-kb construct, suggesting the absence of net activation or repression in that region. However, the 126-bp deletion in B0.4 kb (to generate construct B0.3-kb) caused greater than 50% decrease in activity, suggesting that a key, putative enhancer(s) is located between B0.3 and B0.4-kb. In accord, the A0.3- and B0.5-kb constructs possessed the critical elements necessary for maximal promoter activity of the $\alpha_1$ and $\beta_1$ sGC promoters, respectively, and were further analyzed.

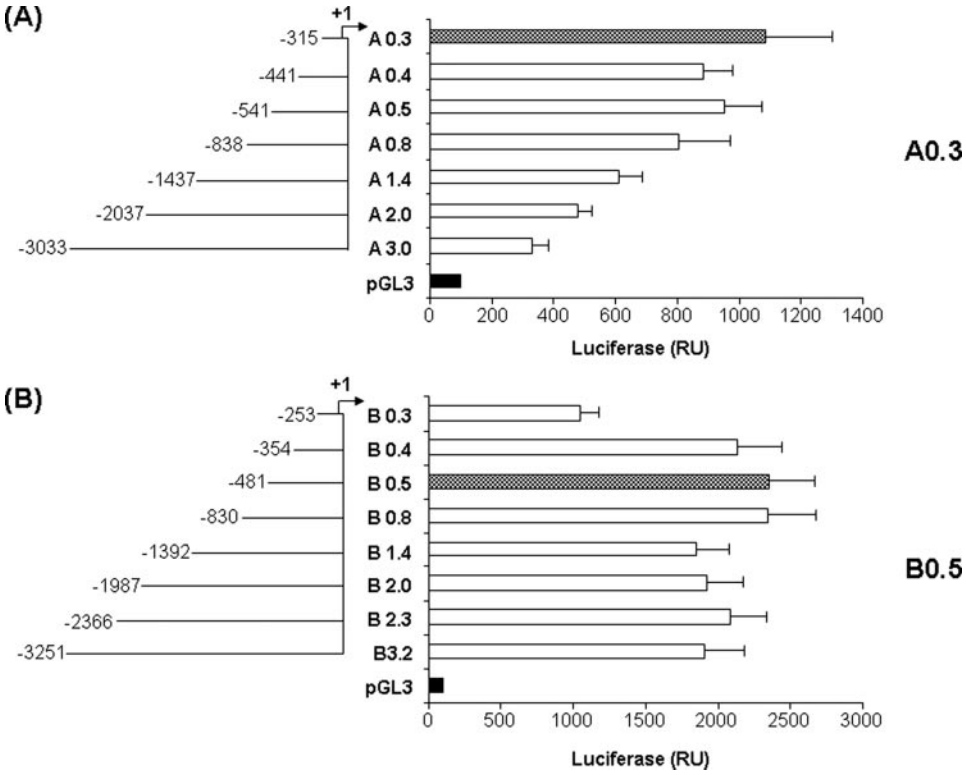


FIGURE 1. **Promoter activity of human $\alpha_1$ (*A*) and $\beta_1$ sGC (*B*) deletion constructs in HASMCs.** The cells were transfected with luciferase reporter constructs containing different size promoter fragments (indicated in bp relative to the transcription start site (+1)). Promoter activity of each construct was measured as firefly luciferase activity normalized to *Renilla* luciferase, and the results are expressed as relative luciferase light units (*RU*). The results are the means ± S.E. ($n \geq 12$). The *hatched bars* indicate the promoter constructs with the greatest activity. pGL3 depicts the promoter-less vector.

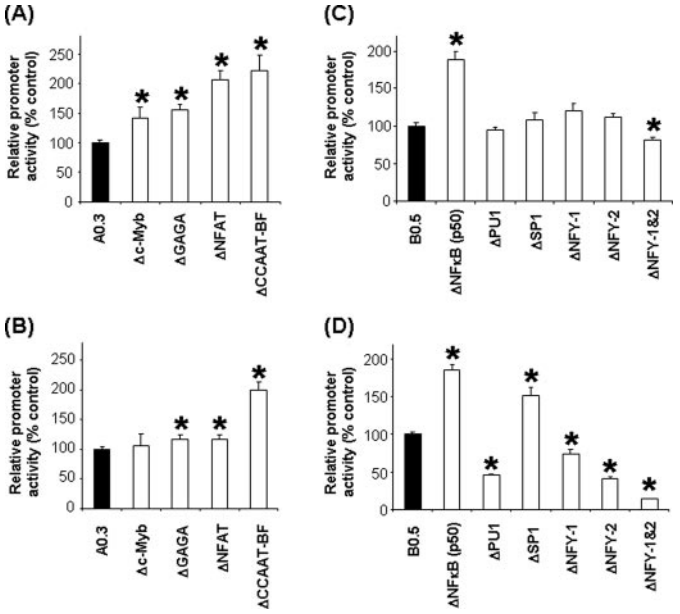


FIGURE 2. **Analysis of putative TFs at the proximal promoter sequence of the human sGC gene under basal conditions in HASMC (*A* and *C*) and COS-7 (*B* and *D*) cells.** *A* and *B*, effects of core deletions of c-Myb, GAGA, NFAT, and CCAAT-BF sites on promoter activity of the A0.3-kb construct. *C* and *D*, effects of core deletions of NF-κB(p50)-, PU1-, SP1-, and NFY-binding sites on promoter activity of the B0.5-kb construct. NFY-1 and NFY-2 indicate the upstream and downstream CCAAT-BF sites with *NFY-1&2* representing the double deletion. Promoter activity was measured as described in Fig. 1. The results are expressed as the means ± S.E. ($n \geq 5$). *, $p < 0.05$.

