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Involvement of Cytochrome c Oxidase Subunit I Gene during Neuronal Differentiation of PC12 Cells

Hyojung Kang, Junmo Chung and Seewoo Lee[1]*

Department of Biology, Ewha Womans University, Seoul 120-750, Korea
[1]Basic Science Division, Samsung Biomedical Research Institute, Seoul 135-230, Korea
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Abstract : It is becoming increasingly evident that significant changes in gene expression occur during the course of neuronal differentiation. Thus, it should be possible to gain information about the biochemical events by identifying differentially expressed genes in neuronal differentiation. The PC12 cell line is a useful model system to investigate the molecular mechanism underlying neuronal differentiation and has been used extensively for the study of the molecular events that underlie the biological actions of nerve growth factor (NGF). In this study, we report an application of the recently described mRNA differential display method to analyze differential gene expression during neuronal differentiation. Using this technique, we have identified several cDNA tags expressed differentially during neuronal differentiation. Interestingly, one of these clones was cytochrome c oxidase subunit I (COX I) gene. The differential expression of COX I gene was confirmed by Northern blot analysis as well as RT-PCR. Southern blot analysis of the genomic DNA of PC12 cells revealed that COX I is a single gene. Induction of the oxidative enzyme might reflect the energy requirement in neuronal differentiation.
Keywords : cytochrome c oxidase, nerve growth factor, PC12 cells, PCR-based differential display

Neuronal differentiation is a complex process depending on both extracellular and intracellular cues. *In vitro* and *in vivo* studies have shown that epigenetic factors, such as transcriptional factors and peptide growth factors, trigger distinct pathways of signal transduction in the target cells (Brown, 1984; Cross and Dexter, 1991). Although the molecular basis for neuronal differentiation has been the focus of extensive investigation, many of the proteins that are uniquely expressed in specific stages of differentiation remain to be isolated. PC12 cells, derived from a rat pheochromocytoma, provide a useful model system to study cell differentiation. This cell line has characteristics of precursor cells for both sympathetic neurons and chromaffin cells. PC12 cells acquire a neuronal phenotype on incubation with neutrophic factors. Nerve growth factor (NGF), among the best characterized, regulates survival, differentiation, and neurotransmitter production of sympathetic neurons and some sensory neurons in the peripheral nervous system and of cholinergic neurons of basal forebrain ganglia in the central nervous system (Levi-Montalcini, 1987; Barde, 1989). The specific receptor for NGF, gp140^{trk}, belongs to the tyrosine kinase receptor family. NGF receptors stimulate a cascade of intracellular signals that control biochemical pathways leading to changes in gene expression (Schlessinger and Ullrich, 1992). This activation leads to a coordinated induction of genes, thought to be ultimately responsible for attainment of the differentiated phenotype (Guardavaccaro *et al.*, 1994). The primary genetic response of the cells exposed to NGF is the rapid and transient induction of a set of genes, called immediate-early genes (IEGs) including *c-fos* (Milbrandt, 1986; Rivera and Greenberg, 1990; Sheng and Greenberg, 1990; Peng *et al.*, 1993), *c-jun* (Greenberg *et al.*, 1985; Wu *et al.*, 1988), *c-myc* (Milbrandt, 1987), Oct-2 (Kendall *et al.*, 1995), and the zinc finger protein NGFI-A (Olsson *et al.*, 1994). The induction of these genes does not require de novo protein synthesis and is likely to be mediated by the posttranslational modification of existing transcription factors. The products of these IEGs are likely to directly or indirectly regulate the expression of the late genes whose expression is induced one or more days after NGF treatment at the time of neurite outgrowth in PC 12 cells. Among IEGs, the proto-oncogene *c-fos* has been the most extensively studied with regard to its transcriptional regulation (Rivera *et al.*, 1990; Sheng *et al.*, 1990; Treisman, 1990 Gabellini *et al.*, 1992; Bad-

