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Increased Expression of Rat Ribosomal Protein L4 mRNA in 5-Azacytidine-Treated PC12 Cells Prior to Apoptosis

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5-Azacytidine (5AzC), a cytidine analogue, is thought to induce apoptosis in fetal neuronal cells and PC12 cells through DNA hypomethylation. However, apoptosis can be inhibited by adding protein synthesis inhibitors, indicating *de novo* protein synthesis may be partially responsible for apoptosis. Therefore, genes expressed just before apoptosis from 5AzC-treated PC12 cells were cloned. cDNA libraries were prepared from both 5AzC-treated and untreated PC12 cells and these libraries were subtracted. One clone overexpressed in 5AzC-treated PC12 cells was obtained, and was identified as the nearly full length (9 nt at 5′ end and 1 nt at 3′ end missing) rat ribosomal protein L4 (rpL4) gene. Time course study of Northern blot analysis in 5AzC-treated PC12 cells revealed that the peak of rat rpL4 gene expression preceded DNA fragmentation. COS-7 cells transfected with different amounts of cDNA from the subtracted clone expressed rat rpL4 dose-dependently. DNA fragmentation in the transfected COS-7 cells occurred proportional to the amount of the cDNA used for transfection. The present study indicates that rat rpL4 gene expression selectively increases in PC12 cells prior to 5AzC-induced apoptosis and that COS-7 cells transfected with and expressing the rat rpL4 gene also undergo apoptosis.

Key Words: **apoptosis; 5-azacytidine; cDNA subtraction; COS-7 cells; PC12 cells; ribosomal protein L4.**

5-Azacytidine (5AzC; C8H12N4O5, MW 244.2), a cytidine analogue, is characterized by replacement of the number 5 carbon atom of the pyrimidine ring by a nitrogen atom, rendering it incapable of accepting a methyl residue during enzymatic methylation of newly synthesized DNA molecules. This effect has been exploited to demethylate DNA to promote gene expression (1). Examples include the mouse non-muscle embryonic cell line, 10T1/2, which changes into muscle cell morphology after treatment with 5AzC (2), and 5AzC-treatment which activates the normally inactive human hypoxanthine/guanine phosphoribosyltransferase gene in mouse-human hybrid somatic cells (3). These events suggest that 5AzC-incorporation during DNA replication hypomethylates DNA which activates previously dormant genes (1, 4). Additionally, highly methylated genes are not able to be transcribed, supporting this theory as well (5). Therefore, DNA methylation is thought to be involved in control of gene expression (6-8).

5AzC induces neuronal apoptosis during embryogenesis (9, 10). Additionally, PC12 cells derived from a rat pheochromocytoma undergo apoptosis after 5AzC-treatment (11). 5AzC-induced apoptotic cell death could be prevented by simultaneous treatment with a protein synthesis inhibitor, cycloheximide (12). These results suggest that 5AzC activates certain dormant genes encoding apoptosis-associated protein(s) through DNA hypomethylation, but inhibition of protein synthesis would prevent PC12 cells from apoptotic death.

In the present study, such apoptosis-related genes were cloned by a cDNA subtraction method, and rat ribosomal protein L4 (rpL4) was identified as a gene with highly increased expression prior to 5AzC-induced apoptosis in PC12 cells. The time course of rat rpL4 expression in PC12 cells after 5AzC-treatment and the ability of this clone to induce apoptosis in transfected COS-7 cells were also examined.

MATERIALS AND METHODS

Cell culture and 5AzC treatment. PC12 22a cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS; Nichirei, Tokyo, Japan), 5% donor horse serum (Nichirei), 25 U/ml penicillin (GIBCO BRL, Grand Island, NY, USA), 25 μg/ml streptomycin (GIBCO BRL) and 25 μg/ml kanamycin

