Complex Regulation of Human Neuronal Nitric-oxide Synthase Exon 1c Gene Transcription

ESSENTIAL ROLE OF Sp AND ZNF FAMILY MEMBERS OF TRANSCRIPTION FACTORS*

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Neuronal nitric-oxide synthase (nNOS) is expressed in a variety of human tissues and shows a complex transcriptional regulation with the presence of nine alternative first exons (1a–1l) resulting in nNOS transcripts with differing 5′-untranslated regions. We previously demonstrated that nNOS exon 1c, one of the predominant transcripts in the human gastrointestinal tract, is driven by a separate promoter (Saur, D., Paehge, H., Schusdziarra, V., and Allescher, H. D. (2000) Gastroenterology 118, 849–858). The present study focused on the quantitative expression of nNOS first exon variants in different human tissues and the characterization of the basal nNOS exon 1c promoter. In human brain, skeletal muscle, colon, and TGW-nu-I neuroblastoma cells, first exon expression patterns were analyzed by quantitative real-time reverse transcription-PCR. In these tissues/cells exon 1c was one of the most abundant first exons of nNOS. By transient transfections of TGW-nu-I and HeLa cells with reporter plasmids containing a series of 5′ and 3′ deletions in the exon 1c regulatory region, the minimal TATA-less promoter was localized within 44 base pairs. Gel mobility shift assays of this cis-regulatory region revealed a high complexity of the basal promoter with a cooperative binding of several transcription factors, like Sp and ZNF family members. When the Sp binding site of the minimal promoter construct was mutated, promoter activity was completely abolished in both cell lines, whereas mutation of the common binding site of ZNF76 and ZNF143 resulted in a decrease of 53% in TGW-nu-I and 37% in HeLa cells. In Drosophila Schneider cells expression of Sp1, the long Sp3 isoform, ZNF66 and ZNF143 potently transactivated the nNOS exon 1c promoter. These results identify the critical regulatory region for the nNOS exon 1c basal promoter and stress the functional importance of multiple protein complexes involving Sp and ZNF families of transcription factors in regulating nNOS exon 1c transcription.

Nitric oxide (NO),1 a ubiquitous multifunctional mediator, is synthesized by nitric-oxide synthases (NOS) during the oxidation of L-arginine to L-citrulline. In the central and peripheral nervous system, skeletal muscle, the mucosal layers of the kidney, testis, and neutrophils, neuronal NOS (nNOS) is the predominant enzyme for the generation of nitric oxide. NO, synthesized by nNOS, acts as neurotransmitter, neuromodulator, or intracellular signaling molecule. It is involved in synaptic plasticity, regulation of gene expression, differentiation, and regeneration and plays an important role in neurodegenerative disorders and stroke as a mediator of neurotoxicity (for review see Refs. 1–4). In the gastrointestinal tract NO generated by nNOS acts as an important mediator of the non-adrenergic non-cholinergic inhibitory innervation of intestinal smooth muscle (5) and as a neuromodulator within the enteric nervous system (6).

Although the transcriptional regulation of the other two NOS enzymes, the calcium-dependent endothelial NOS and the calcium-independent inducible NOS, are extensively studied (for review see Refs. 3, 7, 8), little is known about the transcriptional regulation of the nNOS gene (3, 4, 9, 10), which is considered to be responsible for the largest proportion of NO in the body (1). Although usually named constitutive, recent observations suggest a tightly regulated gene expression of nNOS in response to different physiological and pathophysiological stimuli, resulting in an up- or down-regulation of nNOS mRNA (3, 9).

Recently nine distinct first exons, called exons 1a–1l, of nNOS mRNA have been identified, leading to nNOS mRNA variants with different 5′-untranslated regions and translational efficiencies (11). The nNOS gene is therefore believed to be one of the most complex genes known in terms of first exon usage and alternative splicing (11–13). It has been shown that nNOS exons 1c (12) and 1f and 1g (13) (formerly called exons 153, 152, and 151, respectively), which show high abundant expression in the human gastrointestinal tract (12), are driven by separate promoters in HeLa cells. The use of multiple alternative promoters allows a cell- and tissue-specific, and site-specific transcriptional regulation of nNOS in different physiological and pathophysiological stages.

An altered expression or biological activity of nNOS has been linked to several physiological conditions, like aging and pregnancy, as well as different pathophysiological conditions and diseases such as ischemia/hypoxia and injuries of the central nervous system, inherited diabetes insipidus, heart failure, arteriosclerosis, achalasia, diabetic gastroparesis, and hypertrophic pyloric stenosis (1–4, 7, 14–17). nNOSs mutant mice, synthase; nNOS, neuronal nitric-oxide synthase; EMSA, electrophoretic mobility shift assay; FAM, 6-carboxy-fluorescein; PBS, fetal bovine serum; GSP, gene-specific primer; RT, reverse transcriptase; SL2 cells, Drosophila Schneider cells; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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1 The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; EMSA, electrophoretic mobility shift assay; FAM, 6-carboxy-fluorescein; PBS, fetal bovine serum; GSP, gene-specific primer; RT, reverse transcriptase; SL2 cells, Drosophila Schneider cells; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
generated by targeted disruption of the nNOS gene by homologous recombination, showed a gastrointestinal phenotype resembling hypertrophic pyloric stenosis with delayed gastric emptying of solids and fluids (18, 19). In addition, these mice have a hypertensive lower esophageal sphincter with impaired relaxation (20). A recent study comprising 27 families with generation of 5′ deletions of pGL3–5891/+49, point mutations of pGL3–5891/+49, and site-directed mutagenesis of pGL3–5891/+49. Boldface letters indicate mutated bases.

| Name               | Sense (S) or Antisense Strand (AS) Primers and TaqMan Probes |
|--------------------|---------------------------------------------------------------|
| ZNF42 (S)          | CGCAGGTCAGGGATGTTAGTAAAGC                                      |
| ZNF42 (AS)         | CTCTCCGATGCTTCCAG                                            |
| ZNF76 (S)          | CAGGTAGAGGATGAGAGAC                                           |
| ZNF76 (AS)         | TGATGAGGCTGGTGAGGAC                                           |
| ZNF143 (S)         | TTATGAGGCTGGGTAGGGAG                                          |
| ZNF143 (AS)        | GATGAGGCTGGGTAGGGAG                                           |
| ZNF42 XhoI (S)     | CAGGTAGAGGATGAGAGAC                                           |
| ZNF42 XhoI (AS)    | TGATGAGGCTGGTGAGGAC                                           |
| ZNF76 BamHI (S)    | CAGGTAGAGGATGAGAGAC                                           |
| ZNF76 BamHI (AS)   | TGATGAGGCTGGTGAGGAC                                           |
| ZNF143 BamHI (S)   | CAGGTAGAGGATGAGAGAC                                           |
| ZNF143 BamHI (AS)  | TGATGAGGCTGGTGAGGAC                                           |
| nNOS exon 1a (S)   | CACCTGGAGCCTCTTAAATGGAG                                      |
| nNOS exon 1b (S)   | CCCATGCTGATCCGTGAGGG                                          |
| nNOS exon 1c (S)   | GCCAGGTTGAGGCCTTTGC                                          |
| nNOS exon 1d (S)   | CTTCCGATGCTTCCAG                                            |
| nNOS exon 1e (S)   | ATGCCAGGGTGAGGCCTTTGC                                         |
| nNOS exon 1f (S)   | GGAGGAGCCTTGGAGGGA                                          |
| nNOS exon 1g (S)   | GCTGAGAGATGGAAGAACTGGG                                         |
| nNOS exon 1h (S)   | TTCCTCGGTGCCCGAGGAG                                          |
| nNOS exon 1i (S)   | GATGAGGCTGGGTAGGGAG                                           |
| nNOS exon 2 (AS)   | GGTAGAGGCTGGGTAGGGAG                                          |
| nNOS exon 2 Probe  | AACCATGCTGATCCGTGAGGG                                        |
| nNOS exon 6 (S)    | ACCCATGCTGATCCGTGAGGG                                        |
| nNOS exon 6 (AS)   | GGTAGAGGCTGGGTAGGGAG                                          |
| nNOS exon 7 (S)    | GGAGGAGCCTTGGAGGGA                                         |
| nNOS exon 7 (AS)   | GGTAGAGGCTGGGTAGGGAG                                          |
| P-5891 (S)         | TGTGAGGCTGGGTAGGGAG                                          |
| P-5891 (AS)        | GGTAGAGGCTGGGTAGGGAG                                          |
| P-1938 (S)         | GGTAGAGGCTGGGTAGGGAG                                          |
| P-1938 (AS)        | GGTAGAGGCTGGGTAGGGAG                                          |
| P-1520 (S)         | GGAGGAGCCTTGGAGGGA                                         |
| P-332 (S)          | GGTAGAGGCTGGGTAGGGAG                                          |
| P-279 (AS)         | CAGCAGTGGAGCGGTCTG                                           |
| P-83 (S)           | CAGCAGTGGAGCGGTCTG                                           |
| P-49 (AS)          | ACCTCCGCCACCTGCCCTTTGC                                      |
| P-90 Sp1/ZNF42-M1  | GTCACCCACTCCCTGAGAG                                         |
| P-90 Sp1-M2 (S)    | GTCACCCACTCCCTGAGAG                                         |
| P-90 Ap2/Ol/M-1 (S)| GTCACCCACTCCCTGAGAG                                         |
| P-90 StaF-M (S)    | GTCACCCACTCCCTGAGAG                                         |
| SDM-Sp1/ZNF42-M1 (S)| GTCACCCACTCCCTGAGAG                                         |
| SDM-Sp1/ZNF42-del (S)| GTCACCCACTCCCTGAGAG                                      |
| SDM-Staf-M (S)     | GTCACCCACTCCCTGAGAG                                         |
| SDM-Staf-del (S)   | GTCACCCACTCCCTGAGAG                                         |
| SDM-StafSp1/ZNF42-M1 (S)| GTCACCCACTCCCTGAGAG                                  |
| SDM-StafSp1/ZNF42-del (S)| GTCACCCACTCCCTGAGAG                                |
| GSP1-ASex 1c (AS)  | GTCACCCACTCCCTGAGAG                                         |
| GSP2-ASex 1c (AS)  | GTCACCCACTCCCTGAGAG                                         |
| AP1                | GTCACCCACTCCCTGAGAG                                         |
| AP2                | GTCACCCACTCCCTGAGAG                                         |

