Bigenomic transcriptional regulation of all thirteen cytochrome c oxidase subunit genes by specificity protein 1

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1. Summary

Cytochrome c oxidase (COX) is one of only four known bigenomic proteins, with three mitochondria-encoded subunits and 10 nucleus-encoded ones derived from nine different chromosomes. The mechanism of regulating this multi-subunit, bigenomic enzyme is not fully understood. We hypothesize that specificity protein 1 (Sp1) functionally regulates the 10 nucleus-encoded COX subunit genes directly and the three mitochondrial COX subunit genes indirectly by regulating mitochondrial transcription factors A and B (TFAM, TFB1M and TFB2M) in neurons. By means of in silico analysis, electrophoretic mobility shift and supershift assays, chromatin immunoprecipitation, RNA interference and over-expression experiments, the present study documents that Sp1 is a critical regulator of all 13 COX subunit genes in neurons. This regulation is intimately associated with neuronal activity. Silencing of Sp1 prevented the upregulation of all COX subunits by KCl, and over-expressing Sp1 rescued all COX subunits from being downregulated by tetrodotoxin. Thus, Sp1 and our previously described nuclear respiratory factors 1 and 2 are the three key regulators of all 13 COX subunit genes in neurons. The binding sites for Sp1 on all 10 nucleus-encoded COX subunits, TFAM, TFB1M and TFB2M are highly conserved among mice, rats and humans.

2. Introduction

Neurons are highly dependent on ATP for their activity and functions [1,2]. Approximately 90 per cent of ATP generated in the brain is synthesized in the mitochondria via oxidative phosphorylation [3]. Eukaryotic cytochrome c oxidase (COX) is the terminal enzyme of the energy-transducing mitochondrial electron transport chain and is embedded in the inner mitochondrial membrane, where it mediates the transfer of electrons from reduced cytochrome c to molecular oxygen. COX also engages in proton pumping in the establishment of an electrochemical gradient for the synthesis of ATP. COX is a complex of 13 subunits; the largest three are encoded in the mitochondrial DNA and the remaining 10 are encoded in the nuclear genome [4]. To form a functional holoenzyme with 1 : 1 stoichiometry of all 13 subunits, exact coordination is essential between the two genomes. The mechanism of regulating this ancient, multi-subunit, bigenomic enzyme critical for cell survival is only beginning to be probed.

Two redox-responsive transcription factors, nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), have been found to be mediators of such bigenomic coordination in neurons. Specifically, they both regulate the expression...
Table 1. EMSA probes. Positions of probes are given relative to TSP. Putative Sp1-binding sites are in boldface. Mutated nucleotide sequences are underlined.

| gene          | position | sequence                                      |
|---------------|----------|-----------------------------------------------|
| COX4i1        | +23/ +43 | F: 5'-TTTGGTGTGTAAGGGCGGCTGCGG-3'             |
|               |          | R: 5'-TTTGGGCGAGCGCGTCCAAC-3'                 |
| COX5a         | -20/ +2  | F: 5'-TTTCTTATATCAGGAGCGGGA-3'                |
|               |          | R: 5'-TTTGGGCGAACGCATTACGACAG-3'              |
| COX5b         | +40/ +59 | F: 5'-TTTGGGAAGGGGGCGGATCCG-3'                |
|               |          | R: 5'-TTTGGCAAGATTGGGGCGACTCC-3'              |
| COX6a1        | -53/ -33 | F: 5'-TTTCTTATATCAGGAGCGGGA-3'                |
|               |          | R: 5'-TTTGGGCGAACGCATTACGACAG-3'              |
| COX6b         | -81/ -61 | F: 5'-TTTGGGAAGGGGGCGGATCCG-3'                |
|               |          | R: 5'-TTTGGCAAGATTGGGGCGACTCC-3'              |
| COX6c         | +185/ +204 | F: 5'-TTTGTAGTGGCGCAAGAGCA-3'                  |
|               |          | R: 5'-TTTGGGGCGGCGAGGCAGA-3'                  |
| COX7a2        | -47/ -28 | F: 5'-TTTGGGCAAGGGCGGAGGCGAAGTGG-3'           |
|               |          | R: 5'-TTTGGCGAACGCATTACGACAG-3'               |
| COX7b         | +183/ +202 | F: 5'-TTTCTATATGGGGAGGCGGATCCG-3'             |
|               |          | R: 5'-TTTGGCAAGATTGGGGCGACTCC-3'              |
| COX7c         | -48/ -29 | F: 5'-TTCAGAGGGGGGCGGATCCG-3'                 |
|               |          | R: 5'-TTTGGCAAGATTGGGGCGACTCC-3'              |
| COX8a         | -49/ -30 | F: 5'-TTTGGGCGGCGAGGCGGAGGCC-3'               |
|               |          | R: 5'-TTTGGGGCGGCGAGGCGGAGGCC-3'              |
| Sp-1 mutant (COX4i1) | +23/ +43 | F: 5'-TTTGGGCAAGGGCGGAGGCGAAGTGG-3'           |
| GM3 synthase  | -58/ -38 | F: 5'-TTTGGGCGGCGAGGCGGAGGCC-3'               |
|               |          | R: 5'-TTTGGGGCGGCGAGGCGGAGGCC-3'              |