Interestingly, the A0.3-kb sequence exhibited only 44% homology with the mouse and rat sequence homologues and the B0.5-kb fragment possessed a 57% homology when compared with the rat sequence. As a consequence, there is likely to be disparate regulation of sGC expression (*i.e.* promoter activity) between species, highlighting the importance of characterizing the activity of the human promoter in human cells.

*Identification of Critical Transcription Binding Sites*—Analysis of putative transcription factor-binding sites in the fragments containing the maximal promoter activity for the human $\alpha_1$ and $\beta_1$ sGC genes was performed using MatInspector software (solution parameters: core similarity, 1.0; matrix similarity optimized) (supplemental Figs. S4*A* and 5*A*) and confirmed by Transcription Element Search Software. In both A0.3-kb and B0.5-kb constructs, putative TF-binding sites for several transcription factors located at the optimal regulatory distance from the transcription start site were investigated. To functionally determine the importance of these TFs for $\alpha_1$ and $\beta_1$ sGC promoter activity, the cores of each TF-binding site were deleted individually in both the A0.3-kb and B0.5-kb basal constructs via site-directed mutagenesis. These deletions did not introduce any new binding sites as confirmed by sequence analysis with MatInspector software. Transcriptional activity of these mutant constructs was assessed in HASMCs and COS-7 cells; TF-binding sites that had a significant effect on sGC promoter activity in HASMCs are shown in supplemental Figs. S4*B* ($\alpha_1$ sGC) and S5*B* ($\beta_1$ sGC).

A0.3-kb fragments harboring deletions for CCAAT-BF-, c-Myb-, GAGA-, and NFAT-binding sites showed significant increases in promoter activity compared with A0.3-kb control in HASMCs (Fig. 2*A*), suggesting that binding of these TFs to their recognition sites has repressor effects on $\alpha_1$ sGC promoter activity in these cells. The effect of c-Myb was abolished in COS-7 cells, suggesting a HASMC-specific effect (Fig. 2*B*). Moreover, the effects of GAGA and NFAT were also reduced in COS-7 cells (Fig. 2*B*), despite still having significant repressor activity. However, CCAAT-BF had a similar effect in COS-7 cells, suggesting that this TF may belong to the basal transcription machinery for $\alpha_1$ sGC gene in many cell types.

B0.5-kb fragments harboring a deletion for an NF-κB(p50)-binding site showed almost 2-fold increase in promoter activity compared with B0.5kb control in HASMCs (Fig. 2*C*). This effect was identical in COS-7 (Fig. 2*D*), suggesting that NF-κB(p50) plays a key repressor role in the basal expression of