**EXPERIMENTAL PROCEDURES**

**Materials**—The cell lines TGW-nu-I (human neuroblastoma) and ME-180 (human cervix carcinoma) were kindly provided to our laboratory by Dr. Esumi (21) and Dr. E. R. Werner (22), respectively. The mammalian expression plasmids pcDNA3 ZNF76 and pcDNA3 143 were a gift from Dr. P. Carbon (23), and CB6-MZF-1 (alternative name CB6-ZNF42), under the control of the cytomegalovirus early promoter, was generously provided by Dr. R. Hromas (24). The plasmid pPac-Sp1, which expresses Sp1, was a gift from Dr. Esumi (21) and Dr. E. R. Werner (22), respectively. The empty control plasmid pPac0, containing only the Dro sophila actin promoter, was generously provided by Dr. R. Tjian (25). The expression vector for Sp2 (pPac-Sp2) was a gift from Dr. J. D. Noti (26), and the expression plasmid for the short isoforms of Sp3 (pPac-Sp3), the long isoform of Sp3 (pPacUSp3), Sp4 (pPac-Sp4), and the β-galactosidase expression plasmid pβ7 were generously provided by Dr. G. Suske (27–30). All cell culture reagents were obtained from Invitrogen (Groningen, the Netherlands).

**TABLE I**

nNOS Exon 1c Basal Promoter Regulation

| Name               | Sequence (5′ to 3′) |
|--------------------|---------------------|
| nNOS exon 1a (S)   | CACCTGGAGCCTCTTAAATGGAG |
| nNOS exon 1b (S)   | CCCATGCTGATCCGTGAGGG  |
| nNOS exon 1c (S)   | GCCAGGTTGAGGCCTTTGC   |
| nNOS exon 1d (S)   | CTTCCGATGCTTCCAG      |
| nNOS exon 1e (S)   | ATGCCAGGGTGAGGCCTTTGC |
| nNOS exon 1f (S)   | GGAGGAGCCTTGGAGGGA    |
| nNOS exon 1g (S)   | GCTGAGAGATGGAAGAACTGGG |
| nNOS exon 1h (S)   | TTCCTCGGTGCCCGAGGAG   |
| nNOS exon 1i (S)   | GATGAGGCTGGGTAGGGAG   |
| nNOS exon 2 (AS)   | GGTAGAGGCTGGGTAGGGAG  |
| nNOS exon 2 Probe  | AACCATGCTGATCCGTGAGGG |
| nNOS exon 6 (S)    | ACCCATGCTGATCCGTGAGGG |
| nNOS exon 6 (AS)   | GGTAGAGGCTGGGTAGGGAG  |
| nNOS exon 7 (S)    | GGAGGAGCCTTGGAGGGA    |
| nNOS exon 7 (AS)   | GGTAGAGGCTGGGTAGGGAG  |
| P-5891 (S)         | TGTGAGGCTGGGTAGGGAG   |
| P-5891 (AS)        | GGTAGAGGCTGGGTAGGGAG  |
| P-1938 (S)         | GGTAGAGGCTGGGTAGGGAG  |
| P-1938 (AS)        | GGTAGAGGCTGGGTAGGGAG  |
| P-1520 (S)         | GGAGGAGCCTTGGAGGGA    |
| P-332 (S)          | GGTAGAGGCTGGGTAGGGAG  |
| P-279 (AS)         | CAGCAGTGGAGCGGTCTG    |
| P-83 (S)           | CAGCAGTGGAGCGGTCTG    |
| P-49 (AS)          | ACCTCCGCCACCTGCCCTTTGC |
| P-90 Sp1/ZNF42-M1  | GTCACCCACTCCCTGAGAG  |
| P-90 Sp1-M2 (S)    | GTCACCCACTCCCTGAGAG  |
| P-90 Ap2/Ol/M-1 (S)| GTCACCCACTCCCTGAGAG  |
| P-90 StaF-M (S)    | GTCACCCACTCCCTGAGAG  |
| SDM-Sp1/ZNF42-M1 (S)| GTCACCCACTCCCTGAGAG  |
| SDM-Sp1/ZNF42-del (S)| GTCACCCACTCCCTGAGAG   |
| SDM-Staf-M (S)     | GTCACCCACTCCCTGAGAG  |
| SDM-Staf-del (S)   | GTCACCCACTCCCTGAGAG  |
| SDM-StafSp1/ZNF42-M1 (S)| GTCACCCACTCCCTGAGAG |
| SDM-StafSp1/ZNF42-del (S)| GTCACCCACTCCCTGAGAG  |
| GSP1-ASex 1c (AS)  | GTCACCCACTCCCTGAGAG  |
| GSP2-ASex 1c (AS)  | GTCACCCACTCCCTGAGAG  |
| AP1                | GTCACCCACTCCCTGAGAG  |
| AP2                | GTCACCCACTCCCTGAGAG  |

D. Saur, J. M. Vanderwinden, M. H. De-Laet, and H.-D. Allescher, manuscript in preparation.
ingen, Netherlands). Polyclonal antibodies against Sp1 (PEP 2 X), Sp2 (K-20 X), Sp3 (t-20 X), Sp4 (V-20 X), NF-κB p65 (C-20 X), NF-κB p50 (C-19 X), Ap-2a (C-17 X) were purchased from Santa Cruz Biotechnologies (Heidelberg, Germany). Consensus and mutant consensus oligonucleotides were obtained from electrophoretic mobility shift assays (EMSA) were obtained from Santa Cruz Biotechnology. Myc probe was from CLONTECH ( Palo Alto, USA) and primers were made by MWG (Ebersberg, Germany). Primers were made by MWG and Taq-Man probes by Applied Biosystems (Weiterstadt, Germany). Restriction endonucleases were obtained from New England BioLabs (Manheim, Germany). (γ-32P)ATP was supplied from Amersham Biosciences, Inc. (Buckingham, UK). The Eberchibich (Poco, Roche Molecular Biochemicals, Mannheim, Germany) and the primers P5891I (sense) and P 49I (antisense) (see Table I for primer sequences). The gel-purified PCR product was blunt end-cloned into the SnaI site of the promoter-enhancerless firefly luciferase reporter gene vector pGL3-Basic (Promega, Manheim, Germany) in the forward (pGL3–5891I/49) and reverse (pGL3+/49–5891) orientation. Reporter gene constructs containing 5′ and 3′ deletions of the promoter region of nNOS exon 1c were generated by PCR, exonuclease III/S1 nuclease digestion, or restriction endonuclease digestion. pGL3–2774/+49, pGL3–1938/+49, pGL3–1520/+49 and pGL3–332/+49 were prepared by PCR and blunt end cloning similar to the preparation of pGL3–5891I/+49, using the sense primers P2774, P1938, P1520, and P323 combined with the common antisense primer P 49. For construction of pGL3–5891I–279, sense primer P5891 was used with antisense primer P279, pGL3–279/+49 was prepared by restriction endonuclease digestion of pGL3–332/+49 with PfuI and Msel, followed by blunt and religation. Additional 5′ deletions of pGL3–332/+49 (pGL3–278I/+49, pGL3–241I/+49, pGL3–174I/+49, pGL3–131I/+49, pGL3–90/+49, pGL3–83I/+49, pGL3–63I/+49, pGL3–48I/+49, pGL3–34I/+49, pGL3–24I/+49, pGL3–14I/+49, pGL3–4I/+49, pGL3–18I/+49) were prepared by digestion of pGL3–332/+49 with PfuI and Msel, and both the Sp1/ZNF42 and Staf binding sites in the longer promoter were mutated, respectively, pGL3–90/+49 Sp1/ZNF42-M1, containing a point mutation of the common Sp1 and ZNF42 binding site, pGL3–90/+49 Sp1I-M2, containing only a mutated Sp1 element, pGL3–90/+49 Ap2I/Of-1-M, containing a mutation of the common Ap2 and Of1 binding site, and pGL3–90/+49 Staf-M, containing a mutated Staf consensus sequence (common binding site for ZNF76 and ZNF143), were prepared by digestion of pGL3–332/+49 with PfuI and Msel. In the latter case, the common ZNF76 and ZNF143 binding sites were mutated by the QuikChange XL site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) exactly as described by the manufacturer with the following primers: SDM-Staf/Sp1/ZNF42-M1 (mutation of the Sp1/ZNF42 binding site), SpMutm1/ZNF42-del (deletion of the Sp1/ZNF42 binding site), SpMutm2/ZNF42-del (mutation of the Staf binding site), SpZNF42-M1 (mutation of the Staf and Sp1/ZNF42 binding sites), and SpMutm2/ZNF42-del (deletion of the Staf and Sp1/ZNF42 binding sites). To construct plasmids that express the transcription factors ZNF42, ZNF76, and ZNF143 from the Drosophila actin promoter, the eukaryotic expression vectors CB6-ZNF42 (alternative name CB6-MZF-1), pcdNA3 ZNF76, and pcDNA3 ZNF143 were used as templates for PCR to generate DNAs containing the complete coding sequences of these transcription factors. Primers were designed to contain the following recognition sites for ZNF76 and ZNF143 contained BamHI restriction sites and a Kozak consensus sequence upstream of the ATG start codon. The XhoI and BamHI restriction sites were used to clone the coding sequences of ZNF42, ZNF76, and ZNF143 into the respective XhoI or BamHI sites of the expression plasmid pFox5, which contains only the Drosophila actin promoter. Integrity of all cloned sequences was confirmed by automated
DNA sequencing (GATC) using an ABI Prism 377 DNA sequencer (Applied Biosystems).