*To whom correspondence should be addressed.
Tel: 82-2-3410-3642, Fax: 82-2-3410-3649

ing *et al.*, 1993). Also, oncogenic products of src and ras mimic the NGF-induced neurite outgrowth (Alema *et al.*, 1985; Bar-Sagi and Feramisco, 1985) and induce the transcription of IEG and late genes (Tomas *et al.*, 1991) in a hierarchical order (D'Arcangelo *et al.*, 1993). Although the nature and the mechanisms of IEGs have been well characterized, those of late genes are far from complete. In this study, we set the goal to isolate the late genes and/or the target genes of IEGs during neuronal differentiation. Using differential display technique, we have identified cytochrome c oxidase subunit I (COX I) gene that is differentially expressed during neuronal differentiation. By identifying late and /or target genes of IEGs, new insights into the roles of these genes during neuronal differentiation will be provided.

Materials and Methods

Cell culture and NGF treatment

The PC12 cells were cultured on poly-L-lysine coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) either in the absence or presence of NGF-7S (Sigma, St. Louis, USA). Cells were synchronized prior to NGF treatment in serum-free media (DME/F12 containing 1.2 mg/ml $NaHCO_3$, 1% penicillin-streptomycin, 5 μg/ml insulin, 30 nM selenium, 0.1 mg/ml BSA, 5 μg/ml apo-transferrin, 100 μM putrescine, 20 nM progesterone: N2 media) for 24 hours and was replaced with N2 media containing 50 ng/ml NGF for hours indicated.

Cell cycle analysis

Cells were rinsed and harvested with PBS at room temperature. Immediately after harvest, clumps of cells were dissociated by repeated pipetting. Cells were pelleted, resuspended in 2 ml of PBS and fixed for 1 h at room temperature in 70% ethanol in final concentration. Cells were washed three times with PBS, resuspended in 200 μl of a solution of ribonuclease A (1 μg/ml PBS, 0.13 mM EGTA: Sigma) for 20 min, then exposed to propidium iodide (15 μl, 1mg/ml: Sigma) for at least 5 min. When necessary, samples were diluted with a similarly diluted solution of propidium iodide in PBS to yield comparable cell concentrations of $\sim 5 \times 10^6$ cells per ml. Cell suspensions were analyzed on a FACS vantage flow cytometer (Becton-Dickinson). The argon-ion laser (Coherent Innova 300) was tuned to 488 nm and operated at a power output of 150 mW in light stabilized mode. Propidium iodide fluorescence was measured using a 575/26 band pass filter. Forward scatter was used as the threshold channel. Pulse area and pulse width of the fluorescence were analyzed using a Becton-Dickinson Modfit program.

Southern and Northern blot analysis

Genomic DNA of PC12 cells was isolated by the method of Dilella and Woo (1987). Two μg of genomic DNA samples digested with BamHI, EcoRI, and HindIII were electrophoresed on neutral 1% agarose gel, capillary blotted to Hybond N+ nylon membrane (Amersham, Buckinghamshire, UK) by using the alkaline blotting technique of Reed and Mann (1985), covalently bound with a Stratalinker model 2400 UV crosslinking device (Stratagene, La Jolla, USA), and hybridized with ^{32}P-labled cDNA 63-2 isolated originally from the differential display. Northern blot analysis was performed according to the method described previously (Lee and Gross, 1993). Total RNA was isolated as originally described by Chomczynski and Sacchi (1987). RNA samples (10 μg per lane) were electrophoresed, transferred onto a Hybond-N+ membrane, and hybridized with the same probe used for Southern blot analysis and a housekeeping gene, L32 probe sequentially.