of all 10 nucleus-encoded COX subunit genes [5–7], and indirectly regulate the three mitochondria-encoded COX subunit genes by activating mitochondrial transcription factors A and B (TFAM, TFB1M and TFB2M) [8,9]. Moreover, both NRF-1 and NRF-2 respond to and are regulated by neuronal activity, and changes in their mRNA and protein levels precede those of COX [6,10–12]. Besides NRF-1 and NRF-2, specificity protein 1 (Sp1) has been postulated to bind to the promoters of a few specificity protein 1 (Sp1) has been postulated to bind to the promoters of a few COX subunit genes [13–20] and TFAM [21,22]. However, none of these putative sites has been functionally characterized. The goal of the present study was to test our hypothesis that Sp1 is another biegomonic coordinator that regulates all COX subunit genes.

By means of multiple approaches, in silico analysis, electrophoretic mobility shift and supershift assays, chromatin immunoprecipitation (ChIP), RNA interference and over-expression experiments, we document in this study that Sp1 functionally regulates all 13 COX subunit genes in neurons.

3. Results

3.1. In silico promoter analysis

Proximal promoters of murine nucleus-encoded COX subunits (4i1, 5a, 5b, 6a1, 6b, 6c, 7a, 7b, 7c and 8a), TFAM, TFB1M and TFB2M genes with DNA sequence 1 kb 5′ upstream and 500 bps beyond 3′ of transcription start points (TSPs) were analysed in silico for potential Sp1-binding sites (table 1). Promoters for COX4i1, 5a, 5b, 6a1, 6b, 7a, 7c and 8a showed a typical Sp1 sequence motif ‘GGGCGG’ or ‘CCCGCC’, whereas COX6b and 7b had an atypical sequence of ‘GGGCGT’ or ‘GGGCGG’.

3.2. In vitro binding of specificity protein 1 to promoters

In vitro electrophoretic mobility shift assays (EMSAs) were carried out using 32P-labelled probes (table 1) to determine the specificity of Sp1 binding to promoters of murine COX subunit genes (figure 1a–c). GM3 synthase promoter with a known Sp1-binding site at position –34/ –35 served as a positive control [23] and it formed specific DNA/Sp1 shift and supershift complexes (figure 1a, lanes 1 and 2, respectively). When an excess of unlabelled probe was added as a competitor, no shift band was formed (figure 1a, lane 3). When labelled oligonucleotides were incubated with Sp1 antibody without HeLa nuclear extract, neither shift nor supershift band was observed (figure 1a, lane 7 for Cox4i1-specific oligo), ruling out non-specific antibody–oligo interactions. As shown in figure 1a–c, all 10 murine COX promoters formed specific DNA/protein shift complexes when incubated with purified HeLa nuclear extract (figure 1a, lanes 4, 9 and 12; figure 1b, lanes 1, 4, 7
had no effect (figure 1 lanes 3, 6 and 9, respectively). Sp1 mutant showed neither and supershift assays. 32P-labelled oligonucleotides, excess unlabelled oligos with Sp1 antibodies alone with no HeLa extract did not yield any band body complex for each of the subunits (figure 1 ((

When excess unlabelled competitor was added, it did not yield any band 

Excess unlabelled but mutated Sp1 site could not compete (a), lane 3). All 10 COX subunit promoter sequences with putative Sp1-binding site showed specific shift and supershift bands that were eliminated by excess unlabelled competitors (a), all lanes and (c), lanes 1–9). Excess unlabelled but mutated Sp1 site could not compete (a), lane 6). Labelled oligos with Sp1 antibodies alone with no HeLa extract did not yield any band (a), lane 7). Labelled mutated Sp1 site on COX4I1 was used as a negative control and it did not yield any band ((c), lanes 10–12).