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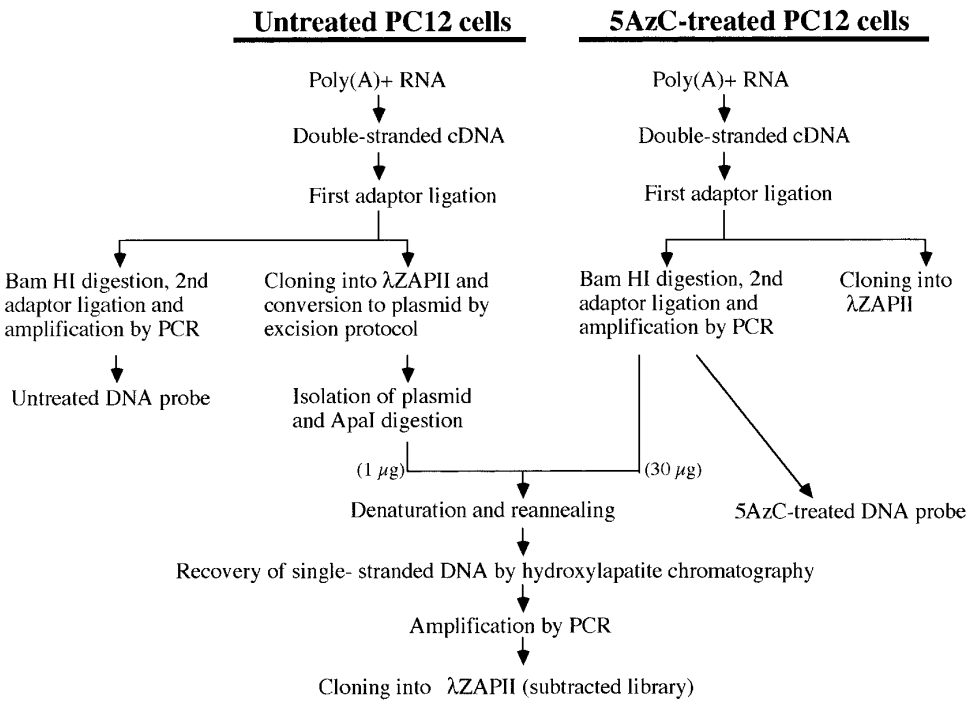

FIG. 1. Schematic outline of the cDNA subtraction procedure.

(GIBCO BRL) at 37°C in 5% CO_2/95% air. After addition of 500 μg/ml 5AzC (Sigma, St. Louis, MO, USA), cells were collected at 1, 3, 6, 12 and 24 hrs for enzyme-linked immunosorbent assay (ELISA) and Northern hybridization, and at 10 hrs for cDNA synthesis. Untreated cells were collected before 5AzC-treatment.

Measurement of DNA fragmentation by enzyme-linked immunosorbent assay (ELISA). To detect DNA fragmentation (apoptotic cell death) of cultured cells, the Cell Death Detection Kit (Boehringer-Mannheim, Mannheim, Germany) was used. 5AzC-treated PC12 cells collected at 1, 3, 6, 12 and 24 hrs or untreated cells were pelleted by centrifugation at 400 g for 5 min. The cell pellets were lysed in the incubation solution included in the kit at 4°C for 30 min. The lysate was centrifuged at 14,000 g for 10 min and the supernatant, including smaller molecule-sized DNA, was collected. Appropriate dilutions of the supernatant were put into wells coated with anti-histone antibodies. After incubation at room temperature for 90 min, the peroxidase-labelled anti-DNA antibody was put into the wells and the wells were incubated at room temperature for 90 min. Color development was done by adding a substrate solution including 2,2′-azino-di-[3-ethylbenzthiazolin-sulfonate] (ABTS). The optical density (OD) of the sample solutions were measured at 400 nm using a spectrophotometer. The OD value was obtained by subtracting OD of the background blank from the raw OD for each sample. The final results were expressed as a ratio to the OD of the living untreated cell control.

Preparation of cDNA libraries from 5AzC-treated and untreated PC12 cells. A PC12 cell cDNA library was prepared using conventional methods (13). Fig. 1 shows the schematic outline of library construction. In brief, cDNA was synthesized from mRNA isolated separately from either 5AzC-treated PC12 cells collected 10 hrs after treatment or untreated cells. The ends of cDNA were blunted with T4 DNA polymerase (cDNA synthesis system plus; Amersham Life Science, Buckinghamshire, England). The first adaptor (EcoRI-BamHI-NotI adaptor; TAKARA, Kyoto, Japan) was ligated to the blunt-end of the cDNAs with T4 DNA ligase (TAKARA). After phosphorylation with T4 DNA polynucleotide kinase (TAKARA), 0.6 kb to 10 kb cDNA fragments were recovered from an agarose gel. After purification, the DNA was ligated into the EcoRI-digested λZAPII phage vector (Stratagene, La Jolla, CA, USA) and packaged using GigapackII Gold packaging extract (Stratagene).