Differential display

Poly $(A)^+$ mRNA was isolated from total RNA using a Oligotex mRNA Mini kit (Qiagen, Chatworth, CA, USA) according to the manufacturer's instruction. DD-PCR was performed according to the protocol described by Liang and Pardee (1992) with a minor modification. Briefly, 100 ng of mRNA samples (pretreated with DNase I to remove genomic DNA contamination) isolated from PC12 cells untreated and treated with NGF (for 12, 24, 48, and 72 h) were reverse-transcribed with 200 U of moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Medison, WI, USA) in the presence of 1 μM of oligo dT primer and 2 μM dNTPs at 42°C for 1 h. The resultant cDNAs were amplified with 90 combinations of the arbitrary and oligo(dT)-anchored primers (Table 1), 2 μM dNTP mix, in the presence of [α-^{33}P]dATP (3000 Ci/mmole; Amersham, Buckinghamshire, UK) and 2 U of Amplitaq DNA polymerase (Clontech, Palo Alto, CA, USA). The PCR was performed for 21 cycles as follows; 1 cycle [94°C for 5 min, 40°C for 5 min, 68°C for 5 min]; 2 cycles [94°C for 2 min, 40°C for 5 min, 68°C for 5 min]; 18 cycles [94°C for 1 min, 60°C for 1 min, 68°C°C for 2 min] and followed by extension at 68°C for an additional 7 min. Samples were separated on a 5% polyacrylamide/ 8 M urea gel and analyzed by autoradiography.

Band recovery

Differentially expressed bands were excised from the

dried gel and soaked in TE buffer followed by boiling at 100°C for 5 min. Reamplifying each band was carried out by PCR with the same primers used in the differential display. The PCR was performed for 20 cycles (94°C for 1 min, 60°C for 1 min, 68°C for 2 min followed by a few extention at 68°C for 7 min). The PCR product was run on a 1% agarose gel, stained with ethidium bromide, and purified using QIAEX gel extraction kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instruction.

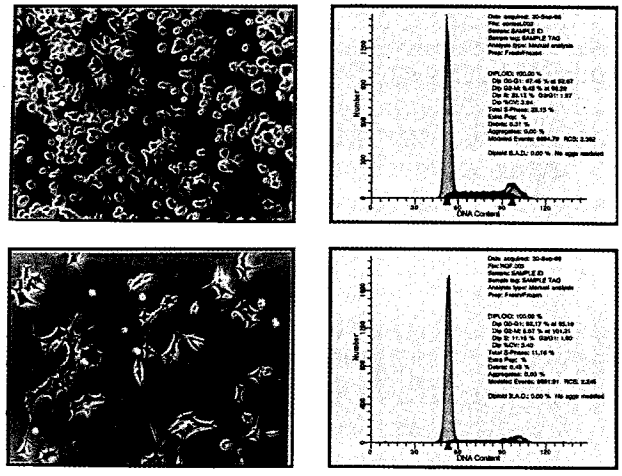
RT-PCR

First strand cDNAs were synthesized from 10 ng each of the mRNA samples with 200 U of MMLV reverse transcriptase, 3' primer (R, Table 1), and 0.5 mM dNTP in the reaction buffer at 42°C for 60 min. PCR was done with 1/10 volume of the first strand cDNA samples, 0.5 μM 5' primer (F, Table 1), 0.2 mM dNTP, and 2.5 U of Amplitaq DNA polymerase (Clontech, USA). The reaction was started by incubation at 94°C for 7 min and the continued for 20 and 25 cycles (92°C, 1 min/ 63C, 1 min/ 72°C, 1 min). PCR-amplified products were then analyzed by ethidium bromide staining after agarose gel electrophoresis.

Results and Discussion

Differentiation of PC12 cell with NGF.

To examine the neuronal differentiation of PC12 cells by NGF, PC12 cells were transferred from Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) to the serum free medium containing 50 ng/ml of NGF. Neuronal differentiation of PC12 cells by NGF was confirmed by flow cytometric analysis as well as microscopic observation. As can be seen in lower panel of A, Fig. 1, NGF treatment resulted in differentiated morphology of PC12 cells, including neurite outgrowth. To test if this morphological change is accompanied by cession of cell division, we performed the cell cycle analysis on PC12 cells untreated and treated with NGF (Fig. 1.B). Cells were fixed and stained for DNA content and subjected to flow cytometric analysis to obtain information on cell cycle distribution. The DNA histogram of the control cell culture displayed a cell cycle distribution typical of continuously proliferating cultures whereas the NGF-treated culture was highly enriched in G1-phase cells (68.2% to 84.4%) at the expense of the S-phase and the G2/M-phase compartments (22.3% to 10.8% and 9.6% to 4.8%, respectively). This indicates that PC12 cells have ceased dividing and differentiated into a mature neuronal phenotype. These morphological changes as well as cell cycle arrest are likely correlated with changes in gene expression during the course of neuronal differentiation.