Cell Culture, Transient Expression, and Reporter Gene Assays—HeLa cells were cultured and transiently cotransfected with the different nNOS exon 1c-pGL3 promoter gene constructs and the herpes simplex virus thymidine kinase promoter-driven Renilla luciferase expression vector pRL-TK (Promega) to normalize for transfection efficiency and cell number essentially as described previously (12). TGW-nu-1 cells were cultured in minimum Eagle’s medium containing 10% fetal bovine serum (FBS), 25 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (37 °C/5% CO₂). 50–60% confluent cells were transiently cotransfected by lipid-mediated transfer using 0.95 μg of plasmid DNA of each pGL3 construct and 0.05 μg of pRL-TK DNA with 6 μl of Plus reagent and 4 μl of LipofectAMINE (Invitrogen) per 3.5-cm dish. Cells were incubated in FBS and antibiotic-free minimum Eagle’s medium containing the lipid-coated DNA complexes for 3 h and in complete medium for an additional 48 h. ME-180 cells were cultured in McCoy’s 5A medium as described before (22). About 60%
confluent cells were transfected with 0.38 μg of each pGL3 test plasmid DNA and 0.02 μg of pRL-TK DNA using 4 μl of Effectene and 3.2 μl of Enhancer (Qiagen) per 3.5-cm dish.

Approximately 2 × 10^6 Drosophila Schneider cells (SL2 cells) were plated the day prior to transfection onto six-well plates and cultured at 25 °C in Media Expression (containing 10% FBS). After washing and addition of fresh medium without FBS, cells were transfected using 9 μl of Cellfectin (Invitrogen) per well along with 1 μg of each pGL3 construct, 0.5 μg of the β-galactosidase expression plasmid pGL7b for normalization of transfection efficiencies, and variable amounts of the expression plasmids pPac-Sp1, pPac-Sp2, pPac-USp3, pPac-Sp3, pPac-Sp4, pPac-ZNF76, and pPac-ZNF143. Variations in the amount of the expression plasmids were compensated with the empty plasmid pPa0 to adjust the total DNA content of the transfection mix to 2 μg per well. After 24 h of transfection, medium was removed and cells were incubated for an additional 24 h in complete medium containing FBS. SV40 promoter/enhancer-directed pGL3-control and promoter-enhancerless pGL3-basic plasmids (both Promega) were used as positive and negative controls in all experiments, respectively.

48 h after transfection, cells were harvested by treatment with lysis buffer (Promega). Total cellular protein was determined by Bio-Rad II protein assay (Bio-Rad). Firefly and Renilla dual-luciferase activity was measured (Promega) with 20 fmol of the indicated [γ-32P]-labeled double-stranded oligonucleotide probes and the ECL system (Amersham Biosciences, Inc.).

**Data Analysis**—Unless otherwise indicated, all data were determined from three independent experiments, each done in triplicate, and are expressed as mean values ± S.D. Comparisons among data sets were made with analysis of variance, followed by Student’s t test. Values of p < 0.05 or less were considered to be statistically significant.

**RESULTS**

**Expression Pattern of nNOS First Exon mRNA Variants**—We analyzed mRNA expression of the nine alternative first exon variants of human nNOS (Fig. 1A) by real-time quantitative RT-PCR (5’ nuclelease assay) in human brain, skeletal muscle, rectum, TGW-nu-1 neuroblastoma cells, ME-180 cervix carcinoma cells, and HeLa cells. In addition, as a parameter for total nNOS mRNA expression, a sequence between exon 6 and exon 7, encoding parts of the oxygenase domain of nNOS (hem binding site that is essential for NOS activity (34)), was amplified. As internal controls GAPDH, HPRT1, and TFP2D mRNA expression were quantified. Real-time RT-PCR of the different first exons revealed high expression of nNOS exon 1c in the investigated human tissues (Fig. 2, A–C) and the TGW-nu-1 cell line (Fig. 2D), whereas ME-180 cells were nNOS exon 1c-negative (Fig. 2E). The other first exons showed varying expression patterns with a cell- and tissue-specific expression (see Fig. 2, A–F). Exon 1f and exon 1g are highly abundant in the brain and rectum and very lowly abundant in skeletal muscle (Fig. 2, A–C, F, where bars are not evident despite positive RT-PCR results in Fig. 2F, values are less than the resolution shown in the figure). In contrast exon 1a is highly expressed in skeletal muscle (Fig. 2B) but missing in TGW-nu-1 cells (Fig. 2D) and lowly abundant in the rectum (Fig. 2C). HeLa cells were nNOS mRNA-negative but showed high expression of the investigated housekeeping genes (data not shown). Fig. 2F summarizes the distribution of alternative first exons of nNOS in the investigated tissues and cell lines. nNOS first exon expression was normalized against GAPDH (Fig. 2, A–E) and showed no significant difference when other housekeeping genes (HPRT1, TFP2D) were used as internal controls (data not shown).

**Cloning of the 5’-Flanking Region of nNOS Exon 1c**—To further determine the partially known promoter sequence of nNOS exon 1c (formerly called exon 1cγ) (12), we used rapid amplification of genomic ends (RAGE) to obtain a 5893-bp DNA fragment of the 5’-flanking regulatory region of exon 1c spanning nucleotides (nt) −5891 to +49 (Fig. 1B). This nucleotide sequence has been deposited in the EMBL/GenBank™ data base with accession number AJ308545. The transcriptional start site of exon 1c was identified previously to be located 84 bp upstream of the exon 1c intron 1 splice junction at an adenine (+1) (Fig. 1, B and C) (12). Computer-based analysis of the sequence immediately upstream of this region using the MatInspector professional software, revealed no putative TATA or CAAT boxes as initiators of transcription, but a putative transcription initiation site with overlapping consensus sequences for Staf (common binding site for ZNF76 and ZNF143 (23)), p53 half site, Olf-1, Ap2, Myc/Max, ZNF42 (alternative title MZF-1 (24)), Sp1 and MAZ between nt −90 and −47 bp relative to the transcription start site. The obtained 5’-flanking region of exon 1c contains in addition the sequence of nNOS exon 1a (nt 3656–3755) derived from EMBL accession number AF049712 (11) or EMBL accession number AF049713 (11) (see Fig. 1B). Exon 1c is located at nt 5892–5975 of the submitted sequence with EMBL accession number AJ308545. Therefore exons 1a, 1b, and 1c are located in close proximity within 2400 bp, similar to the genomic structure of exons 1f and 1g (former called exons 1cγ and 1cδ) of human (13) and exons 1b and 1c of rat nNOS (35).