and 10 and figure 1c, lanes 1, 4 and 7, respectively). These complexes were displaced by competition with excess unlabelled probes (figure 1a, lanes 5, 10 and 13; figure 1b, lanes 2, 5, 8 and 11 and figure 1c, lanes 2, 5 and 8, respectively), but was not displaced with unlabelled mutant Sp1 probe (figure 1a, lane 6). Supershift assays using anti-Sp1 antibodies produced a supershift band of DNA/Sp1/antibody complex for each of the subunits (figure 1a, lanes 8, 11 and 14; figure 1b, lanes 3, 6, 9 and 12 and figure 1c, lanes 3, 6 and 9, respectively). Sp1 mutant showed neither shift nor supershift complexes, and unlabelled oligos also had no effect (figure 1c, lanes 10, 12 and 11, respectively).

3.3. In vivo occupancy of the promoters by specificity protein 1

ChIP assays were performed to verify possible Sp1 binding to all 10 COX promoters in vivo. β-actin exon 5 (Actb) served as a negative control, whereas GM3 synthase with a known Sp1-binding site [23] served as a positive control. As a negative control, another immunoprecipitation from the same stock of cell lysate was done using anti-nerve growth factor receptor (NGFR) antibodies. Polymerase chain reactions (PCRs) targeting regions of 10 COX subunit promoters surrounding putative Sp1-binding sites were carried out in parallel on chromatin immunoprecipitated from N2a cells. A 0.5 per cent dilution of input chromatin (i.e. prior to immunoprecipitation) was used as a standard to indicate the efficiency of the PCRs.

The proximal promoters of all 10 nucleus-encoded COX subunits (4I1, 5a, 5b, 6aI, 6b, Actb, 7a2, 7b, 7c and 8a promoters revealed that all 10 COX promoter DNA coding genes were co-immunoprecipitated with Sp1. Mitochondrial transcription factors (TFAM, TFB1M and TFB2M) also co-immunoprecipitated with Sp1 antibody. Reactions targeting GM3 synthase promoter was used as a positive control, and β-actin (Actb) was used as a negative control.

3.4. Effect of Sp1 knock-down

Transfection with vectors containing Sp1 shRNA resulted in an 83 per cent decrease in the level of Sp1 mRNA (p < 0.001, figure 3b) and an 82 per cent decrease in Sp1 protein (p < 0.001, figure 3a). Silencing of Sp1 led to a decrease in the mRNAs of the three mitochondrial COX subunits (p < 0.001 for all, figure 3b), three mitochondrial transcription factors (p < 0.001 for all, figure 3b) and 10 nucleus-encoded COX subunits (p < 0.001 for all, figure 3b) relative to scrambled vectors. Silencing of Sp1 also led to a 55 per cent decrease in the protein level of COxl

Figure 1. In vitro binding of Sp1 on COX subunit genes as measured with EMSA and supershift assays. 

32P-labelled oligonucleotides, excess unlabelled oligos specific for each promoter as competitors, excess unlabelled mutant Sp1 as competitors, HeLa extract and Sp1 antibodies are indicated by a + or a − sign. Arrowheads indicate Sp1 shift and supershift complexes. The positive control, GM3 synthase, shows specific shift and supershift bands that were eliminated by excess unlabelled competitors (a), lane 1) and supershift ((a), lane 2) bands. When excess unlabelled competitor was added, it did not yield any band (a), lane 3). All 10 COX subunit promoter sequences with putative Sp1-binding site showed specific shift and supershift bands that were eliminated by excess unlabelled competitors (a), lanes 9–14; (b), all lanes and (c), lanes 1–9). Excess unlabelled but mutated Sp1 site could not compete (a), lane 6). Labelled oligos with Sp1 antibodies alone with no HeLa extract did not yield any band (a), lane 7). Labelled mutated Sp1 site on COX4I1 was used as a negative control and it did not yield any band ((c), lanes 10–12).