(A) (B)

Fig. 1. Differentiation of PC12 cells with NGF. (A). PC12 cells untreated and treated with NGF were visualized by phase-contrast microscopy. After transfer of PC12 cells from Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) to the serum free meium containing 50 ng/ml of NGF, neurite outgrowth was induced. Morphological change in PC12 cells during NGF-induced neuronal differentiation is conspicuous (lower panel). Both pictures were taken on an Olympus IMT-2 microscope with 40X objectives. Photos were taken with a Kodak Ektachrome P400 film and printed photographically. (B). PC12 cells untreated (upper panel) and treated with NGF for 24 h (lower panel) were analyzed on a Becton-Dickinson FACS vantage flow cytometer. Propidium iodide fluorescence was measured using a 575/26 band pass filter. The percentage of cells with fractional DNA content and the cell cycle distributions are estimated using Becton Dickinson Modfit program.

Identification of differentially expressed genes by PCR-based differential display

NGF causes a rapid induction of a set of IEGs and the products of these IEGs are likely to directly or indirectly regulate the expression of the late genes. Thus, by identifying differentially expressed genes induced one or more days after NGF treatment, we can gather information about the biochemical events in neuronal differentiation. To investigate differentially expressed late genes in neuronal differentiation, the differential display technique was applied to the mRNAs of the PC12 cells untreated and treated with NGF. Cells were synchronized prior to NGF treatment in serum free media (N2, see Materials and Methods) for 24 h and was replaced with N2 media containing 50 ng/ml NGF as indicated. Total RNA was isolated as originally described by Chomczynski and Sacchi (1987) and poly $(A)^+$ mRNA was isolated from total RNA using a Oligotex

Table 1.

Primers used for this study

1. Differential display

P1:5'-ATTAACCCTCACTAAATGCTGGGGA-3'
P2:5'-ATTAACCCTCACTAAATCGGTCATAG-3'
P3:5'-ATAACCCTCACTAAATGCTGGTGG-3'
P4:5'-ATAACCCTCACTAAATGCTGGTAG-3'
P5:5'-ATTAACCCTCACTAAAGATCTGACTG-3'
P6:5'-ATTAACCCTCACTAAATGCTGGGTG-3'
P7:5'-ATTAACCCTCACTAAATGCTGTATG-3'
P8:5'-ATTAACCCTCACTAAATGGAGCTGG-3'
P9:5'-ATTAACCCTCACTAAATGTGGCAGG-3'
P10:5'-ATTAACCCTCACTAAAGCACCGTCC-3'

T1:5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
T2:5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
T3:5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
T4:5'-CATTATATGCTGAGTGATCTTTTTTTTTCA-3'
T5:5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
T6:5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
T7:5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
T8:5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
T9:5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

2. RT-PCR and DNA sequencing

S: 5'-GGAACTATCAACTTTATCAC-3'
F: 5'-CCTATGTTCGTAAACGGTTG-3'
R: 5'-TATTGGGTTATAGCAGGGGG-3'

mRNA Mini kit (Qiagen, Chatworth, CA, USA) according to the manufacturer's instruction. Messenger RNAs prepared from unstimulated and NGF stimulated (for 12, 24, 48, and 72 h) PC12 cells were reverse-transcribed into cDNAs. The resultant cDNAs were amplified with 90 combinations of the arbitrary and oligo (dT)-anchored primers (Table 1). Five cDNA bands were identified as differentially expressed in NGF stimulated PC12 cells. Fig. 2 shows the representative differential display result obtained with P6/T3, P10/T8, and P8/T5 primer combinations. One particular cDNA fragment (63-2) was identified to be up-regulated to a high degree in NGF treated (24 h) but not in untreated PC12 cells (compare lane 4 and 5 in Fig. 2). The cDNA fragment was recovered from the sequencing gel, reamplified by PCR using P6/T3 primer set, and subcloned into the TA-cloning vector pCRII (Invitrogen, San Diego, CA, USA). Double-stranded plasmid-DNA was sequenced in both directions according to the method of Kraft *et al.* (1988) using the sequencing primer (Table 1), M13 forward, and reverse primers. The identity of the differentially expressed gene was determined by searching GenBank and EMBL databases. Sequence homology analysis (BLAST search) has revealed that differentially expressed cDNA has 100% sequence homology to the rat cytochrome c oxidase subunit I (Fig. 3).