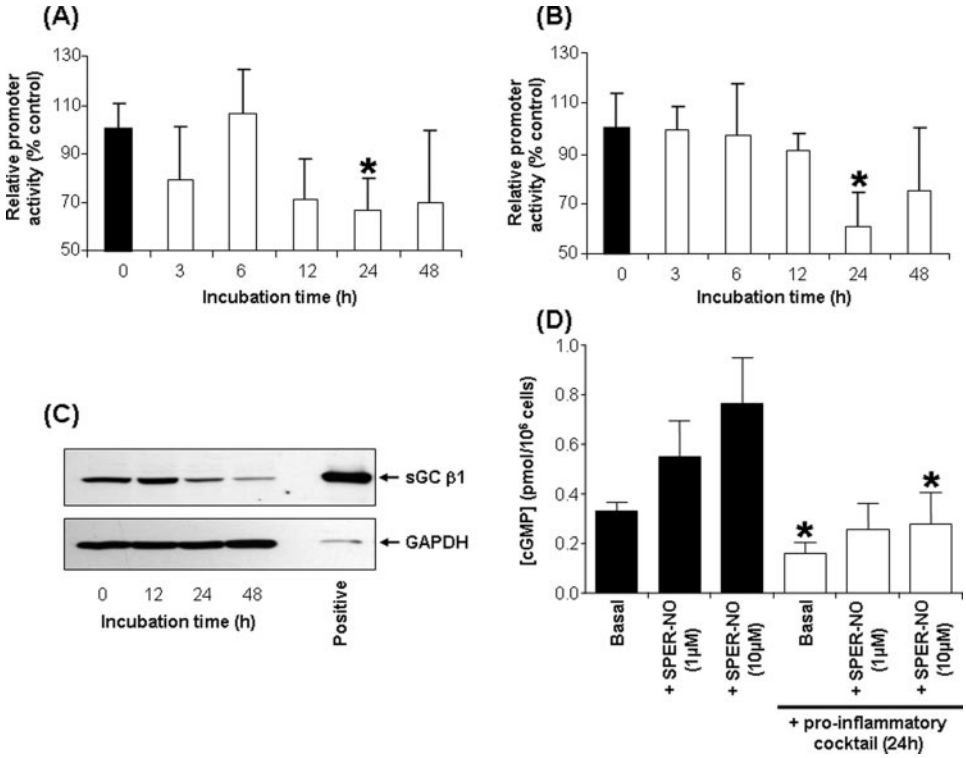


FIGURE 3. Temporal effects of a pro-inflammatory mixture on A0.3-kb (A) and B0.5-kb (B) promoter activity in HASMCs. *Black bars*, basal conditions; *empty bars*, pro-inflammatory conditions. Promoter activity was measured as described in the legend to Fig. 1. The results are expressed as the means ± S.E. ($n \geq 3$). *, $p < 0.05$. *C*, expression of sGC $\beta_1$ protein determined by Western blot in HASMC under basal and pro-inflammatory conditions (0–48 h of incubation with mixture). The data are representative of at least three similar experiments. *D*, basal and NO-stimulated increases in cGMP in HASMC in the absence and presence of pro-inflammatory mixture (24 h; $n = 40$). *, $p < 0.05$ *versus* the corresponding response in the absence of pro-inflammatory mixture.

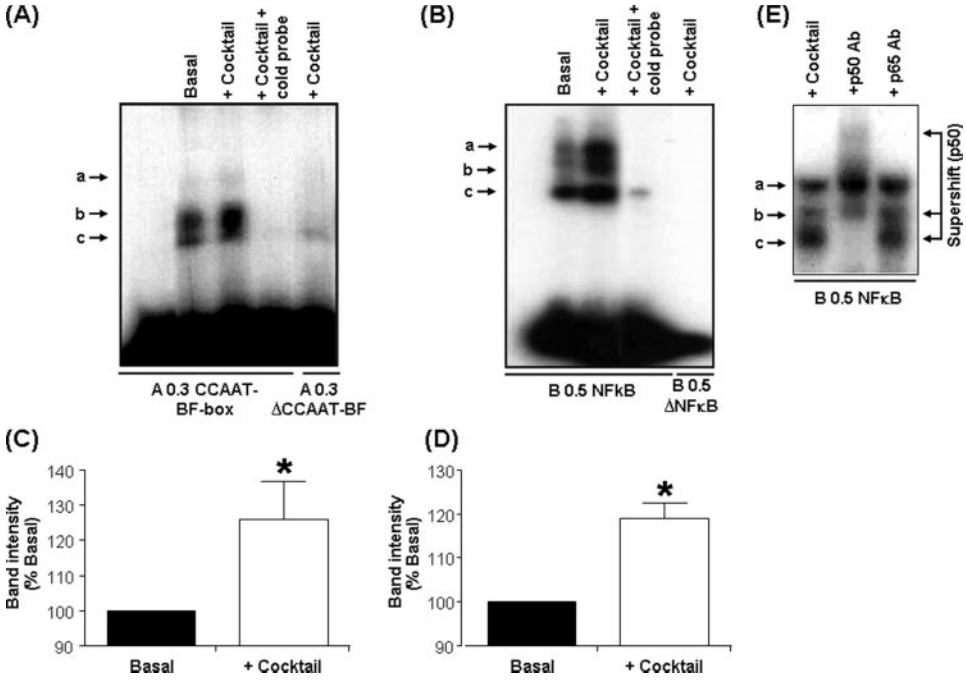


FIGURE 4. Electrophoretic mobility shift assay (A and B) with densitometric quantification (C and D) of HASMC nuclear extracts under basal and pro-inflammatory conditions (2h of incubation with the mixture) was performed using labeled primers carrying the NF-κB or CCAAT-BF binding sites or the mutated version (ΔNF-κB or ΔCCAAT-BF). For competition experiments, a 100× excess of unlabeled double-stranded oligonucleotide (cold probe) was added to the binding reaction. A supershift at the NF-κB site was demonstrable using a p50, but not p65, antibody (*E*). The results are expressed as the means ± S.E. ($n \geq 3$). *, $p < 0.05$.

$\beta_1$ sGC, independent of cell type. In contrast, a B0.5-kb fragment harboring deletions for both NFY-binding sites showed a small reduction in B0.5-kb promoter activity in HASMCs, suggesting a slight activator effect of this TF (Fig. 2*C*). The activator effect of NFY was much stronger in COS-7 (Fig. 2*D*) and in a BE2 human neuroblastoma cell line (34), suggesting that a cell-specific effect of NFY is not so apparent in HASMC. PU1 and SP1 TFs showed activator and repressor effects, respectively, in COS-7 cells but not in HASMCs, again suggesting cell-specific regulation of sGC promoter activity.