Figure 2. In vivo ChIP assays for Sp1 binding to nucleus-encoded COX subunit and mitochondrial transcription factor (TFAM, TFB1M and TFB2M) promoters. PCR reactions were performed on N2a cell chromatin precipitated with anti-Sp1 antibodies (Sp1 lanes) or anti-nerve growth factor receptor p75 antibodies (negative control, NGFR lanes). Control reactions were performed with input chromatin (input lanes). PCR products targeting COX4I1, 5a, 5b, 6aI, 6b, 6c, 7a2, 7b, 7c and 8a promoters revealed that all 10 COX promoter DNAs co-immunoprecipitated with Sp1. Mitochondrial transcription factors (TFAM, TFB1M and TFB2M) also co-immunoprecipitated with Sp1 antibody. Reactions targeting GM3 synthase promoter was used as a positive control, and β-actin (Actb) was used as a negative control.
3.5. Effect of Sp1 over-expression

Transfection with vectors over-expressing Sp1 led to an 80-fold increase in Sp1 mRNA \((p < 0.001, \text{figure 4b})\) and a fivefold increase in Sp1 protein \((p < 0.001, \text{figure 4d})\). Over-expression of Sp1 caused an increase in the mRNAs of three mitochondrial COX subunits \((p < 0.001 \text{ for all, figure 4b})\), three mitochondrial transcription factors \((p < 0.001 \text{ for all, figure 4b})\) and 10 nuclear-encoded COX subunits \((p < 0.001 \text{ for all, figure 4b})\). Over-expression of Sp1 also led to a 55 per cent increase in the protein level of Cox1 \((p < 0.001, \text{figure 4a})\) and a 40 per cent increase in the level of Cox4i1 protein \((p < 0.01, \text{figure 4a})\).

3.6. Effect of depolarization and impulse blockade

To determine if the expression of COX subunit genes was affected by depolarizing stimulation, cells were subjected to 20 mM of KCl for 5 h after incorporating Sp1 shRNA. The KCl regimen was found previously to activate COX gene expression in primary neurons [10,11]. As shown in figure 5, depolarization stimulation resulted in a significant increase in the mRNA levels of Sp1 \((p < 0.001, \text{figure 5a})\), three mitochondrial COX subunits \((p < 0.001 \text{ for all, figure 5a})\), three mitochondrial transcription factors \((p < 0.001 \text{ for all, figure 5a})\) and 10 nucleus-encoded COX subunits \((p < 0.001 \text{ for all, figure 5a})\). With Sp1 silencing, both mitochondria-encoded and nucleus-encoded COX subunits as well as the three mitochondrial transcription factors failed to respond to KCl.

Tetrodotoxin (TTX) at 0.4 \(\mu\)M has been shown to decrease the levels of COX subunit mRNAs as well as COX enzyme activity \textit{in vivo} and in primary neurons [24–26]. To evaluate the effect of impulse blockade on COX subunits, N2a cells were treated with TTX for 3 days. TTX reduced the mRNA levels of Sp1 \((p < 0.001, \text{figure 5b})\), three mitochondrial COX subunits \((p < 0.001 \text{ for all, figure 5b})\), three mitochondrial transcription factors \((p < 0.001 \text{ for TFAM and TFB1M}; p < 0.05 \text{ for TFB2M, figure 5b})\).
and 10 nucleus-encoded COX subunits \( (p < 0.001) \) for COX5a, 6a1, 7a2, 7c and 8a; \( p < 0.01 \) for COX6a1, 5b, 6b and 7b; \( p < 0.05 \) for COX6c; figure 5b). This indicates an overall suppressive effect of TTX on all COX subunit gene expression in neurons. On the other hand, reductions in the mRNA levels of Sp1 \( (p < 0.001, \) figure 5b), three mitochondrial COX subunits \( (p < 0.001 \) for all), three mitochondrial transcription factors \( (p < 0.001 \) for all, figure 5b) and 10 nucleus-encoded COX subunits \( (p < 0.001 \) for all, figure 5b) were rescued in cells that over-expressed Sp1 (figure 5b).

3.7. Homology of specificity protein 1-binding sites

The functional Sp1-binding sites on 10 nucleus-encoded COX subunits, TFAM, TFB1M and TFB2M are conserved among humans, mice and rats (figure 6). Sp1 sites had a relatively high degree of homology (60–100%; mostly 80–90%) among the three species.