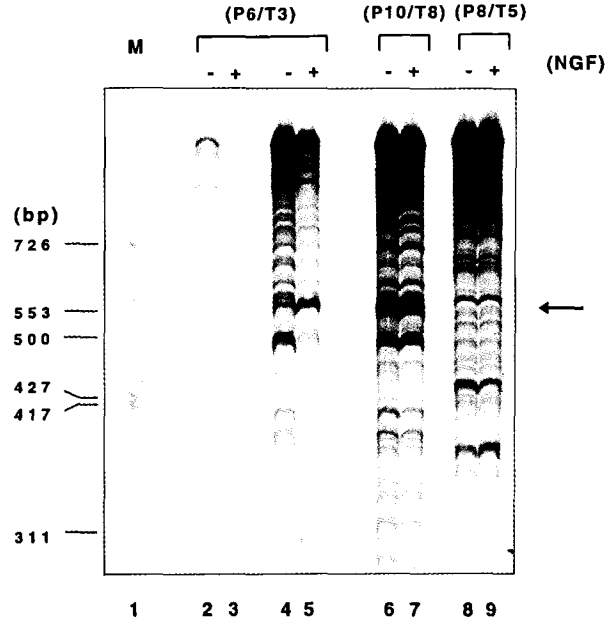


Fig. 2. Differential display of mRNA isolated from PC12 cells stimulated with and without NGF. The mRNAs isolated from PC12 cells untreated (upper panel) and treated with NGF were reverse-transcribed and the resultant cDNAs were amplified with the arbitrary and oligo(dT)-anchored primers. In this experiment, P6/T3, P10/T8, and P8/T5 primer combinations were used for the amplification. PCR products were resolved in an 8M urea/5% polyacrylamide DNA sequencing gel. Lane 1, end-labeled Hae III digested ϕx174 DNA; lanes 2, 4, 6, and 8: unstimulated; lanes 3, 5, 7, and 9: stimulated with NGF for 24 h. The band indicated with an arrow shows a marked induction on the differential displaying gel and was named 63-2.

Differential expression of COX I gene

To confirm differential expression of the cDNA 63-2, the RT-PCR was carried out with the mRNA-derived cDNA extracted from PC12 cells of unstimulated and NGF-stimulated for 12, 24, 48, and 72 h. To avoid the promiscuity of the arbitrary primers, a new set was designed from the sequence of rat COX I gene (5' primer; (F), 3' primer; (R), Table 1). First strand cDNAs were synthesized from each mRNA with a 3' primer, R