**Materials**—Nuclear extracts (60 μg of protein per lane) from transfected SL2 cells and untransfected TGW-nu-1 and HeLa cells were separated by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad) as previously described (12, 31). Blots were probed with Sp1, Sp2, and Sp3 antibodies diluted 1:1000. Signal detection of the immunoreactive bands was facilitated by enhanced chemiluminescence using the ECL system (Amersham Biosciences, Inc.).
Fig. 2. Quantitative analysis of human nNOS mRNA expression by real-time RT-PCR in human brain (A), skeletal muscle (B), rectum (C), TGW-nu-I neuroblastoma cells (D), and ME-180 cervix carcinoma cells (E). A set of nine forward primers, specific for the nine alternative first exons of nNOS (exon 1a–1i) were used with a common exon 2-specific reverse primer and an internal exon 2-specific 6-carboxyfluorescein (FAM)-labeled TaqMan probe. For primer locations see arrows in Fig. 1A. As a parameter for total nNOS mRNA expression, a pair of exon 6- and exon 7-specific primers present in all known nNOS cDNAs were used with an exon 7-specific internal FAM-labeled probe. Relative amounts of transcripts were calculated using standard curves and dividing the expression levels of the different nNOS variants by the expression levels of the GAPDH housekeeping gene measured in the same RNA preparation. Results shown are the mean ± S.D. of one (pooled RNA obtained from CLONTECH; A and B) and three independent RNA isolations (C–E). Individual cDNA samples were analyzed in triplicate with a given pair of primers. F, distribution of alternative first exon variants of human nNOS in the brain, skeletal muscle, rectum, TGW-nu-I cells, ME-180 cells, and HeLa cells as determined by real-time RT-PCR. +++ indicates a high, ++ a moderate, and + a low expression level; − indicates negative RT-PCR results (where bars are not evident (A–E) despite positive RT-PCR results, values are less than the resolution of the figure).
Fig. 3. Functional analysis of the human nNOS exon 1c promoter in TGW-nu-I (A), HeLa (B), and ME-180 (C) cells. 5' and 3' deletions were introduced into the nNOS exon 1c 5'-flanking region, and the indicated DNA fragments were ligated into the promoter-/enhancerless firefly
and human with a sequence homology of 100% between nt = 84 and -41 relative to the transcription start site of human exon 1c and nt 320 and 363 of rat nNOS exon 1b (EMBL accession number AF008911) (35). Note that the nomenclature of alternative first exons of nNOS is different in rat and human with human exon 1c corresponding to rat exon 1b (11, 35).

Identification of the Basal Promoter Region of nNOS Exon 1c—Previously, we showed that exon 1c (designated exon 1c3) of nNOS is driven by a separate promoter within 332 bp upstream of the transcription start site in HeLa cells (12). However, neither the minimal promoter, nor the major transcription factors that regulate basal transcriptional activation were elucidated. To examine the sequence that is necessary for basal transcription of nNOS exon 1c, we analyzed various 5’ and 3’ deletions of the 5’-flanking region of exon 1c by reporter gene assays. The 5.9-kb promoter fragment of exon 1c (see Fig. 1B), obtained by 5’-RAGE, was cloned 5’ to the firefly luciferase reporter gene of the pGL3-basic plasmid, resulting in pGL3-5891/+49. Different 5’ and 3’ deletions of the exon 1c promoter were generated by PCR, or restriction endonuclease and exonuclease III digestion of the pGL3-5891/+49 and pGL3-332/+49 plasmids (Fig. 1, B and C). These constructs were transiently transfected into nNOS-exon 1c-positive TGW-nu-I neuroblastoma cells (see Fig. 2, D and F) and nNOS-negative HeLa cells (see Fig. 2F), as determined by quantitative real-time RT-PCR. After transfections of TGW-nu-I and HeLa cells with pGL3-5891/+49, we obtained a 13.7- and 9.1-fold increase of the normalized promoter activity compared with that of promoter/enhancerless pGL3-basic, respectively (Fig. 3, A and B). After deletion of 3117 bp from full-length pGL3-5891/+49, a decrease of 57% (p = 0.008) in promoter activity could be observed in TGW-nu-I cells (Fig. 3A). In contrast, an increase of 67% (p = 0.007) was evident in HeLa cells (Fig. 3B). For both cell lines, progressive deletions from -2774/+49 to -1520/+49 did not show a significant change of transcriptional activity. Further deletion to -332/+49 resulted in a decrease of ~44% (p = 0.012) in TWG-nu-I cells, whereas promoter activity showed no significant change in HeLa cells. These observations suggest the presence of cis regulatory sequences exhibiting positive effects on the promoter activity between -5891 and -2774 and between -1520 and -332 specifically in TGW-nu-I cells, whereas sequences between -5891 and -2774 negatively affect promoter activity in HeLa cells. This argues for a cell-specific regulation of nNOS exon 1c promoter activity by cis acting elements in the upstream 5’-flanking region of exon 1c.

Up to 242 bp (to position -90) could be further deleted in HeLa cells without a significant change of transcriptional activity. In contrast, in TGW-nu-I cells, we observed a drop in activity of ~33% (not significant) between -278 and -241, an increase between -241 and -131 of ~72% (p < 0.01), and an additional profound rise of ~108% (p < 0.001) after deletion of sequences between -131 and -90. However, deletion of an additional 27 bp (to position -63) abolished promoter activity completely in both cell lines (p < 0.001). Values seen for this -63/+49 construct, as well as for further deletions up to +18/+49, essentially reflected those of pGL3-basic. The antisense construct of pGL3-5891/+49, named pGL3 + 49/−5891, showed only background activity. As positive control the SV40 promoter/enhancer-directed pGL3-control vector was used in TGW-nu-I (Fig. 3A) and HeLa cells (data not shown).

These data demonstrate that the basal promoter for exon 1c is disrupted by deletion to -63 relative to the exon 1c transcription start site, and therefore, they strongly suggest that the promoter resides within the predicted region between -90 and -47. Interestingly, adjacent upstream 5’ regions regulate this basal promoter activity differentially in nNOS-positive TGW-nu-I cells and nNOS-negative HeLa cells, demonstrating distinct mechanisms of activation in these two cell lines.

When 350 bp was removed from the 3’-end of pGL3-5891/+49, a -5891/−279 construct resulted that lacks the minimal promoter and the transcription start site of exon 1c but contains the putative promoters and transcription start sites of exon 1a and exon 1b. After transient transfection of this construct, normalized promoter activity showed just background activity in exon 1a and exon 1b mRNA-negative TGW-nu-I cells and in nNOS mRNA-negative HeLa cells (Fig. 3, A and B). These results demonstrate that the upstream 5’-flanking region of exon 1c, containing exons 1a and 1b and their putative promoters, can influence transcription of exon 1c but does not activate transcription of exon 1a and/or exon 1b in both cell lines under the investigated conditions.

Because exon 1c promoter constructs are active in endogenous nNOS-negative HeLa cells, we further investigated this discrepancy by reporter gene assays using nNOS-positive but exon 1c-negative ME-180 cells. After transient transfection...
with different nNOS exon 1c pGL3 promoter constructs, we observed a moderate, but significant increase in relative luciferase activity over that of promoter/enhancerless pGL3-basic (Fig. 3C). Therefore, endogenous nNOS-negative HeLa cells and nNOS exon 1c-negative ME-180 cells are able to transactivate nNOS exon 1c reporter constructs.