Preparation of the subtracted cDNA library. cDNA from 5AzC-treated PC12 cells was digested with BamHI and the second adaptor (F-J adaptor) (13) containing BamHI and EcoRI restriction sites was ligated to the BamHI-digested DNA. The second adaptor-ligated cDNA was amplified by polymerase chain reaction (PCR) using oligonucleotides from the second adaptor site as primers. pBluescript SK phagemid clones from the λZAPII cDNA library of untreated PC12 were purified by ultracentrifugation in a cesium chloride-ethidium bromide density gradient (14). The phagemid clones were digested with ApaI (TAKARA). One microgram of PCR-amplified cDNA from 5AzC-treated PC12 cells and 30 μg of cDNA phagemid from untreated PC12 cells were hybridized in a reaction mixture (0.12 M sodium phosphate buffer pH 7.4, 0.6 M NaCl, 5 mM EDTA, 0.1% SDS) at 65°C for 24 hrs. Unpaired, single stranded DNA was isolated by hydroxylapatite chromatography (Bio-gel; Bio-Rad, Richmond, CA, USA) and amplified by PCR using the aforementioned oligonucleotides as primers. Amplified DNA was packed into the λZAPII vector as described previously.

Selection of a specific clone showing increased expression after 5AzC-treatment. Duplicate filters (Hybond-N; Amersham Life Science) were prepared from the agar plates on which the subtracted library was cultured. The filters were differentially screened with cDNA probes prepared from either 5AzC-treated or untreated PC12 cells. These probes were radio-labelled with $\alpha[^{32}P]$-dCTP (Amersham Life Science) using a random primer labelling kit (Boehringer-Mannheim). Of 6.4 × 104 clones, 11 clones that showed stronger hybridization to the 5AzC-treated probe than with the untreated probe were selected. To restrict clone selection further, blotting membranes (Pall Biodyne B; Pall, East Hills, NY, USA) to which total RNA from 5AzC-treated or untreated PC12 cells had been transferred were probed with the 11 clones, and only one clone that definitely increased after 5AzC-treatment was obtained (clone 8).

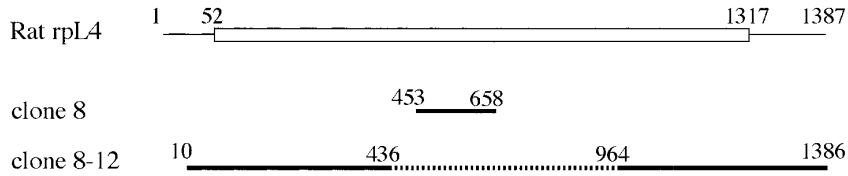

FIG. 2. Schematic diagram of rat rpL4, clone 8 and clone 8-12 structure. The open box indicates the ORF in rat rpL4. Thick lines represent sequence data determined in the present study, which are identical to rat rpL4. The dotted line represents undetermined sequence.

The sequence of clone 8 was determined using the Auto Read sequencing kit (Pharmacia, Uppsala, Sweden) according to Sanger's method (15).

Screening of the cDNA library from 5AzC-treated PC12 cells. Membrane filters prepared from the 5AzC-treated cDNA library were screened with $\alpha[^{32}P]$-dCTP (Amersham Life Science)-labelled clone 8 probe. Of 6.4×104 clones, 2 clones (clone 8-12 and clone 8-16) showing strong hybridization signals were selected. Sequences of both 5′ and 3′ regions of clone 8-12 were determined using the sequencing kit described previously.