4. Discussion and conclusions

Using multiple molecular and biochemical approaches, including in silico analysis, electrophoretic mobility shift and supershift assays, ChIP assays, RNA interference and over-expression assays, we documented that Sp1 transcription factor regulates all 10 nucleus-encoded COX subunit genes directly and three mitochondrial-encoded COX genes indirectly by regulating the three transcription factors important in the transcription of mitochondrial DNA. Knocking down Sp1 resulted in a significant reduction in the expression of all 10 nucleus-encoded and three mitochondria-encoded COX subunit genes, and TFAM, TFB1M and TFB2M genes in neurons. Over-expression of Sp1 resulted in a significant upregulation of Sp1 itself, all 10 nucleus-encoded and three mitochondria-encoded COX subunit genes, and TFAM, TFB1M and TFB2M genes in neurons. The expression of all COX subunits and their transcription factor, Sp1, are tightly coupled to neuronal activity. The Sp1-binding sites on promoters of nuclear COX subunit, TFAM, TFB1M and TFB2M genes are conserved among mice, rats and humans. It is noteworthy that COX6b and 7b had an atypical Sp1-binding motif (‘GGCGGT’ and ‘GGCGCA’, respectively), whereas other COX promoters as well as those for TFAM, TFB1M and TFB2M all had a typical Sp1-binding sequence.

Sp1 is the first C\( _{2}\)H\( _{2}\)-type zinc finger transcription factor to be isolated and cloned from mammalian cells. Sp1 recognizes CG-rich sequences in CpG islands and was initially recognized as a constitutive transcription activator of housekeeping genes and other TATA-less genes. Sp1 is ubiquitously expressed, and at least 12 000 Sp1 typical binding sites have been found in the human genome, most of which have been associated with genes involved in many cellular functions [27–30]. Knock-out of Sp1 is embryonically lethal [27,28]. Earlier studies have shown the presence of Sp1-binding sites on the promoter regions of some COX subunit genes. These include COX4I in humans, mice and rats [14,31,32], COX5b in mice and humans [16,18,20] and COX6a1 in rats [13]. Putative Sp1-binding sites have also been reported on nucleus-encoded TFAM gene [21,22]. However, functional characterization has not been done on any of these sites. The present study documented that Sp1 bigenomically regulates all 13 COX subunit genes, making it an important regulator of genes involved in the
production of cellular energy. The expression of Sp1 itself is tightly regulated by neuronal activity. Likewise, the regulation of all COX subunit genes and mitochondrial transcription factors is strongly dictated by neuronal activity, consistent with our earlier studies [10,11,24,26]. Depolarizing stimulation failed to increase their expression in the presence of Sp1 siRNA, and Sp1 over-expression rescued their downregulation induced by impulse blockade. These findings substantiate the important role of Sp1 in regulating COX subunit genes in an activity-dependent manner.

COX is a critical terminal enzyme of the mitochondrial electron transport chain, without which oxidative metabolism cannot be carried to completion and ATP cannot be generated [1,2,4]. The three mitochondria-encoded COX subunits (COX1, COX2 and COX3) form the catalytic core of the enzyme, whereas the 10 nucleus-encoded subunits reportedly serve modulatory functions [33,34]. COXIV binds ATP directly and regulates COX activity based on the intra-mitochondrial ATP/ADP ratio [35,36]. COX5b and COX6a are also involved in the regulation of COX activity [37,38]. COX5a has a binding site for 3,5-diiodothyronine (T2) and relieves the inhibition of COX enzyme induced by intra-mitochondrial ATP [39]. COX6b is involved in the stabilization of the dimeric COX enzyme, and Cox6c provides a low-affinity binding site for cytochrome c [40]. Three of the nuclear subunit proteins (6a, 7a and 8a) have tissue-specific isoforms and presumably regulate COX activity in different tissues based on tissue-specific requirements [33,41]. The functions of subunits Cox7a, 7b, 7c and 8a are not known with certainty.

The mechanism of regulating this multi-subunit, bigenomic and multi-chromosomal protein poses a major challenge. Recently, we demonstrated that all 10 nucleus-encoded subunits of COX are regulated by the transcription factors NRF-1 and NRF-2 [5–7]. Both factors also regulate indirectly the three mitochondria-encoded COX subunit genes by activating TFAM, TFB1M and TFB2M important for mitochondrial DNA transcription and replication [8,9]. The present study revealed that Sp1 is the third transcription factor known thus far to functionally regulate all 10 nucleus-encoded and three mitochondrial-encoded COX subunit genes in neurons in vitro and in vivo. Moreover, our recent study showed that all of the nucleus-encoded COX subunit transcripts and those for TFAM, TFB1M and TFB2M are all transcribed in the same transcription factory [42].