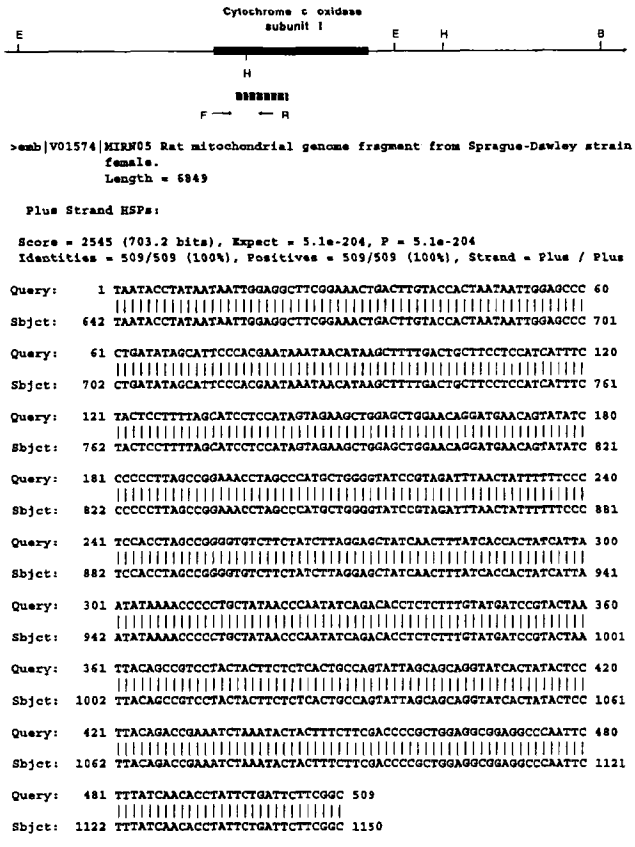


Fig. 3. Comparison of the DNA sequences of the cDNA 63-2 with Sprague-Dawley rat cytochrome c oxidase subunit I. The identity of the differentially expressed gene was determined by searching GenBank and EMBL databases. Sequence homology analysis (Blast search) has revealed that differentailly expressed cDNA 63-2 has 100% sequence homology to cytochrome c oxidase subunit I. The alignments were produced using the BLAST program of the National Center for Biotechnology Information network server.

and PCR were applied by addition of 5' primer, F. PCR-amplified products were then analyzed by ethidium bromide staining after agarose gel electrophoresis. PCR products using primers from the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GPDH) were used as an internal control. Among the results of 12, 24, 48, and 72 h, that of 24 h was the most conspicuous (data not shown). Fig. 4 shows differential expression of COX I gene in PC12 cells treated with NGF for 24 h (compare lanes 2 and 3). To verify differential expression of the COX I gene further, Northern blot analysis was applied to RNA isolated from the cells treated with NGF for 24 h. Total RNA samples (10 μg per lane) were electrophoresed, transferred onto a Hybond-N+ membrane, and hybridized with the ^{32}P-labeled cDNA 63-2 reamplified by PCR and a housekeeping gene, L32 probe sequentially. Autoradiogram confirms the differential expression exhibited earlier in the mRNA differential display (Fig. 5).

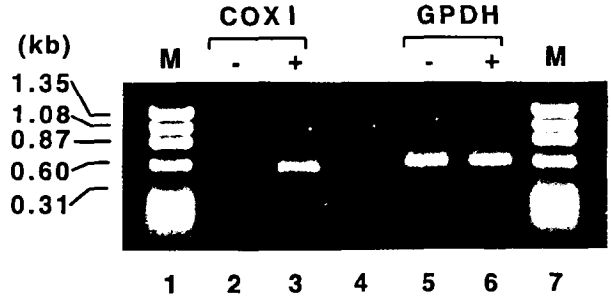


Fig. 4. RT-PCR with primers specific for the COX I gene. RT-PCR was performed with mRNA isolated from the PC12 cells untreated and treated with NGF for 24 h. RT-PCR with house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GPDH) primer was used as an internal control. The primer set was designed from the sequence information of cytochrome c oxidase subunit I gene: Lane1 and 7, Hae III digested ϕx174 DNA marker; Lane 2, performed with mRNA isolated from the untreated PC12 cells; lane 3, from the PC12 cells treated with NGF for 24 h; Lane 4, negative control; lane 5 and 6, GPDH for internal control.

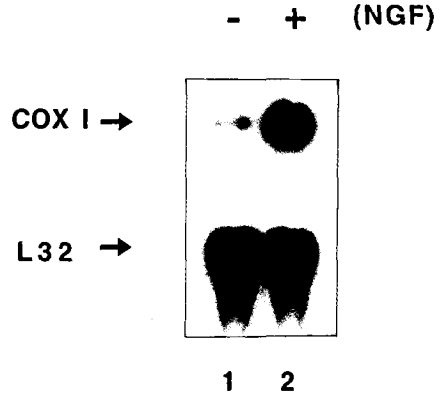


Fig. 5. Northern blot analysis. Northern blot analysis of COX I gene expression in PC12 cells untreated and treated with NGF. Total RNA (10 μg per lane, loaded in the order: lane 1, undifferentiated and lane 2, differentiated PC12 cells) was resolved by electrophoresis, transferred to nylon membrane, and hybridized to COX I and the housekeeping gene, L32 probes sequentially. The L32 mRNA expression was relatively constant in the experimental conditions and therefore used for standardizing the samples loaded in each lane.