Northern hybridization. Total RNA extracted from 5AzC-treated PC12 cells collected at 1, 3, 6 and 12 hrs following 5AzC-treatment or from untreated cells was separated on a 1% agarose electrophoretic gel containing 18 % formaldehyde and then transferred to a nylon membrane (Pall Biodyne B). The blotting membrane was incubated in prehybridization solution (5× SSC (1× SSC = 1.5 M NaCl, 0.15 M sodium citrate), 2.5× Denhart's solution, 0.1% SDS, 5 mM EDTA and 100 μg/ml salmon sperm DNA) at 42°C for 2 hrs. Hybridization using approximately 1.4 kbp of clone 8-12 labelled with $\alpha[^{32}P]$-dCTP (Amersham Life Science) was done at 42°C for 16 hrs. The membrane was washed in 2×SSC containing 0.1% SDS at 50°C for 60 min, 0.5×SSC at 50°C for 30 min and 0.5×SSC at room temperature for 10 min, respectively. The hybridization signal was detected autoradiographically on X-ray film (BIOMAX MR; Eastman Kodak, Rochester, NY, USA). The membrane was also incubated with a 983 bp DNA fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. Densitometry was performed on a computer (Power Macintosh 8100; Apple Computer, Cupertino, California) using image-analyzing free software (NIH Image ver. 1. 55).

Transfection of COS-7 cells with the 8-12 subtracted cDNA clone. The 8-12 cDNA clone obtained from subtraction cloning was inserted into the pCIneo vector (Promega, Madison WI) at the downstream of the CMV promoter. A sequence encoding hemoagglutinin (HA) was ligated to the C terminal end of the insert for detection by Western analysis. Several different concentrations of the construct were transfected into COS-7 cells using the LipofectAMINE (BRL, Rockville, MD) system. The cells were cultured in DMEM containing 10% FBS for 48 hrs. Total soluble proteins were isolated in RIPA buffer.

COS-7 cells were also transfected with graded concentrations of the cloned DNA (8-12) using Fugene6 system (Boehringer-Mannheim) to detect apoptosis. The transfected cells were cultured for 48 hrs in the medium and total DNA was collected to investigate DNA fragmentation.

Western analysis. Total proteins from lysed COS-7 cells were electrophoretically separated and transferred to a nitrocellulose membrane. The membrane was treated with rabbit anti-HA antibody (Boehringer) and then with peroxidase-conjugated anti-rabbit IgG antibody (Jackson Immuno Research, West Grove, PA). Reactive proteins were detected using chemiluminescence substrate (Pierce, Rockford, IL).

RESULTS

Isolation and characterization of a gene showing increased expression by 5AzC-treatment. Eleven clones were selected from the subtracted cDNA library, enriched for genes showing increased expression after 5AzC-treatment. Northern band identified with a probe derived from clone 8 gave a much more intense signal to RNA from 5AzC-treated cells than to RNA from untreated cells (data not shown), indicating increased expression of clone 8 gene after 5AzC-treatment. A GenBank search revealed that the partial sequence of this 206 bp probe was identical to a portion of rat ribosomal protein L4 gene (rpL4) (16), from nt 453 to nt 658 (Fig. 2). Further screening of the 5AzC-treated cDNA library with clone 8 as probe resulted in two additional clones (clones 8-12 and 8-16) showing strong hybridization signals. The sequences of 427 bases of 5′ region and 423 bases of 3′ region of clone 8-12 were identical to those of nt 10 to nt 436 and nt 964 to nt 1386 of rat rpL4, respectively (Fig. 2). Thus, clone 8-12 covers nearly the full length of the rat rpL4 gene, which includes the open reading frame (ORF), nt 52 to nt 1317.

5AzC-induced DNA fragmentation (apoptosis) of PC12 cells detected by ELISA. The ELISA OD ratio of 5AzC-treated PC12 cells to untreated PC12 cells 1 hr after 5AzC-treatment was 1.06; 3 hrs was 1.38; 6 hrs was 1.99; 12 hrs was 2.68; and 24 hrs was 1.18 (Fig. 3), indicating the amount of fragmented DNA peaked about 12 hrs after 5AzC-exposure, but decreased approximately to the base line level by 24 hrs after exposure.