The coordinated regulation of all 13 COX subunit genes is likely to be complex and multifactorial, requiring various transcription factors and coactivators. The mechanism of coordinating multiple transcription factors can be complementary, concurrent and parallel or a combination of complementary and concurrent/parallel. In the case of NRF-1 and NRF-2, our recent study has shown that both NRF-1 and NRF-2 regulate these genes in a parallel and concurrent manner [43]. Moreover, double knock-down of NRF-1 and Sp1 or of NRF-2 and Sp1 in primary neurons affected cell survival (Dhar and Wong-Riley 2012, unpublished data), indicating important roles of these factors in activating key metabolic genes that regulate cell growth, functioning and survival. Sp1-binding sites are in close proximity to those of NRF-1 and NRF-2 (Dhar and Wong-Riley 2012, unpublished data). Thus, Sp1 may physically interact with NRF-1 and/or NRF-2 in regulating some or all of the COX subunit genes. Future studies will be directed at evaluating such possible protein–protein interactions and possible colocalization of these factors in the same or different transcription factories. In addition to transcription factors, a transcriptional coactivator, peroxisome proliferator-activated receptor gamma coactivator 1a (PGC-1a), is also likely to be involved in the regulation of COX in neurons. PGC-1a coactivates NRF-1 and NRF-2 in the control of mitochondrial biogenesis [44,45] and its response to changes in neuronal activity precedes those of NRF-1 and NRF-2 [10,46].

Figure 6. Aligned partial sequences of promoters for nucleus-encoded COX subunits and mitochondrial transcription factors (TFAM, TFB1M and TFB2M) from mice (M), rats (R) and humans (H). Conserved putative Sp1-binding sites are shown in solid boxes. Sp1-binding sites in mice are shown in bold and underlined.
In conclusion, our multi-faceted investigation revealed that Sp1, in addition to NRF-1 and NRF-2, is a critical regulator of all 13 COX subunit genes in neurons ([5–7], the present study).

5. Experimental procedures

5.1. In silico analysis of promoters of murine COX subunit genes

DNA sequences surrounding the TSPs of all 10 nucleus-encoded COX subunit genes, and TFEAM, TFB1M and TFB2M were derived from the mouse, rat and human genome database in GenBank. These promoter sequences encompassed 1 kb upstream and up to 500 bps downstream (excluding protein-coding sequence) of the TSP of each subunit gene analysed. These sequences were aligned using MEGALIGN, DNASTAR LASERGENE software. Putative Sp1-binding sequences ‘GGGCGG’ or ‘CCCGCC’ were searched using DNASTAR LASERGENE software. Regions of high homology were selected for experimental analyses.

5.2. Cell culture

Murine neuroblastoma (N2a) cells (ATCC, Manassas, VA, USA) were grown in Dulbecco’s modified Eagle’s medium supplemented with 50 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin (Invitrogen, Carlsbad, CA, USA) and 10 per cent foetal bovine serum (Sigma, St Louis, MO, USA) at 37°C in a humidified atmosphere with 5 per cent CO₂.

5.3. Electrophoretic mobility shift and supershift assays

EMSAs to assay possible binding of Sp1 on all 10 COX subunit promoters were carried out with methods as described previously [7]. Briefly, oligonucleotide probes with putative Sp1-binding site on each promoter (table 1, based on in silico analysis) were synthesized, annealed and labelled by a Klenow fragment (Invitrogen) fill-in reaction with [α⁻³²P] dATP (50 μCi/200 ng; Perkin-Elmer, Shelton, CT, USA). Each labelled probe was incubated with 2 μg of calf thymus DNA and 5 μg of HeLa nuclear extract (Promega, Madison, WI, USA) and processed for EMSA. Supershift assays were also performed, and in each reaction, 1–1.5 μg of Sp1-specific antibodies (polyclonal goat antibodies, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the probe/nuclear extract mixture and incubated for 20 min at room temperature. For competition, 100-fold excess of unlabelled oligonucleotides were incubated with nuclear extract before adding labelled oligonucleotides. Shift reactions were loaded onto 4 per cent polyacrylamide gel and run at 200 V for 2.5 h in 0.25× Tris/borate/EDTA (TBE) buffer. Results were visualized by autoradiography. Mouse GM3 synthase with Sp1-binding site at position −34/−55 was designed as previously described [23] and used as a positive control. Sp1 mutants with mutated sequences as shown in table 1 were used as negative controls.