**Identification of Transcription Factors Binding to the Basal nNOS Exon 1c Promoter**—Computer-based sequence inspection of the GC-rich (66%) and TATA-less basal promoter region between −90 and −47 of exon 1c indicated a variety of putative cis-regulatory elements, like Staf (consensus binding site for ZNF143 and ZNF76 [23]) (−85 to −64), p53 half-site (−79 to −70), Oif-1 (−78 to −57), Ap2 (−73 to −62), Myc/Max (−61 to −48), ZNF42 (alternative title MZF-1) (−70 to −63), and a low affinity GC box with binding sites for Sp1 (−69 to −57) and MAZ (−67 to −59). To assess transcription factor binding, a double-stranded 44 bp 32P-labeled oligonucleotide (−90/−47) (see Table II for all oligonucleotides used in gel shift experiments, except consensus and mutant consensus oligonucleotides for Ap2, Myc-Max, NF-I, YY1, NFκB, p53, Sp1, USF-1, which were purchased from Santa Cruz Biotechnologies), including these elements, was used in electrophoretic mobility shift assays (EMSAs). A series of five shifted protein-DNA complexes were observed after incubation of TGW-nu-I nuclear extracts with the labeled probe (Fig. 4A, lane 2), whereas only two protein-DNA complexes were evident using HeLa nuclear extracts (Fig. 4B, lane 2). These complexes were competed with a 100-fold molar excess of unlabeled −90/−47 probe, establishing binding specificity (Fig. 4, A and B, lane 3). Shifted bands without competition after addition of the unlabeled −90/−47 probe were considered as nonspecific binding and were marked by an asterisk in Fig. 4 (A–D). Using a 100-fold molar excess of an unlabeled Sp1 consensus oligonucleotide (containing the binding site for the transcription factors Sp1, Sp3, and Sp4) complexes I/III (TGW-nu-I cells, Fig. 4A, lane 7) and complex I (HeLa cells, Fig. 4B, lane 7) were completely competed, and complex II (both cell lines) was partially competed. There was no further competition of complex II, when a 200- and 500-fold molar excess of unlabeled Sp1 consensus oligonucleotides was used (both cell lines, data not shown). No competition of the complexes was observed with a 100- and 200-fold molar excess of a commercial mutant Sp1 oligonucleotide (Fig. 4, A and B, lane 8). Using TGW-nu-I nuclear extracts a slight reduction in the protein-DNA complexes I, II, and III was seen with a −90/−63 oligonucleotide where the GC box is disrupted (Fig. 4A, lane 4) and with a −90/−47 oligonucleotide in which the Sp1 binding site was mutated (Fig. 4A, lane 5), whereas complexes IV and V were completely competed by these two oligonucleotides. With HeLa cell nuclear extracts complex I was slightly and complex II was completely competed using the −90/−63 and −90/−47 Sp1-Mut competitors (Fig. 4B, lanes 4 and 5). Furthermore, no competition of any complex was observed using an unrelated YY1 consensus oligonucleotide (TGW-nu-I cells, Fig. 4A, lane 23; HeLa cells, Fig. 4B, lane 21). When protein-DNA complexes were incubated with antibodies against Sp1, Sp2, Sp3, and Sp4 or combinations of these antibodies, supershifts of different complexes were observed with TGW-nu-I and HeLa cell nuclear extracts (Fig. 4, C and D). For the Sp1 antibody a supershift of complex I/III (TGW-nu-I cells) and complex I (HeLa cells), for the Sp2 antibody a supershift of complex I (TGW-nu-I and HeLa cells), for the Sp3 antibody a supershift of complex I/II/III (TGW-nu-I cells) and complex II (HeLa cells) was observed whereas for the Sp4 antibody no supershift was seen. A combination of Sp1, Sp2, and Sp3 antibodies led to different supershifts as shown in Fig. 4C for TGW-nu-I cells and Fig. 4D for HeLa cells. When all three antibodies were added to the binding reaction, complexes I/II/III (TGW-nu-I cells) and complexes I/II (HeLa cells) were supershifted with a nearly complete abrogation. Using antibodies against goat and rabbit IgG, no supershift of any complex was evident (data not shown). Taken together these results demonstrate that Sp1, Sp2, and Sp3 can interact with the 44-bp minimal promoter of exon 1c. To further characterize the retarded complex II obtained with TGW-nu-I and HeLa cells, which was not completely competed by an Sp1 consensus oligonucleotide, and the unaffected complexes IV and V (TGW-nu-I cells), consensus oligonucleotides for the transcription factors Staf (ZNF76 and ZNF143), p53, Oif-1, Ap2, Myc/Max, ZNF42, MAZ, NF-κB, NF-1, YY1, and USF were used. Myc/Max, NF-1, USF, p53 (data not shown), and YY1 (Fig. 4A, lane 23; Fig. 4B, lane 21) had no effects on nucleoprotein complex formation. Unspecific competition of the retarded protein-DNA complexes V (MAZ, Fig. 4A, lane 20) and IV/V (Oif-1, Fig. 4A, lanes 11 and 12) was evident using TGW-nu-I cell nuclear extracts and a 100-fold excess of unlabelled consensus and mutated consensus oligonucleotides for MAZ and Oif-1. In contrast, oligonucleotides containing Staf, ZNF42, Ap2, and NF-κB binding sites were specific in competition studies. An oligonucleotide with a Staf binding site (consensus sequence for ZNF76 and ZNF143) competed partially complex II (both cell lines, Fig. 4, A and B, lane 9), whereas ZNF42 consensus oligonucleotides competed complexes I/III/IV/V (TGW-nu-I cells, Fig. 4A, lane 15) and complex I (HeLa cells, Fig. 4B, lane 15). Addition of oligonucleotides containing mutations in the Staf and ZNF42 binding sites failed to compete protein-DNA complexes (Fig. 4, A and B, lane 16). A combination of Sp1 and Staf consensus oligonucleotides resulted in a complete competition of complex II (both cell lines, Fig. 4, A and B, lane 13), whereas Sp1 combined with other consensus oligonucleotides like Oif-1 (Fig. 4, A and B, lane 14) and ZNF42 (data not shown) showed the same degree of competition as Sp1 alone. This observation suggests a cooperative binding of Sp and ZNF76/143 transcription factors to the basal nNOS exon 1c promoter.

Consensus oligonucleotides for Ap2 and NF-κB showed a
FIG. 5. Mutagenesis of transcription factor binding sites decreases transcription from the nNOS exon 1c minimal promoter. TGW-nu-I (A, C, E) and HeLa (B, D, F) cells were transiently cotransfected with pRL-TK and different wild type or mutant reporter gene constructs containing targeted substitutions or deletions in transcription factor binding sites as described under “Experimental Procedures.” To adjust for transfection efficiency, the firefly luciferase activity of the test plasmids was corrected for Renilla luciferase activity (pRL-TK). Data are expressed as percent luciferase activity relative to the respective “full-length” wild type pGL3 construct and represent the means ± S.D. of three independent experiments in triplicate. A and B, the percent luciferase activities of wild type pGL3−90/+49, 5′-deleted pGL3−83/+49, and pGL3−48/+49, the promoter/enhancerless pGL3-basic vector, or mutant pGL3−90/+49 reporter gene constructs containing the indicated targeted substitutions in the binding site of Sp1 (pGL3−90/+49 Sp1-M2), the common binding sites of Sp1 and ZNF42 (pGL3−90/+49 Sp1/ZNF42-M1), Ap2 and Olf1 (pGL3−90/+49 Ap2/Olf1-M), ZNF76 and ZNF143 (called Staf binding site; pGL3−90/+49 Staf-M) are plotted for TGW-nu-I (A) and HeLa (B) cells. C and D, the percent luciferase activities of wild type pGL3−5891/+49, 5′-deleted pGL3−5891−48/+49, pGL3-basic vector, and the indicated mutant pGL3−5891/+49 constructs containing targeted substitutions or deletions in the Sp1/ZNF42, Staf, and both the Sp1/ZNF42 and Staf binding element are plotted for TGW-nu-I (C) and HeLa (D) cells. E and F, the percent luciferase activities of wild type pGL3−332/+49, 5′-deleted pGL3−332−48/+49, pGL3-basic vector, and the indicated mutant pGL3−332−49 constructs containing targeted substitutions or deletions in the Sp1/ZNF42, Staf, and both the Sp1/ZNF42 and Staf binding element are plotted for TGW-nu-I (E) and HeLa (F) cells.
clear reduction in the protein-DNA complexes I/II/III (TGW-nu-I cells, Fig. 4A, lanes 21 and 22) and complex I (HeLa cells, Fig. 4B, lanes 17 and 20), whereas mutated oligonucleotides failed to compete (Fig. 4B, lane 18 and data not shown). However, using Ap2 and NF-κB subunits p50 and p65 antibodies, we were unable to observe a supershift or abrogation of any complex (data not shown). The causal mechanism of this observation is unclear. Ap2 and NF-κB may not be able to bind to the minimal promoter of exon 1c autonomously and thus do not play a direct role in exon 1c basal promoter activation. Therefore, competition of the retarded bands in the gel shift assays could be due to protein-protein interactions of Ap2/NF-κB with other nuclear factors whose DNA binding affinity and specificity could be increased by the presence of Ap2 or NF-κB. Such mechanisms have been demonstrated recently (36, 37), and therefore Ap2 and NF-κB could participate in the formation of multiple protein complexes that enhance or repress the transactivation potential of other transcription factors like Sp1.

To determine transcription factor binding in the absence of Sp and GC box activity, a 22-bp 32P-labeled oligonucleotide with a disrupted GC box and Sp1 binding site was used in EMSAs. A series of four and six shifted protein-DNA complexes was observed after incubation with TGW-nu-I (Fig. 4E, lane 2) and HeLa (Fig. 4F, lane 2) cell nuclear extracts, respectively. These complexes were completely competed with a 100-fold molar excess of unlabeled −90/−63 probe (Fig. 4, E and F,
Fig. 6. Sp1, the long isoform of Sp3, ZNF76, and ZNF143 transactivate the human nNOS exon 1c promoter in Drosophila Schneider cells. SL2 cells were cotransfected with the indicated nNOS exon 1c-promoter/pGL3-luciferase reporter gene constructs and/or empty pPac expression vector as described under “Experimental Procedures.” Luciferase activity was assayed 48 h after transfection and normalized for total cellular protein values of lysed SL2 cells. Data are expressed as fold induction of normalized luciferase activity relative to that obtained following cotransfection of the pGL3 reporter plasmids with empty pPac0, which does not express Sp or ZNF proteins. Values represent means ± S.D. of three independent experiments in triplicate. Where bars and/or error bars are not evident, values are less than the resolution of the figure. Variations in the amount of the expression plasmids were compensated with the empty plasmid pPac0 to keep the total DNA content of pPac plasmids constant (0.5 μg per transfection).