*Effect of Pro-inflammatory Conditions on sGC Promoter Activity in HASMCs*—To examine whether vascular inflammation modulates sGC expression in HASMCs, human $\alpha_1$ and $\beta_1$ sGC promoter activity was examined in the presence of a pro-inflammatory mixture using the A0.3-kb (Fig. 3*A*) and B0.5-kB (Fig. 3*B*) constructs. Pro-inflammatory conditions significantly reduced both the $\alpha_1$ and $\beta_1$ sGC promoter activity at 24 h. Moreover, Western blot analysis revealed a clear, time-dependent decrease in sGC $\beta_1$ expression following exposure of the HASMC to the pro-inflammatory mixture (Fig. 3*C*) that was mirrored by a significant reduction in both the basal and NO-stimulated sGC activity (Fig. 3*D*).

As shown in Fig. 2, CCAAT-BF and NF-κB exerted the most pronounced effect on $\alpha_1$ and $\beta_1$ sGC promoter activity, respectively, among the TFs studied. To determine whether these transcription factors bind to the target DNA sequences on the promoter, nuclear extracts from HASMCs were analyzed by EMSA. In terms of the $\alpha_1$ sGC promoter, three complexes were detected using the oligonucleotide probe containing −93 to −66 bp region (CCAAT-BF oligonucleotide) upstream of the transcriptional start site (Fig. 4*A*). The specificity of these complexes was demonstrated by a reduction of the

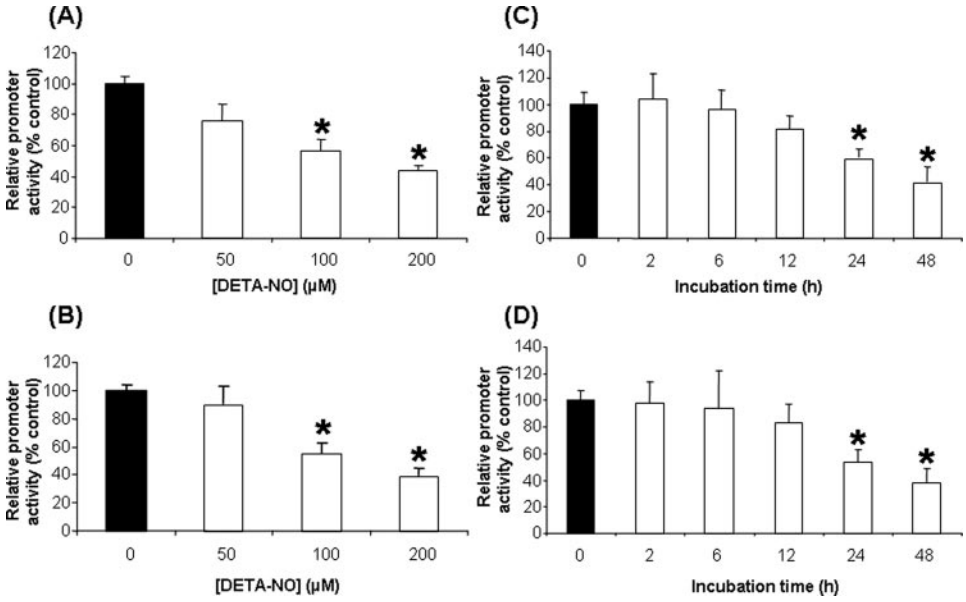


FIGURE 5. **Concentration- (*A* and *B*) and time- (*C* and *D*) dependent effects of the NO donor DETA-NO (0–200 μM and 0–48-h incubation) on A0.3 (*A* and *C*) and B0.5 (*B* and *D*) promoter activity in HASMCs.** Promoter activity was measured as described in the legend to Fig. 1. The results are expressed as the means ± S.E. ($n \geq 3$). *, $p < 0.05$ *versus* response in the absence of DETA-NO.

intensity of all three bands in the presence of an excess of unlabeled probe. When the nuclear extract derived from HASMCs treated with the pro-inflammatory mixture was analyzed, an increase in the intensity of two of these complexes was observed (Fig. 4*C*), suggesting that vascular inflammation increased the binding of CCAAT-BF to the proximal $\alpha_1$ sGC promoter. The deletion of a single *c* (*g*) of the core of the CCAAT-BF site (position −82; supplemental Fig. S4) abolished the formation of two of the three complexes, highlighting the importance of that single base for the binding of the CCAAT-BF to the target promoter.