5.4. Chromatin immunoprecipitation assays

ChIP assays were performed similar to those described previously [7]. Briefly, approximately 750 000 N2a cells were used for each immunoprecipitation and were fixed with 1 per cent formaldehyde for 10 min at room temperature. ChIP assay kits (Upstate, Charlottesville, VA, USA) were used with minor modifications. Following formaldehyde fixation, cells were resuspended in a swelling buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 1% NP-40 and protease inhibitors added right before use) and homogenized 10 times in a small pestle Dounce tissue homogenizer (7 ml). Nuclei were then isolated by centrifugation before being subjected to sonication. The sonicated lysate was immunoprecipitated with either 0.2 μg of Sp1 polyclonal rabbit antibodies (H-225, sc-14027; Santa Cruz Biotechnology) or 2 μg of NGFR p75 polyclonal goat antibodies (C20, sc-6188; Santa Cruz Biotechnology). Semi-quantitative PCR was performed using 1/20th of precipitated chromatin. Primers targeting promoter sequences near TSP of COX subunit genes were designed (table 2). Mouse GM3 synthase promoter previously found to have Sp1 binding [23] was used as a positive control, and exon 5 of β-actin gene was used as a negative control (table 2). PCR reactions were carried out with the EX Taq hot-start polymerase (Takara Mirus Bio, Madison, WI, USA) with the following cycling parameters: 30 s denaturation at 94°C, 30 s annealing at 59.5°C and 20 s extension at 72°C (32–36 cycles per reaction). All reactions were hot-started by heating to 94°C for 120 s. Use of hot-start polymerase and PCR additives significantly improved the quality and reproducibility of ChIP. PCR products were visualized on 2 per cent agarose gels stained with ethidium bromide.

5.5. Knock-down of Sp1 with shRNA and KCl treatment

Sp1 shRNA plasmids for mouse were obtained from Santa Cruz Biotechnology (sc-29488-SH) as a pool of three target-specific lentiviral vectors with H1 promoter and puromycin resistance. Each vector encompassed 19–25 nt (plus hairpin) shRNAs designed to knock-down gene expression of Sp1. Control shRNA plasmid-A (Santa Cruz Biotechnology; sc-108062) or scrambled shRNA served as negative controls. N2a cells were plated in 60-mm dishes at a density of 5 to 8 × 10⁵ cells/dish. They were cotransfected 3 days post-plating with Sp1 shRNA at 1 μg per dish via Lipofectamine 2000. Empty vectors or scrambled shRNA vectors alone were used at the same concentrations as vectors with shRNA against Sp1. Puromycin at a final concentration of 0.5 μg ml⁻¹ was added to the culture medium on the second day after transfection to select for purely transfected cells. Green fluorescence was observed to monitor transfection efficiency. Transfection efficiency for N2a cells ranged from 40 to 75 per cent. Transfected cells were selected by the addition of puromycin. This antibiotic selection yielded essentially 100 per cent transfected cells. After 48 h of transfection, cells were stimulated with 20 mM KCl in the culture media for 5 h as previously described [10,11]. Cells were harvested and processed for the isolation of RNAs and proteins.

5.6. Sp1 over-expression and tetrodotoxin treatment

The human Sp1 cDNA clones were obtained from Open Biosystems (Lafayette, CO, USA). The Sp1 cDNA was cloned into pcDNA Dest40 vector using Gateway Multisite Cloning kit (Invitrogen) and according to the manufacturer’s instructions. N2a cells were plated in six-well plates at a density of 5 to 8 × 10⁶ cells per well. They were transfected 1 day post-plating with 2 μg Sp1-containing plasmids using JetPrime
reagent (Polyplus Transfection, New York, NY, USA). Empty vectors were used as a control and at the same concentration as vectors with Sp1 insert. Geneticin 500 mg ml⁻¹ (Invitrogen) was added to the culture medium on the second day after transfection for the selection of transfected cells. Transfection efficiency for N2a cells ranged from 65 to 75 per cent. Transfected cells were selected using 250 mg ml⁻¹ Geneticin. This antibiotic selection yielded essentially 100 per cent transfected cells. N2a cells transfected with Sp1 were treated with 0.4 mM TTX in the culture media for 3 days. Cells were harvested and processed for the isolation of RNAs and proteins.

5.7. RNA isolation and cDNA synthesis

Total RNA was isolated by RNeasy kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Three micrograms of total RNA were treated with DNase I and purified by phenol–chloroform. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions.