A. Drosophila Schneider cells were cotransfected with 1.0 μg of pGL3–90/49, 5'-deleted pGL3–48/+49, and the promoter-enhancerless pGL3-basic luciferase reporter plasmid along with 0.5 μg of the expression plasmids pPac-Sp1, pPac-USp3, pPac-ZNF76, pPac-ZNF143, and pPac-ZNF42, and cotransfection of 1.0 μg of pGL3–90/63 along with increasing concentrations (0.025–0.5 μg) of the expression plasmids pPac-Sp1, pPac-USp3, pPac-ZNF76, pPac-ZNF143, and pPac-ZNF42 into SL2 cells reveals a dose-dependent transactivation of the minimal nNOS exon 1c promoter for Sp1, the long isoform of Sp3, ZNF76, and ZNF143. C, specific Sp and ZNF family members of transcription factors exert a differential activation of the nNOS exon 1c promoter. Combinations of the expression plasmids pPac-Sp1, pPac-Sp2, pPac-Sp3, pPac-USp3, pPac-Sp4, pPac-ZNF76, pPac-ZNF143, and pPac-ZNF42, and empty pPac0 were cotransfected along with 1.0 μg of the wild type pGL3–90/49 into SL2 cells. 0.25 μg of DNA of each indicated pPac expression plasmid was used for transfections.

Using a 100-fold molar excess of an unlabelled Sp1 consensus oligonucleotide, the signal intensity of the protein-DNA complexes II and III were amplified, and the signal intensity of complex IV was reduced by TGG-nu-I cell nuclear extracts (Fig. 4E, lane 4). In contrast, complete competition of complex I was observed using HeLa cell nuclear extracts (Fig. 4F, lane 4). When a ZNF42 consensus oligonucleotide was used, complex IV was completely competed and complexes II and III were shifted or competed resulting in a retarded band between complex II and III in TGG-nu-I cells (Fig. 4E, lane 5), whereas no competition was observed with a mutated ZNF42 consensus oligonucleotide (Fig. 4E, lane 6). The significance of the described observation remains unclear but demonstrates that ZNF42 is able to modulate transcription factor binding to the −90/−63 sequence. In HeLa cells ZNF42 consensus oligonucleotides resulted in only an unspecified competition of complex III (Fig. 4F, lanes 5 and 6). A Staf consensus oligonucleotide competed complex I/II/III (TGG-nu-I cells, Fig. 4E, lane 9) and complex I/II/IV/V (HeLa cells, Fig. 4F, lane 9). However, complex VI of Fig. 4F (HeLa cells) could not specifically be competed by any of the used consensus oligonucleotides, indicating that an as yet unknown transcription factor present in HeLa cells is able to bind to the −90 to −63 sequence of the exon 1c promoter. No competition was observed with mutant Staf (Fig. 4, E and F, lane 10), mutant Sp1 (data not shown), and unrelated YY1 (Fig. 4E, lane 7, Fig. 4F, lane 8) consensus oligonucleotides using nuclear extracts from both cell lines.

Collectively, these results identify a multiplicity of protein-DNA complexes within the nNOS exon 1c basal promoter involving Sp1, Sp2, Sp3, and members of the ZNF family of transcription factors. These factors have differential effects on nucleoprotein complex formation. Some of them increase, whereas others decrease, band intensities, suggesting modulatory effects on protein-DNA interactions.

Confirmation of Transcription Elements and Promoter Transactivation by Mutagenesis and Transient Transfections—To further clarify the role of the different cis regulatory elements in the basal nNOS exon 1c promoter, reporter gene constructs for pGL3–90/49 containing point mutations in the Sp1/ZNF42 (pGL3–90/+49 Sp1/ZNF42-M1), Sp1 (pGL3–90/+49 Sp1-M2), Ap2/Olf-1 (pGL3–90/+49 Ap2/Olf-1-M), and different combinations and amounts of pPac-Sp1, pPac-Sp2, pPac-Sp3, pPac-USp3, pPac-Sp4, pPac-ZNF76, pPac-ZNF143, pPac-ZNF42, or empty pPac0 expression vector as described under “Experimental Procedures.” Luciferase activity was assayed 48 h after transfection and normalized for total cellular protein values of lysed SL2 cells. Data are expressed as fold induction of normalized luciferase activity relative to that obtained following cotransfection of the pGL3 reporter plasmids with empty pPac0, which does not express Sp or ZNF proteins. Values represent means ± S.D. of three independent experiments in triplicate. Where bars and/or error bars are not evident, values are less than the resolution of the figure. Variations in the amount of the expression plasmids were compensated with the empty plasmid pPac0 to keep the total DNA content of pPac plasmids constant (0.5 μg per transfection).

A. Drosophila Schneider cells were cotransfected with 1.0 μg of pGL3–90/49, 5'-deleted pGL3–48/+49, and the promoter-enhancerless pGL3-basic luciferase reporter plasmid along with 0.5 μg of the expression plasmids pPac-Sp1, pPac-USp3, pPac-ZNF76, pPac-ZNF143, and pPac-ZNF42, and cotransfection of 1.0 μg of pGL3–90/63 along with increasing concentrations (0.025–0.5 μg) of the expression plasmids pPac-Sp1, pPac-USp3, pPac-ZNF76, pPac-ZNF143, and pPac-ZNF42 into SL2 cells reveals a dose-dependent transactivation of the minimal nNOS exon 1c promoter for Sp1, the long isoform of Sp3, ZNF76, and ZNF143. C, specific Sp and ZNF family members of transcription factors exert a differential activation of the nNOS exon 1c promoter. Combinations of the expression plasmids pPac-Sp1, pPac-Sp2, pPac-Sp3, pPac-USp3, pPac-Sp4, pPac-ZNF76, pPac-ZNF143, and pPac-ZNF42, and empty pPac0 were cotransfected along with 1.0 μg of wild type pGL3–90/49 into SL2 cells. 0.25 μg of DNA of each indicated pPac expression plasmid was used for transfections.
Staf (pGL3–90/+49 Staf-M) binding sites were constructed (see Fig. 5, A and B). These constructs were transiently expressed in TGW-nu-I (Fig. 5A) and HeLa cells (Fig. 5B), and promoter activities were compared with that of the wild-type plasmid pGL3–90/+49. Furthermore, 5′-deleted pGL3–83/+49, pGL3–48/+49, and promoter/enhancerless pGL3-basic were used as controls. Mutation of the common Sp1/ZNF42 binding site, as well as the Sp1 binding site, alone abolished promoter activity of nNOS exon 1c to that of pGL3-basic in both cell lines (Fig. 5, A and B). In TGW-nu-I cells, the pGL3–90/+49 promoter construct containing a mutated Staf element resulted in a 53% decrease in luciferase activity compared with the pGL3–90/+49 wild type construct (Fig. 5A), whereas a decrease of 37% was detected in HeLa cells (Fig. 5B). In TGW-nu-I and HeLa cells, constructs of pGL3–90/+49 with mutations in the Ap2/Olf-l binding site were 94% and 98.5% as active as wild type pGL3–90/+49, respectively, indicating that the Ap2 and Olf-l consensus binding sites in the exon 1c minimal promoter play no role for basal transcription (Fig. 5, A and B). Taken together, these data clearly demonstrate the critical cis-acting roles for Sp and ZNF binding motifs in the exon 1c minimal promoter.

To investigate the importance of this basal promoter region for the transactivation of the longer reporter constructs pGL3–5891/+49 and pGL3–332/+49, site-specific mutations and deletions in the Sp and Staf binding sites were introduced into the wild type plasmids. After transient expression in TGW-nu-I cells, promoter activities of all mutated/deleted pGL3–5891/+49 plasmids reflected that of pGL3-basic (Fig. 5C). In HeLa cells, the construct pGL3–5891/+49 StafSp1/ZNF42-M1, which contains a double mutation of the Staf and Sp1/ZNF42 binding sites, abolished promoter activity completely, and the construct pGL3–5891/+49 StafSp1/ZNF42-del, with a deletion of both sites, was 11% as active as wild type pGL3–5891/+49 (Fig. 5D). Plasmids containing a mutation (pGL3–5891/+49 Sp1/ZNF42-M1) or deletion (pGL3–5891/+49 Sp1/ZNF42-del) of the Sp1/ZNF42 site displayed a drop of 87% and 79% in functional promoter activity relative to the wild type construct, respectively, and mutation (pGL3–5891/+49 Staf-M) and deletion (pGL3–5891/+49 Staf-del) of the Staf element resulted in a decrease of 68% and 71%, respectively (Fig. 5D). The same mutations and deletions were incorporated in to the pGL3–332/+49 construct. After transient expression in TGW-nu-I and HeLa cells, relative functional reporter activities revealed no important differences compared with that of the mutations/deletions in the setting of the full-length –5891/+49 construct (Fig. 5, E and F).

Collectively, these data clearly indicate the major importance of the GC-rich region between –90 and –47 relative to the exon 1c transcription start site to promote nNOS exon 1c expression.