For the $\beta_1$ sGC promoter, interactions within the NF-κB(p50)-binding region were also assessed using a NF-κB oligonucleotide encompassing −138 to −109 bp region of this promoter (supplemental Fig. S5*B*). Three complexes were formed after incubation of the NF-κB probe with HASMC nuclear extracts. These complexes were no longer formed in the presence of an excess of unlabeled probe, confirming specificity (Fig. 4*B*). Interestingly, a supershift of the complexes was only observed when we used an anti-p50 antibody but not anti-p65 (Fig. 4*E*), suggesting that the smaller NF-κB subunit (perhaps as a p50 homodimer) is pivotal in regulating sGC $\beta_1$ expression; this finding dovetails well with the observed increase in sGC $\beta_1$ promoter activity in the construct with deleted p50 consensus site(s) and with previous reports detailing a predominantly repressor activity of p50 homodimers (38, 39). When the nuclear extract derived from HASMCs treated with the pro-inflammatory mixture was analyzed, an increase in the intensity of the complex bands was observed (Fig. 4*D*), suggesting that vascular inflammation increased the binding of the NF-κB(p50) factor to the proximal $\beta_1$ sGC promoter. The deletion of the 4-bp core (*gggg*) for the NF-κB factor-binding site disrupted the formation of two complexes and reduced the formation of the third one, indicating that the *gggg* motif is necessary for its DNA interaction. Furthermore, an important role for NF-κB in regulating the expression of the $\beta_1$ subunit was supported by a parallel reduction in enzyme protein expression and activity (Fig. 3, *C* and *D*).

*Effect of NO on sGC Promoter Activity in HASMCs*—To assess the effect of the sGC ligand NO on expression of the enzyme in HASMCs (to establish the existence of a feedback loop in terms of enzyme expression, which is well established to occur biochemically (30, 40)), human $\alpha_1$ and $\beta_1$ sGC promoter activity was examined in the presence of the NO donor DETA-NONOate (releases NO spontaneously in aqueous solution (41)). Pilot studies revealed that for the $\alpha_1$ subunit, the promoter activity was inhibited to the greatest extent in the A0.3 construct, suggesting that key NO-dependent repressors are located in this region of the promoter. For $\beta_1$ sGC, maximal inhibition was observed in the B0.5 construct, suggesting that this area of the promoter contains the critical NO-responsive repressor element(s). Subsequent studies were therefore conducted using these constructs. Fig. 5 shows that NO exerted a concentration-dependent inhibition of both $\alpha_1$ and $\beta_1$ sGC promoter activity. This effect was maximal at 200 μM DETA-NO, which results in an approximate ambient NO concentration of 200 nM (42). Moreover, the inhibition of promoter activity by NO followed a time course very similar to that observed under pro-inflammatory conditions with maximal effect observed at 24–48 h (Fig. 5).

To determine whether the repressor activity of NO was cGMP-dependent or independent, further experiments were conducted using the A0.3 and B0.5 constructs as models of promoter function. Here, the NO-dependent inhibition of both the $\alpha_1$ and $\beta_1$ sGC promoter was not reversed in the presence of the sGC blocker ODQ (5 μM) nor mimicked by the cGMP analogue 8-Br-cGMP (300 μM; Fig. 6, *A* and *B*). Thus, it appears that the inhibitory effect of NO on sGC promoter activity and expression is mediated directly, rather than via activation of the enzyme (and cGMP production).

In an attempt to identify putative transcription factors that might mediate the repressor effects of NO, A0.3, and B0.5 constructs with targeted mutations in specific TF-binding sites were analyzed for their responsiveness to NO. Interestingly, the activity of the A0.3 fragment with a deleted CCAAT-BF site was no longer sensitive to inhibition by DETA-NO (Fig. 6*C*), intimating that this response element is involved in mediating the negative feedback effect of NO on sGC expression. In contrast, deletion of the same site in the B0.5 construct did not alter promoter activity in the presence of NO.