For quantification, the autoradiograms obtained with each probe were scanned with a Dage MTI CCD72X camera and the intensity of the region of interest was measured with the MCID software program (Imaging research Inc. Michigan City, IN. USA) on Intel Alt server computer. The COX I gene transcripts level of NGF treated sample was more than 8 times higher than that of control.

Southern blot analysis of COX I gene

To investigate the genomic arrangement and the copy number of COX I gene, Southern blot analysis

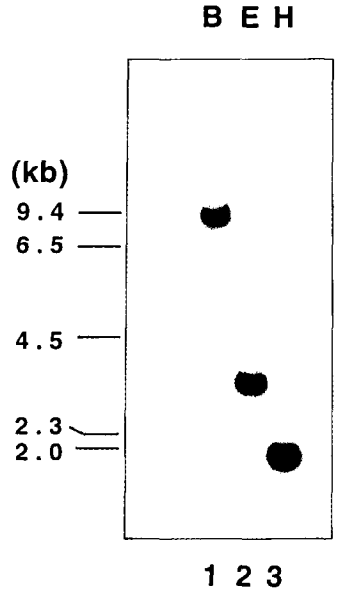


Fig. 6. Southern blot analysis of COX I gene. Genomic DNA samples isolated from the PC12 cells were digested with BamHI, EcoRI, and HindIII (lane 1, lane 2, and lane3, respectively), electrophoresed on neutral 1% agarose gel, capillary blotted onto a Hybond-N+ nylon membrane (Amersham), and hybridized with the ^{32}P-labeled, reamplified, cDNA 63-2 probe.

was employed to the genomic DNA of PC12 cells. Genomic DNA was isolated by the protocol described by Dilella and Woo (1987). Two μg of genomic DNA samples digested with BamHI, EcoRI, and HindIII were electrophoresed on neutral 1% agarose gel. Electrophoretically separated DNA samples were capillary blotted to Hybond N+ nylon membrane by using the alkaline blotting technique of Reed and Mann (1985), covalently bound with a Stratalinker model 2400 UV crosslinking device (Stratagene, La Jolla, CA, USA), and hybridized with the same probe used for Northern blot analysis. As can be seen in Figure 6, each digestion renders the single band with similar intensity, which indicates that COX I is a single gene. The restriction map of COX I gene, based on Southern blot analysis, is provided in Fig. 3.

The eukaryotic cytochrome c oxidases all contain three mitochondrially encoded subunits (I, II, and III) and up to ten nucleus-encoded subunits. It is the terminal enzyme complex of the respiratory chain, coupling the transfer of electron from cytochrome c to molecular oxygen with the concomitant production of a proton electrochemical gradient across the inner mitochondrial membrane. Numerous data suggest that mitochondrial activity is involved in the regulation of cell growth and differentiation (Kim *et al.*, 1995; Stevens *et al.*, 1995; Chariot *et al.*, 1996). A study investigating the changes in mitochondrial activity in avian myoblast cultures undergoing differentiation or in BrdU-treated, differentiation-deficient cells has shown that precocious differentiation events are associated with a stimulation of cytochrome oxidase activities (Rochard *et al.*, 1996). In addition, when larvae of the cichlid fish *Oreochromis mossambicus* were analyzed at different developmental stages, cytochrome c oxidase activity in the mitochondria of neuronal perikarya increased during development which parallels the differentiation of the area octavolateralis (Paulus *et al.*, 1993). These possibly reflect the increasing energy demand during development and differentiation. Similar energy demand might explain the increased expression of COX I gene during neuronal differentiation. As most mitochondrial genes are transcribed as a single large transcript, it will be interesting to examine whether other mitochondrial subunit genes, COX II and COX III are coordinately increased with COX I gene during neuronal differentiation.

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