Activation of the nNOS Exon 1c Promoter in Drosophila Schneider Cells—To further determine whether members of the Sp and ZNF families of transcription factors functionally interact with the basal promoter of exon 1c, transient transfection experiments were performed with Drosophila Schneider cells (SL2 cells), which lack many mammalian transcription factors, like Sp-related proteins (25, 26), ZNF76 and ZNF143 (23, 38). Expression constructs under the control of an SL2-specific promoter for Sp1 (pPac-Sp1), Sp2 (pPac-Sp2), the long (pPac-USp3) and short isoforms of Sp3 (pPacSp3), Sp4 (pPacSp4), ZNF42 (pPac-ZNF42), ZNF76 (pPac-ZNF76), ZNF143 (pPac-ZNF143) and empty pPac0 were cotransfected along with reporter vectors (pGL3) under the control of different nNOS exon 1c promoters. Normalized luciferase activities for the pGL3 reporter constructs were compared with that in cotransfections with the empty SL2 expression vector pPac0. As shown in Fig. 6A cotransfection of pGL3–90/+49 with pPac-Sp1 induced a 10.4-fold increase in relative luciferase activity over cotransfection with pPac0. In contrast, values slightly above those of pGL3-basic (representing basal luciferase activity) were observed after cotransfection of SL2 cells with pPacSp1 and pGL3–48/+49, which lacks the GC box of the basal exon 1c promoter (Fig. 6A). A 14.8-fold increase was seen, when the long isoform of Sp3 (pPac-USp3) was cotransfected with pGL3–90/+49, whereas cotransfection experiments with pGL3–48/+49 again showed only background activity (Fig. 6A). Cotransfection of pPac-ZNF76 and pPac-ZNF143 along with pGL3–90/+49 resulted in a 11.6- and 6.8-fold transactivation of pGL3–90/+49, respectively, whereas induction of pGL3–48/+49 by ZNF76 and ZNF143 reflected that of pGL3 basic (Fig. 6A). No transactivation of pGL3–90/+49 and pGL3–48/+49 promoter activity was seen after cotransfection with pPac-ZNF42, indicating that ZNF42 is not able to induce the basal nNOS exon 1c promoter autonomously (Fig. 6A). Likewise, there was no significant stimulation of normalized luciferase activity over that of pGL3-basic, when either pPacSp2, pPacSp3 encoding the short isoforms of Sp3, or pPacSp4 was cotransfected with pGL3–90/+49 or pGL3–48/+49 (data not shown). Fig. 6B shows the effects of increasing amounts of pPac-Sp1, pPac-USp3, pPac-ZNF76, pPac-ZNF143, and pPac-ZNF42 on pGL3–90/+49 luciferase activity. Sp1, the long isoform of Sp3, ZNF76, and ZNF143 exhibited a dose-dependent transactivation of pGL3–90/+49, whereas pGL3–48/+49 was again not activated. Increasing amounts of pPac-ZNF42 had no effect on transactivation of pGL3–90/+49 (Fig. 6B).

In combination experiments (Fig. 6C), the stimulatory effects of Sp1 and the long Sp3 isoform (21.3-fold induction), as well as Sp1 and ZNF143 (15.3-fold induction) were additive, whereas Sp1 combined with ZNF76 (28.4-fold induction) and full-length Sp3 combined with ZNF76 (27.2-fold induction) resulted in a potentiation of nNOS exon 1c basal promoter transactivation. Cotransfection of pPac-Sp1 with pPacSp3 or pPacSp4, as well as cotransfection of pPac-USp3 with Sp2, Sp4, ZNF143, or ZNF42, had no effect on promoter activation of pGL3–90/+49 (Fig. 6C). However, combining pPac-Sp1 with pPacSp2 or pPacZNF42, pPac-USp3 with pPacSp3, pPac-ZNF76 with pPac-ZNF42, and pPac-ZNF143 with pPac-ZNF42 resulted in a slight, but statistically significant decrease of nNOS exon 1c promoter activity (Fig. 6C). Thus ZNF and Sp family members are able to exert positive and negative effects on the transactivation of the nNOS exon 1c minimal promoter.

Expression of Sp1, Sp2, isoforms of Sp3, and Sp4 in SL2 cells after transient transfection with the different pPac plasmids was verified by Western blot analysis of SL2 cell nuclear extracts using selective antibodies against Sp1, Sp2, Sp3, and Sp4 (data not shown). Where no antibodies were available (ZNF42, ZNF76, and ZNF143), expression in SL2 cells after transient transfection was confirmed using EMSAs. Binding of expressed ZNF42, ZNF76, and ZNF143, as well as Sp1 and the short and long isoforms of Sp3 to the minimal exon 1c promoter (90/–47) and the respective consensus oligonucleotides (ZNF42, Staf, Sp1) was seen in gel shift assays using nuclear extracts from SL2 cells transiently transfected with the different expression plasmids (pPacSp1, pPac-USp3, pPacSp3, pPacZNF42, pPac-ZNF76, pPac-ZNF143) (data not shown). In contrast to Sp1 and Sp3 isoforms, expressed Sp2 and Sp4 proteins showed no binding to the exon 1c minimal promoter. In addition, there was no binding of expressed Sp2 to the Sp1 consensus oligonucleotide as previously described (26, 27, 30, 39) (data not shown). Control experiments showed that Sp1, Sp2, and Sp3 proteins and ZNF42, ZNF76, and ZNF143 mRNAs are expressed in TGW-nu-I and HeLa cells, enabling
the regulation of nNOS exon 1c promoter activity in mammalian cells (data not shown). In contrast to Sp1, Sp2, Sp3, ZNF76, and ZNF143, mRNA for ZNF42 was not detectable in human brain, skeletal muscle, and rectum by RT-PCR, indicating that ZNF42 plays no role in the transcriptional regulation of nNOS exon 1c in these tissues under the investigated conditions. This is in agreement with previous findings, demonstrating that ZNF42 is a specific transcriptional regulator of myeloid differentiation (24).

In summary, these results identify Sp1, full-length Sp3, ZNF76, and ZNF143 as potent transcriptional activators of the nNOS exon 1c promoter, whereas the short isoforms of Sp3 and ZNF42 exhibit a specific repressive effect on Sp/ZNF-mediated transcriptional activation.

**DISCUSSION**

A variety of human nNOS mRNA variants have been described recently (3, 4, 11–13, 40). Among these, transcripts with different untranslated first exons are generated by alternative promoter usage (12, 13). Real-time quantitative RT-PCR was used to determine the expression patterns and the quantitative distribution of nine alternative first exons of nNOS (exons 1a–1i) (11) and revealed a cell- and tissue-specific expression with exon 1c being one of the predominant variants in human brain, skeletal muscle, rectum, and TGW-nu-I neuroblastoma cells. Because exon 1c is highly expressed in human brain and skeletal muscle, it seems responsible for the largest proportion of nNOS mRNA in the body. Furthermore, nNOS exon 1c mRNA expression is significantly reduced in the pyloric sphincter of patients with infantile hypertrophic pyloric stenosis. Therefore the transcriptional regulation of this variant is of special interest.

To characterize the structure and the expression regulation of nNOS exon 1c, we cloned its genomic 5′-flanking region and analyzed the basal promoter. A 5939-bp genomic 5′-flanking DNA fragment of exon 1c, which contains in addition nNOS exon 1a and exon 1b, was obtained by 5′-RAGE PCR. By 5′ and 3′ deletion analysis of reporter plasmids, using nNOS exon 1c RNA-positive TGW-nu-I, nNOS-negative HeLa and nNOS mRNA-positive, but exon 1c-negative ME-180 cells, the minimal promoter was localized to position −90 to −47, relative to the transcription start site of exon 1c. This region is highly conserved between different species with a sequence homology of 100% between rat (nt 320 to 363 of EMBL accession number AF008911) and man (nt −84 to −41 of exon 1c). Such a high degree of conservation argues for an important, biologically conserved function of this transcriptional control region (41).

Interestingly, nNOS exon 1c reporter plasmids were active in HeLa and ME-180 cells that lack endogenous nNOS exon 1c mRNA. This indicates that cell-specific expression of nNOS exon 1c is mediated by distinct mechanisms that cannot influence transcription of the investigated reporter gene constructs. Such cell-specific transcriptional control mechanisms can be due to repressive cis-acting elements that inhibit transcription in distinct cell types. They are often localized within the upstream 5′-flanking region or within the first intron of a gene, as shown for the growth-associated protein 43 (42). Therefore additional sequences in the far upstream region or in the first intron may mediate cell-specific expression of nNOS exon 1c. In addition the chromatin structure and acetylation state that predicts DNA sequence accessibility plays an important role in cell-specific gene regulation (for review see Ref. 43). Therefore condensation and thus silencing of the nNOS gene could also be responsible for the lack of endogenous nNOS expression in HeLa cells.