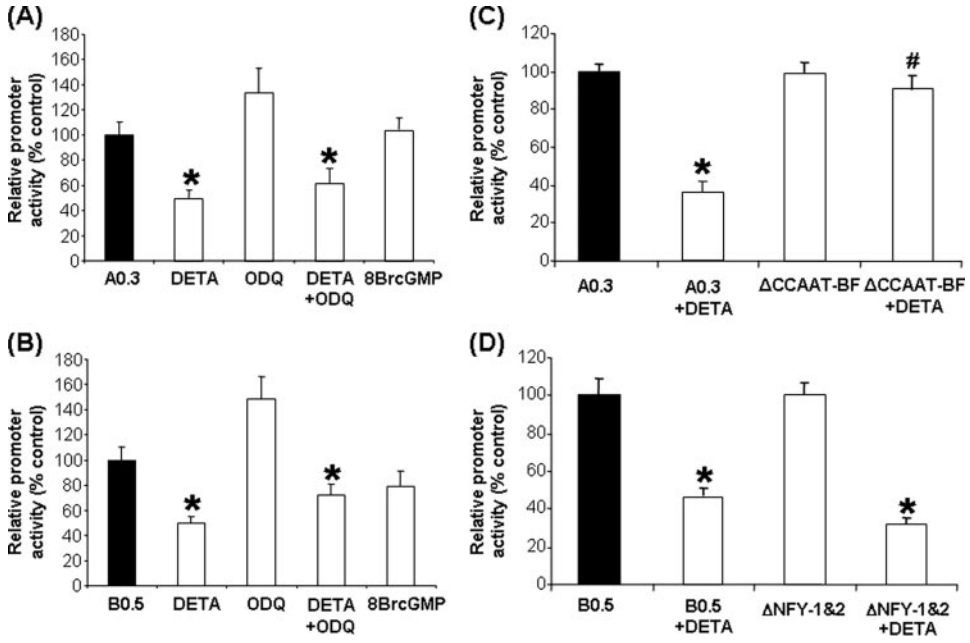


FIGURE 6. **Effect of the sGC inhibitor ODQ (5 $\mu$M) and cGMP analogue 8Br-cGMP (300 $\mu$M) and core deletions of CCAAT-BF and NFY on NO-mediated (DETA-NO, 200 $\mu$M; 24 h of incubation) inhibition of A0.3 (*A* and *C*) and B0.5 (*B* and *D*) promoter activity in HASMC.** Promoter activity was measured as described in the legend to Fig. 1. The results are expressed as the means ± S.E. ($n \geq 3$). *, $p < 0.05$ *versus* respective control. #, $p < 0.05$ *versus* A0.3 + DETA. In *C* and *D*, promoter activity in the absence of DETA is normalized to 100%.

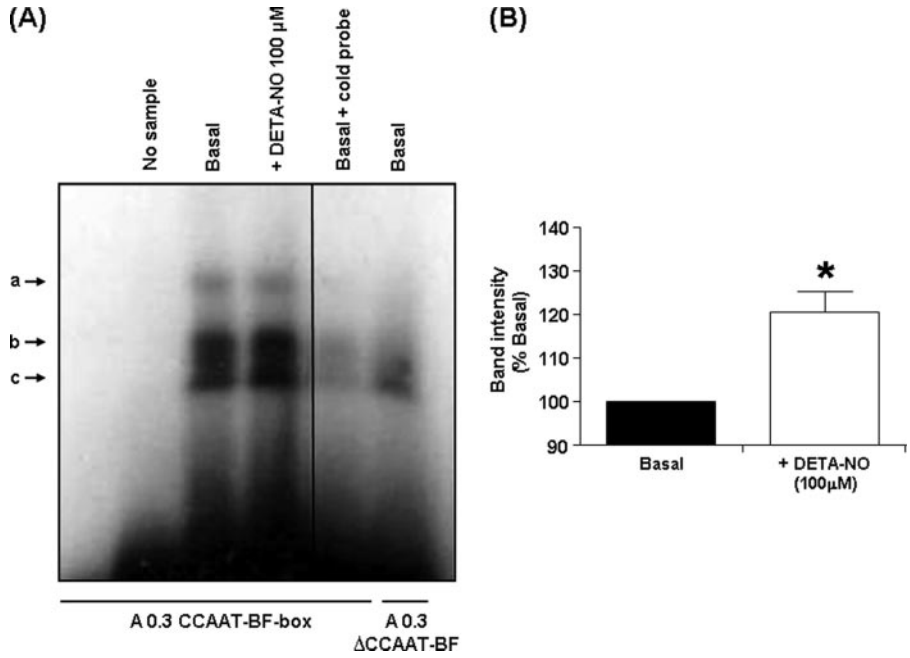


FIGURE 7. **Electrophoretic mobility shift assay (*A*) with densitometric quantification (*B*) of HASMC nuclear extracts under basal conditions and in the presence of 100 $\mu$M DETA-NO (2-h incubation) was performed using labeled primers carrying the CCAAT-BF site or its mutated version (ΔCCAAT-BF).** For competition experiments, a 100× excess of unlabeled doubled-stranded oligonucleotide (cold probe) was added to the binding reaction. The results are expressed as the means ± S.E. ($n \geq 3$). *, $p < 0.05$.

Finally, we confirmed the importance of NO in inhibiting sGC promoter activity via the CCAAT-BF site, by EMSA. In accord with the luciferase assay data, DETA-NO (100 $\mu$M) caused an increase in the intensity of the three CCAAT-BF complexes observed that was essentially absent when using probes with a dysfunctional CCAAT-BF site (Fig. 7). However, despite attempting to produce a supershift in the CCAAT-BF complexes observed with specific antibodies to two putative CCAAT-BFs (*e.g.* CBF-B, MAZ), we were unable to observe such a change (data not shown), suggesting that additional CCAAT-BFs are likely involved.

## DISCUSSION

Activation of sGC is essential for NO-mediated regulation of vascular smooth muscle reactivity (5) and proliferation (6), microvascular permeability (7, 8), and the reactivity/adherence of platelets (9) and leukocytes (8, 11). As such, under physiological circumstances activation of sGC is fundamental to cardiovascular homeostasis and maintains an important cytoprotective/anti-thrombotic influence. Moreover, because inappropriate sGC activity may be responsible for several features of cardiovascular pathologies including blood pressure dysregulation (*i.e.* smooth muscle reactivity), capillary edema, and cellular recruitment, dysfunction of sGC is likely to have at least an equivalent impact on (patho)physiology as inappropriate NO production; changes in the expressional regulation (and activity) of this enzyme are therefore likely to exert considerable influence on the progression of disease. Despite this obvious importance, there exists scant information concerning the physiological regulation and pathological alterations of sGC expression. To address this deficit, we have characterized the human $\alpha_1$ and $\beta_1$ sGC promoter regions in human aortic smooth muscle cells.