5.8. Real-time quantitative polymerase chain reaction

Real-time quantitative PCRs were carried out in a Cepheid Smart Cycler Detection system (Cepheid, Sunnyvale, CA, USA). SyBr Green (BioWhittaker Molecular Application, Rockland, ME, USA) and EX Taq real-time quantitative PCR hot-start polymerase were used following the manufacturer’s protocols and as described previously [7]. Primer sequences are shown in table 3. PCR runs: hot start 2 min at 95°C, denaturation 10 s at 95°C, annealing 15 s according to the Tm of each primer and extension 10 s at 72°C for 15–30 cycles. Melt curve analyses verified the formation of single desired PCR product. Rat 18S was used as an internal control, and the 2⁻¹ΔΔCT method [47] was carried out for the relative amount of transcripts.

Table 2. Primers for ChIP assays. Positions of amplicons are given relative to TSP.

| gene     | sequence F: | sequence R: | amplicon length (bp) |
|----------|-------------|-------------|----------------------|
| COX4i1   | 5'-GAAAAAGTCTGCCGGAAG-3' | 5'-GTACCTGCAAGGGCGAG-3' | 284                  |
| COX5a    | 5'-CCACCAGGAAATGTTACTA-3' | 5'-AGCGAGCGGCGGTGAG-3' | 237                  |
| COX5b    | 5'-GGCTGTGTAGACCTCCCA-3' | 5'-AGCTGTAGTACCTCCAG-3' | 200                  |
| COX6a1   | 5'-GCTGACAGCAGGGAGATG-3' | 5'-GACCCATCAGGGAACTCA-3' | 215                  |
| COX6b    | 5'-GCCCGACCAAAATAAAAGGA-3' | 5'-TAGCAAAAGCGCAATATGCA-3' | 196                  |
| COX6c    | 5'-GTTGAAGGCTGAGGAGAGA-3' | 5'-GCAAATACAGGGGGAACG-3' | 299                  |
| COX7a2   | 5'-GCTTTCCTTCCATGTGATT-3' | 5'-AAACGCAACCTCCTCTACG-3' | 219                  |
| COX7b    | 5'-GATGTCCTTCCGCTACAGAA-3' | 5'-CTAGCTTCCCTCCCAGTGA-3' | 194                  |
| COX7c    | 5'-ACATTTCCACAAATCTCATG-3' | 5'-ATGCCGTACACCTACTC-3' | 189                  |
| COX8a    | 5'-TGGGACAAAGTGTCCTCAT-3' | 5'-GCCAGGTCTGACACGA-3' | 213                  |
| TFAM     | 5'-GTTGCAACAGGGCGGAC-3' | 5'-CACGATCAGACGGGAATCC-3' | 331                  |
| TFB1M    | 5'-CTGAAAGAATTAATGCGAGG-3' | 5'-GGGACGCTTGGGAAAGAG-3' | 340                  |
| TFB2M    | 5'-GGAGTCGGACACCTCTACG-3' | 5'-GATTTCACCAACCACTA-3' | 290                  |
| GM3 synthase | 5'-CACATCTCTCCTGCAGGAG-3' | 5'-AATTCACCCGGAGAATG-3' | 198                  |
| β-actin exon 5 | 5'-CTCTTCTCCAGCCCTTC-3' | 5'-CAGATGTAAGCGTGAAGT-3' | 187                  |
5.9. Western blot

Proteins from Sp1 shRNA and over-expression samples along with appropriate controls were loaded onto 10 per cent SDS-PAGE gel and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad). Subsequent to blocking, blots were incubated in primary antibodies against Sp1 (1:1000; Santa Cruz), COX1 (1:1000; Molecular Probes, Life Technologies) and polyclonal antibodies that recognize mainly COX4i1 (1:1000) [48]. β-Actin (1:3000; Sigma) served as a loading control. Secondary antibodies used were goat anti-rabbit and goat anti-mouse antibodies (Vector Laboratories, Burlingame, CA, USA). Blots were then reacted with the enhanced chemiluminescent (ECL) reagent (Pierce, Rockford, IL, USA) and exposed to autoradiographic film (RPI, Mount Prospect, IL, USA). Quantitative analyses of relative changes were done with an Alpha Imager (Alpha Innotech, San Leandro, CA, USA).

5.10. Statistical analysis

Significance among group means was determined by analysis of variance (ANOVA). Significance between two groups was analysed by Student’s t-test. p-values of 0.05 or less were considered significant.

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