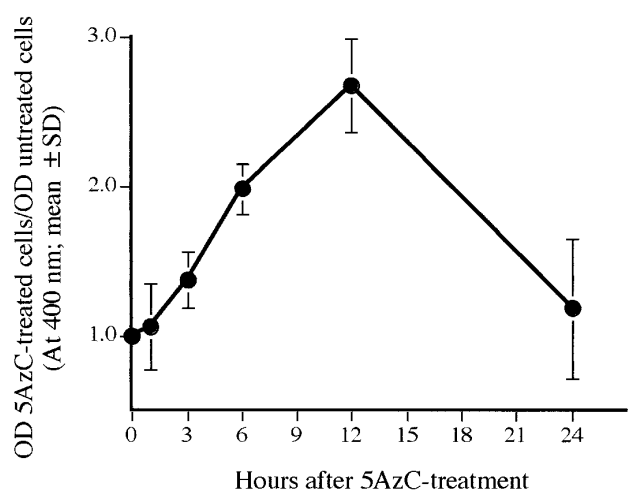

FIG. 3. DNA fragmentation (apoptosis) of PC12 cells after 5AzC-treatment measured by ELISA.

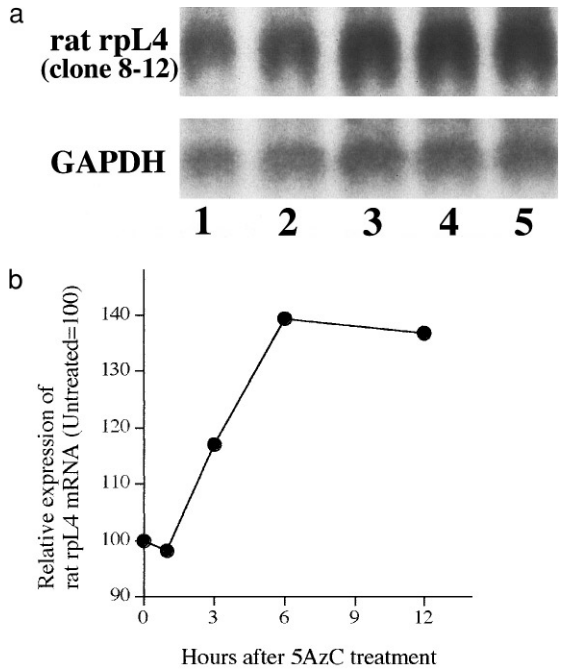

FIG. 4. Northern analysis of 5AzC-treated PC12 cells using clone 8-12 as a probe. Lanes 1, 2, 3, 4 and 5 represent rat rpL4- and GAPDH-specific bands 0, 1, 3, 6 and 12 hrs after 5AzC-treatment, respectively. (b) Expression of rat rpL4 mRNA after compensation by that of endogenous GAPDH.

Changes of rat rpL4 expression after 5AzC-treatment. Northern membranes prepared from total RNA of either 5AzC-treated or untreated PC12 cells were hybridized with clone 8-12 probe (Fig. 4a). The signal level of rat rpL4 band was standardized using GAPDH internal expression as a reference control. The relative signal intensities of the band at 0, 1, 3, 6 and 12 hrs after 5AzC-treatment were 100, 98, 117, 139 and 137, respectively (Fig. 4b). Consequently the expression of rat rpL4 increased until 6 hrs after 5AzC-treatment, and this high level was kept until at least 12 hrs after treatment.

DNA fragmentation (apoptosis) of rat rpL4-transfected COS-7 cells. Dose-dependent expression of rat rpL4 protein was observed after 48 hrs incubation in COS-7 cells transfected with rat rpL4 using 1 or 5 μg of cDNA (Fig. 5). However, the expression was reduced when 10 μg of cDNA was used for transfection.

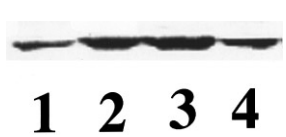

FIG. 5. Western analysis of the proteins from COS-7 cells transfected with clone 8-12. The inserted clone was tagged with the HA protein sequence. The membrane was probed with an anti-HA antibody. Zero (Lane 1), 1 (Lane 2), 5 (Lane 3) and 10 (Lane 4) mg of the cDNA (clone 8-12) was transfected into COS-7 cells.