Analysis of the human $\alpha_1$ and $\beta_1$ sGC gene promoters revealed some intriguing aspects of transcriptional regulation of these genes and differences in the promoter consensus core elements. Both sGC genes have a TATA-less promoter and show CCAAT-BF sites in direct ($\alpha_1$ sGC) or reverse ($\beta_1$ sGC) orientation ~80 bp upstream from the putative transcription start site, in agreement with the canonical position for eukaryotic promoters (43). Classically, the following three-way combinations are highly favorable in human promoter regions to facilitate gene transcription: BRE-Inr-MTE and/or TATA-Inr-MTE (37); yet, neither combination was detected in either the $\alpha_1$ or

$\beta_1$ sGC promoter. However, because the $\alpha_1$ sGC promoter exhibits two potential initiation modules, an alternative transcription start site 51 bp downstream given by the Inr-DPE module (and characteristic of TATA-less promoters) (44) cannot be ruled out. Surprisingly, an Inr element is not present in the human $\beta_1$ sGC promoter.

Functional analysis of constructs containing different size fragments of the $\alpha_1$ and $\beta_1$ sGC 5′-flanking regions showed a slightly different profile of activity between both subunit promoters in HASMCs. Despite $\alpha_1$ sGC showing the highest promoter activity in the A0.3-kb construct, suggesting repressor elements localized further upstream, the situation was different for $\beta_1$ sGC. Here, the B0.5-kb construct showed the highest promoter activity, and the presence of several enhancers and repressors functioning at a distance from this regulatory region was detected. The fact that B0.5-kb activity was comparable with B0.8kb activity in HASMCs but has been shown to be over 3-fold lower than B0.8 kb in BE2 cells (34), suggests a cell-specific regulation of the crucial promoter region necessary to achieve the maximum $\beta_1$ sGC promoter activity in HASMCs. This cell-specific effect is also highlighted by the fact that B0.4-kb activity is almost the same as B0.5 kb and 2-fold higher than B0.3 kb in HASMCs, intimating strong activator elements located in the −253 to −354 region, not observed in BE2 cells (34). Thus, A0.3-kb and B0.5-kb constructs demonstrated the highest promoter activity in HASMCs, indicating that the regulatory promoter of sGC has all the necessary elements for maximum promoter activity in the 300–500-bp region upstream of the transcription start site.

We also focused on the identification of principal TFs responsible for the transcriptional regulation of $\alpha_1$ and $\beta_1$ sGC in HASMCs. A multiplicity of putative transcription factor-binding sites predicted by MatInspector software are clustered in both A0.3-kb ($\alpha_1$ sGC) and B0.5-kb ($\beta_1$ sGC) promoter fragments, all of which have been shown to play an important role in the transcription of a wide variety of eukaryotic genes. GATA (GATA-binding factor, typical of the cardiovascular system), GFI1 (growth factor independence I; typical of the hematopoietic system), NFY (ubiquitous), and PBX (homeodomain factor; typical of the hematopoietic system) have consensus sequences in both promoters, and some of these factors may have a regulatory role in the co-expression of both $\alpha_1$ and $\beta_1$ sGC genes; this is particularly pertinent for the GFI1 (both $\alpha_1$ and $\beta_1$ sGC) and PBX ($\alpha_1$ sGC) sites, which are positioned between the CCAAT-BF site and the transcription start site and therefore are likely to play key roles in expressional regulation of sGC in hematopoiesis. Indeed, the $\alpha_1$ and $\beta_1$ sGC promoter regions contain no less than 20 consensus sequences for TFs reported to play a role in regulating hematopoiesis, supporting a key role for sGC in this process (34).

Functional analysis with a luciferase $\alpha_1$ sGC reporter construct containing a c-Myb, GAGA, NFAT, or CCAAT-BF core deletion in the A0.3-kb promoter fragment demonstrated that the integrity of these sites is very important for transcription repression in HASMCs. The c-Myb and to a lesser extent the GAGA and NFAT repressor effects were not observed in COS-7 cells, intimating a vascular smooth muscle cell-specific effect. In fact, c-Myb plays a crucial role in arterial smooth muscle cell proliferation (associated with $G_1$/S cell cycle transition), as occurs in many vasculopathies (45) and NFAT is associated with cardiac morphogenesis, vasculogenesis, and vascular smooth muscle hypertrophy (46); the physiological importance of NO-sGC signaling in curbing these proliferative processes fits with the ability of such TFs to suppress sGC promoter activity and thereby promote pathogenesis. However, it is noteworthy that when two other NFAT sites located at −174 and −161 in $\alpha_1$ sGC were mutated, they did not significantly alter $\alpha_1$ sGC promoter activity.