To detect transcription factors binding to the basal nNOS exon 1c promoter, EMSAs, including competition and supershift analysis were performed. They identified Sp and ZNF family members of transcription factors as the critical factors regulating the exon 1c −90/−47 basal promoter region. For example, Sp1 and isoforms of Sp3 are binding to the low affinity GC box (GGGAGGGG). Although this motif contains an A in place of the consensus C, it has been demonstrated to bind transcription factors of the Sp family, which activate or repress transcription substantially (30, 41, 44). Because the minimal promoter of nNOS exon 1c is GC-rich, TATA-less, and Sp-regulated, it resembles those for constitutively expressed genes, like dihydrofolate reductase, endothelial Nos, or the serotonin 1a receptor (41, 45–47). The regulation of such promoters is poorly understood. It has been shown that GC-rich promoter regions, lacking a canonical TATA box, can bind Sp1 molecules that interact with multiple components of the transcriptional machinery (for review see Ref. 48), and therefore Sp1 plays a critical role in the assembly of the transcription initiation complex (45). A number of transcription factors has been demonstrated as acting in combination with Sp1 or promoting its displacement from the same or an overlapping site (28, 30, 37, 49). Among these, Sp2, Sp3, and Sp4 belong to the same transcription factor family (27, 28, 30). They share a highly homologous DNA binding domain, but only Sp1, Sp3, and Sp4 recognize the classic Sp1 GC-box in vitro with similar affinities (27, 30, 39). Sp3 mRNA encodes for different isoforms, arising from alternative translation initiation sites (50). These isoforms of Sp3 have been found to exert both activating and inhibiting effects on gene transcription (26, 28, 30, 49–53). In contrast, Sp1 is typically an activator of transcription (30, 48). Because Sp1, Sp2, and isoforms of Sp3 are ubiquitous expressed (28), this could explain why the investigated reporter plasmids under the control of the nNOS exon 1c minimal promoter are expressed in nNOS exon 1c-negative HeLa and ME-180 cells. When the effects of Sp transcription factors on the exon 1c promoter were examined in Drosophila Schneider cells that lack Sp, ZNF76, and ZNF143 binding activity (23, 25, 26, 38), Sp1 and the long isoform of Sp3 potently stimulated transcription, whereas Sp2 and the short isoforms of Sp3 and Sp4 were transcriptionally inactive on their own. Expressed Sp1 and the short and long isoforms of Sp3 were able to bind to the exon 1c minimal promoter region in gel shift assays, whereas the Sp2 and Sp4 proteins did not form protein-DNA complexes. However, an Sp2-specific antibody resulted in a supershift of complex I in EMSAs using TGW-nu-I and HeLa cell nuclear extracts. This suggests that Sp2 is not able to bind to the minimal promoter of exon 1c autonomously. The supershift of the retarded band I in the gel shift assays could be due to protein-protein interactions of Sp2 with other nuclear factors, not present in SL2 cells, resulting in an increase in DNA binding affinity and specificity of Sp2 (52). Such mechanisms have been described for a variety of transcription factors like GATA members and the p65 subunit of NF-κB (37, 54). In cotransfection experiments the transactivating effects of expressed Sp1 and full-length Sp3 were completely additive, whereas Sp2 selectively repressed Sp1-mediated transcriptional activation of the nNOS exon 1c basal promoter. This was a moderate effect with a decrease from a 8.4- to a 6.7-fold induction of luciferase activity. However, this observation demonstrates a specific and differential modulation of promoter transactivation by different members of the Sp family of transcription factors. A similar effect was observed for the combination of short and long isoforms of Sp3, where the short isoform selectively repressed transactivation of the exon 1c basal promoter by full-length Sp3 (decrease from a 13.9- to a 7.5-fold induction). The mode of action could be due to formation or disruption of protein-protein (self-)interactions and multimerization that increase or decrease the DNA binding
affinity of the respective transcription factors or to competition for common DNA recognition sites (30, 37, 49, 50, 52, 54). In contrast to Sp2 and the short isoforms of Sp3, Sp4 did not alter promoter transactivation by Sp1 and the long isoform of Sp3. In addition to Sp transcription factors, we demonstrated that exon 1c promoter activity is regulated by different members of the ZNF family of transcription factors. ZNF76, ZNF42, and ZNF143 specifically bind to the minimal exon 1c promoter in gel shift experiments, and cotransfection of ZNF76 or ZNF143 resulted in a strong induction of the exon 1c promoter in Drosophila Schneider cells, whereas ZNF42 by itself was transcriptionally inert. Combining ZNF and Sp transcription factors in SL2 cells resulted in differential effects on nNOS exon 1c promoter activity. ZNF76 potentiated the stimulatory effects of Sp1 and the long isoform of Sp3, whereas ZNF143 combined with Sp1 showed an additive effect. In contrast, no effect was seen when ZNF143 was coexpressed with the long isoform of Sp3. ZNF42 significantly repressed exon 1c promoter transactivation of ZNF76, ZNF143, and Sp1. These findings demonstrate a differential regulation of the nNOS exon 1c promoter by Sp and ZNF family members of transcription factors and suggest highly coordinated protein-protein interactions and protein-DNA binding to overlapping cis-acting elements. This observation was confirmed by the results of gel shift competition studies. Complete competition of the retarded complex II obtained with TGW-nu-I cell nuclear extracts could be demonstrated only by using a combination of a 100-fold molar excess of unlabelled Sp1 and Staf (ZNF76 and ZNF143 binding site) oligonucleotides, whereas Sp1 or Staf alone were not able to compete this band completely.

The involvement of members of the Sp and ZNF families of transcription factors on transactivation of the basal human nNOS exon 1c promoter was verified by mutating the consensus sequences and transfecting different reporter constructs into HeLa and TGW-nu-I cells. The mutated GC box completely abolished promoter activity of all tested exon 1c reporter plasmids in TGW-nu-I cells and reduced promoter activity by ≈78% or more in HeLa cells. Mutation of the Staf (ZNF76 and ZNF143) binding site decreased relative luciferase activity of the minimal promoter construct (pGL3–90/+49) by ≈53% in TGW-nu-I and ≈37% in HeLa cells. This effect was even more pronounced in the longer promoter constructs pGL3–322/+49 and pGL3–589/+49 with a drop of ≈76 and ≈90%, respectively, in TGW-nu-I cells and ≈68 and ≈60%, respectively, in HeLa cells. Collectively, these data stress the essential role for Sp and Staf binding motifs for the initiation of transcription from the TATA less human nNOS exon 1c promoter.

Little is known about the role of the ZNF transcription factor family members ZNF76 (55) and ZNF143 (56) in transactivation of mRNA promoters. Both transcription factors resemble the Drosophila Kruppel segmentation gene product due to the presence of repeated Cys2-His2 zinc finger domains that are connected by conserved sequences. They contain seven tandemly repeated zinc fingers, showing high homology to the Xenopus laevis transcriptional activator Staf (23), originally identified in Xenopus as the transactivator of the tRNAase gene (38). In addition Xenopus Staf possesses the capacity to stimulate expression from TATA-box containing RNA polymerase II mRNA promoters (23, 57). ZNF76 and 143 are highly identical and functionally equivalent to the X. laevis Staf and, therefore, believed to be the human homologues (23). Both transcription factors are expressed in a broad range of tissues (23), including human brain, skeletal muscle, rectum, TGW-nu-I, and HeLa cells, and are able to bind tightly to Staf consensus-responsive elements present in the X. laevis tRNAase and the human U6 promoter with identical affinities (23). When expressed in Drosophila Schneider cells, lacking ZNF76 and ZNF143 activity (23, 38), both factors activated transcription from the TATA-box containing thymidine kinase mRNA promoter through the Staf binding site (23). Here we demonstrate for the first time that the human homologues of Staf can stimulate transcription from a TATA-less promoter in Drosophila Schneider cells on their own. In addition, we demonstrate a synergistic activation of transcription by combinations of Sp1, full-length Sp3, ZNF76, and ZNF143, whereas ZNF42, Sp2, and the short isoforms of Sp3 repressed transcriptional activation in certain cases. It has been suggested that the cell content of distinct transcription factors is a critical factor influencing the transcription of TATA-less promoters (49). Therefore, differences in the cellular levels of Sp1, Sp2, Sp3 isoforms, ZNF26, ZNF76, and ZNF143 in different cell and tissue types or changes of the cellular levels in response to physiological and pathophysiological conditions (30, 51, 58) could conceivably exert profound effects on the expression of nNOS exon 1c. This adds another level of complexity and cell specificity to the already intricate regulation of nNOS gene expression resulting from the use of multiple promoters (3, 4, 10, 12, 13, 40).

In conclusion, our results demonstrate that nNOS exon 1c is the predominant nNOS mRNA variant in the body. The minimal exon 1c promoter could be localized to 44 bp, with a cooperative binding of several transcription factors of the Sp and ZNF families. This portends many possibilities for tissue- and cell-specific control in response to cellular requirements.

Recent findings support a complex and tightly regulated gene expression of nNOS, showing profound changes in different physiological and pathophysiological states (1–4, 14, 16, 17, 21, 35). However, the causal mechanisms are unknown, and therefore the results of this study could provide insight into the roles of changes of nNOS gene transcription, including possible therapeutical strategies for increasing nNOS gene expression.

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