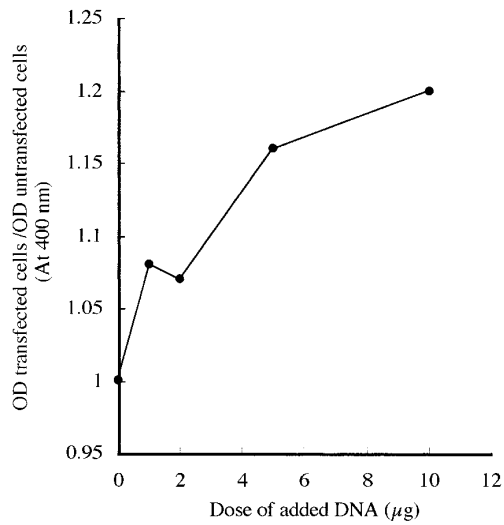

FIG. 6. DNA fragmentation (apoptosis) of the transfected COS-7 cells after 5AzC-treatment measured by ELISA.

DNA fragmentation of the transfected COS-7 cells was determined by ELISA. The OD ratio after 48 hrs incubation were 1.00, 1.08, 1.07, 1.16 or 1.20 in the cells to which 0, 1, 2, 5, or 10 μg cDNA was used for trasnfection, respectively (Fig. 6).

DISCUSSION

5AzC induces DNA demethylation which may consequently activate certain dormant genes (1, 4). Hossain *et al.* (11, 12) reported that 5AzC induces apoptosis in PC12 cells and that cycloheximide rescues these cells from 5AzC-induced apoptosis. Therefore, it can be presumed that certain apoptosis-related genes are expressed by 5AzC-treatment through a mechanism of DNA demethylation. On this basis, a cDNA subtraction between mRNAs from 5AzC-treated and untreated PC12 cells was performed and the mRNAs reached the maximal level just before DNA fragmentation. As the result, the rat rpL4 gene was isolated as a gene that increased its expression after 5AzC-treatment. The results of Northern hybridization using clone 8-12 (nearly full length of rat rpL4) as a probe revealed that the expression of rat rpL4 markedly increased after 5AzC-treatment and that the peak time of gene expression precedes DNA fragmentation. This suggests that rat rpL4 is expressed prior to apoptosis, and that the protein might be involved in an apoptotic cascade. The results of the COS-7 cell transfection with rat rpL4 cDNA also support the involvement of this gene in the 5AzC-induced apoptotic cascade.

Ribosomes are known as specialized intracytoplasmic multimolecular particles involved in protein biosynthesis. They are built up of approximately 80 protein species in the eukaryotes. Ribosomal protein L4 is

the fourth largest protein (molecular weight 47,280) which comprises the large ribosomal subunit, but the precise function of the protein is still unknown (17). The promoter region of ribosomal protein genes in higher eukaryotes have almost identical sequences including a CpG-rich region (18). Such CpG islands are considered to be crucial for the activity of the promoter (19). Thus, it is likely that the expression of rat rpL4 might be epigenetically regulated through DNA methylation; that is, demethylation of rat rpL4 promoter sequence after 5AzC-incorporation may activate the expression of the gene.

A previous study dealing with genomic hybridization of ribosomal protein genes revealed that multiple copies of the gene are present in the mammalian genome (20). The family of ribosomal protein genes consists of a single functional gene and several inactive pseudogenes, and the pseudogenes are highly methylated at their 5′ region (21, 22). Therefore, it is also likely that hypomethylation of the 5′ region through 5AzC-incorporation would activate the dormant pseudogenes.

Expression of rpL4 was reported to increase in HeLa cells after the administration of diethylmaleate (23). Diethylmaleate decreases the level of endogenous reduced glutathione, provokes oxidative stress, and finally induces apoptosis in human neutrophils (24). Therefore, rpL4 may participate in an apoptotic cascade in either a direct or indirect manner. In addition, Neumann and Krawinkel (25) reported that constitutive expression of human ribosomal protein L7 in Jurkat cells led to arrest in G1 of the cell cycle and induced apoptosis as a consequence of cell-to-cell contact, further supporting involvement of ribosomal proteins in mammalian cell apoptosis.

Further studies are in progress to elucidate the mechanism through which ribosomal proteins induce apoptosis in PC12 or COS-7 cells.

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