Functional analysis with a luciferase $\beta_1$ sGC reporter construct containing a NFY core deletion in the B0.5-kb promoter fragment revealed that this site is critical for transcription activation in HASMCs, although this effect was less evident than in COS-7 cells (this study) and BE2 cells (34). Similar analysis also showed that the integrity of an NF-κB(p50) site is very important for transcription repression control, in both HASMCs and COS-7 cells, suggesting that this TF is globally important in constitutive gene expression.

To corroborate the importance of these TFs in regulation of sGC promoter activity, we conducted EMSAs to confirm the interaction of CCAAT-BF and NF-κB with the sGC promoter region. It is clear from these data that both CCAAT-BF and NF-κB(p50) bind strongly to the $\alpha_1$ and $\beta_1$ sGC promoter constructs, respectively, and that the effects of these TFs on sGC promoter activity are specific. A CCAAT-BF site has been found to be prevalent in the promoters of cell cycle-regulated eukaryotic genes (47) and is known to be essential for cell cycle-dependent activation and repression of several mammalian genes (48–50). In addition to the NFY site reported by Sharina *et al.* (34), a second upstream site was also shown to have an important role, highlighting the role NFY plays in $\beta_1$ sGC transcription. Moreover, the presence of the two closely situated NFY sites in the immediate promoter is in agreement with the idea that expression of $\beta_1$ sGC gene could be regulated during the cell cycle (34).

Many cardiovascular diseases, particularly atherosclerosis, are now recognized to constitute a chronic inflammation of the blood vessel wall. Because NO-sGC-cGMP signaling exerts an important vasoprotective, anti-atherogenic effect, elucidation of the expressional regulation of sGC in a pro-inflammatory environment is likely to provide further insight into the pathogenesis of vascular disease. To mimic such a pathological scenario in the vasculature, we examined the effect of pro-inflammatory conditions on sGC promoter activity in HASMCs. A0.3-kb and B0.5-kb fragments were analyzed in the presence of a pro-inflammatory mixture. Under such conditions, sGC A0.3-kb and B0.5-kb promoter activity at 24 h was significantly reduced, demonstrating a coordinated regulation of both $\alpha_1$ and $\beta_1$ sGC gene expression. The fact that the strongest repressors for $\alpha_1$ ad $\beta_1$ sGC (CCAAT-BF and NF-κB, respectively) exhibit enhanced DNA binding under pro-inflammatory conditions might explain, at least in part, the reduction in $\alpha_1$ and $\beta_1$ sGC promoter activity observed under this "pathogenic" environment. Moreover, analysis of protein expression confirmed that the decreased promoter activity in the presence of pro-inflammatory mixture was mirrored by reductions in both sGC

$\beta_1$ protein expression and activity, providing a key link between NF-κB and regulation of sGC bioactivity.

We also examined the effect of NO *per se* to mediate a feedback loop and prevent sGC promoter activity/expression. Using the NO donor DETA-NO, we demonstrated that NO exerts a concentration-dependent reduction in $\alpha_1$ and $\beta_1$ sGC promoter activity. In these studies, the inhibitory effect of NO peaked at 200 μM DETA-NO, which results in an ambient NO concentration of ~200 nM (42), more akin to those associated with inducible NO synthase activity and pathological conditions (rather than generated by constitutive NO synthase). Moreover, the effects of NO in this respect appear to be cGMP-independent, implying direct effects on TF activity (as has been shown for NF-κB (51) and HIF-1α (52)). These studies also revealed that a CCAAT-BF site is pivotal to the inhibitory effects of NO, at least in terms of the $\alpha_1$ subunit, because deletion of this site leads to insensitivity to NO. Because several TFs bind this site, it is difficult to pinpoint which might be responsible; despite attempts to use EMSA-based supershifts to derive information concerning the identity of this TF, we were unable to elucidate the mechanism further (although we did obtain negative results for CBF-B and MAZ, suggesting that it is not either of these factors).

The inflammation- and NO-based reduction in sGC promoter activity and protein expression might be likely to represent a common negative feedback regulation of sGC expression in cardiovascular disorders associated with high output NO production, such as septic shock. Here, it would be appropriate for sGC expression to be down-regulated to offset the excessive NO levels and thereby reverse the associated, life-threatening hypotension. These data also suggest that the reduction in sGC mRNA levels reported in vascular smooth muscle cells in response to LPS/interleukin-1β (27), tumor necrosis factor-α/LPS (53), and NO donors (54, 55) is likely to be due, at least in part, to a direct down-regulation in sGC promoter activity via NF-κB(p50)- and CCAAT-BF-dependent pathways.

In sum, these data provide a systematic, comparative analysis of human sGC promoter regulation in HASMCs and has identified potentially important factors regulating human sGC expression within a cell system relevant to cardiovascular physiology and (patho)physiology. These observations suggest that expressional regulation of human sGC $\alpha_1$ and $\beta_1$ subunit expression, both coordinated and individually, is likely to play an important role in the (patho)physiological regulation of enzyme activity. As such, these findings represent a significant advance in the understanding of expressional regulation of the genes encoding sGC subunits and provide insight into potential pathogenic mechanisms that result in aberrant NO-sGC signaling.

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