CHAPTER-II: METHODS

A. Preparation of RNA

B. Northern Blotting

C. Subtraction Library

D. Differential Display

E. Rat Brain cDNA Library

F. Cell Culture

IIA. PREPARATION OF RNA

Healthy *Fischer 344* rats (Harlan-Sprague-Dawley, Indianapolis, IN) were used. The rat reaches puberty by about 8 weeks and is senescent by 24 months of age (Sapolsky 1992). In order to study embryonic development, pregnant animals at different stages of gestation, timed from the appearance of the vaginal plug, were sacrificed. Postnatal animals were decapitated and brain quickly dissected out and placed in liquid nitrogen. In some experiments, the heart, lungs, liver, spleen, kidneys, skin and muscle were also removed for study. Embryonic animals were removed following decapitation of the mother. Early gestation embryos (E7-10) were studied whole, the cephalic half of mid-gestation embryos (E11-14) were used, and in late gestation (E16-19), it was possible to selectively dissect the brain out for study.

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987; Joseph et al 1993). Animals were decapitated, the brain, and in some experiments other tissues, were dissected out and placed immediately in liquid nitrogen. The frozen tissue was crushed with mortar and pestle in the continuing presence of liquid nitrogen. Ten ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was added to the crushed sample, homogenized using a glass-teflon homogenizer (Eberbach, Aim Arbor, MI), and kept on ice. Thereafter, 0.2 M sodium acetate pH 4.0, 10 ml of water saturated phenol and 2 ml of chloroform-isoamyl alcohol (49:1) were added to the sample, vortexed, kept on ice for 15 mm, and centrifuged (10,000 x g for 20 mm) at 4°C. The aqueous phase was precipitated with an

equal volume of isopropanol, the resulting pellet suspended in 0.3 ml of the denaturing solution, transferred to a microcentrifuge tube and precipitated again with isopropanol. The final RNA pellet was washed with 75% ethanol and dissolved in 0.5% SDS.

IIB. NORTHERN BLOTTING

Total RNA was isolated by the single step guanidinium thiocyanate-phenol-chloroform method and prepared for Northern blotting (Joseph et al 1993). To 20 μg of RNA (in a volume less than 3.5 μl) was added 3 μl of 10x running buffer (0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA, pH 7.0), 3.5 μl formaldehyde, 10 μl of formamide, and volume made up to 20 μl with diethylpyrocarbonate-treated water. One μl of 1.25 mg/ml of ethidium bromide was also added. The preparation was denatured at 65°C for 15 mm, and 5 μl of loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue) added.

Electrophoresis was carried out on 1% agarose-3.7% formaldehyde using a buffer containing 0.02 M MOPS, 5 mM sodium acetate and 1 mM EDTA pH 7.0. Before transfer, the gel was photographed under UV light. RNA was transferred onto a nylon membrane (Micron Separations, Westboro, MA) using 10x SSC (1.5 M NaC1, 0.15 M sodium citrate, pH 7.0) by overnight capillary action. After transfer, the membrane was washed with 5x SSC, UV cross linked for 2 min (Hoefer Scientific, San Francisco, CA), and baked at 80°C for 1 hour. The membrane was pre-hybridized in a solution containing 1% bovine serum albumin, 7% SDS, 0.5 M sodium phosphate pH 7.0 and 1 mM EDTA, at 65°C for 1 hour. Hybridization was continued overnight in the same solution with the addition of 1 x 10^6 cpm/ml of random-primed ^{32}P-labelled probe. The membrane was washed in a solution containing 1% SDS, 40 mM sodium phosphate pH 7.0 and 1 mM EDTA, at room temperature for 15 mm, and then at 65°C for 30 min (two times). The washed membrane was exposed to film (Kodak X-OMAT AR)

between intensifying screens, at -70°C. Thereafter, the blot was stripped by boiling for 20 min in a solution containing 1% SDS and 0.lx SSC, and re-hybridized with *β-actin* or *glyceraldehyde 3-phosphate dehydrogenase (G3PDH)* to control for the amount of RNA applied in the different lanes.

IIC. SUBTRACTION LIBRARY

The studies to identify genes selectively expressed in the developing rat brain were initiated by constructing a subtracted library. Using this technique, genes selectively expressed in the developing brain could be identified by removing or subtracting away those genes that are expressed both in the developing and adult brain. A low ratio hybridization-subtraction cDNA library was prepared as described by other investigators (Fornace et al 1986, 1988; Sargent 1987). Adapting this technique, cDNA prepared from neonatal rat brain was hybridized with an excess of mRNA from adult rat brain, and sequences present in both age groups were subtracted away, *negative selection.* Further enrichment was achieved by hybridization with mRNA from neonatal brain, *positive selection,* and discarding unhybridized sequences (**FIGURE-II-1**).

A litter of one day old rat pups (n = 12) and the mother were sacrificed by decapitation. Their brains were quickly dissected out, immersed in liquid nitrogen, ground, homogenized, and total RNA extracted by the acid guanidinium thiocyanate phenol chloroform method. Thereafter, mRNA was separated on oligo-dT columns (Pharmacia, Piscataway, NJ), and analyzed on an agarose gel to ensure that ribosomal RNA was removed. Twenty five μg of mRNA from neonatal rat brain *(Neonatal* mRNA) was used to synthesize ^{32}P-labelled cDNA in the presence of reverse transcriptase, oligo-dT and random primers.

SUBTRACTION HYBRIDIZATION LIBRARY

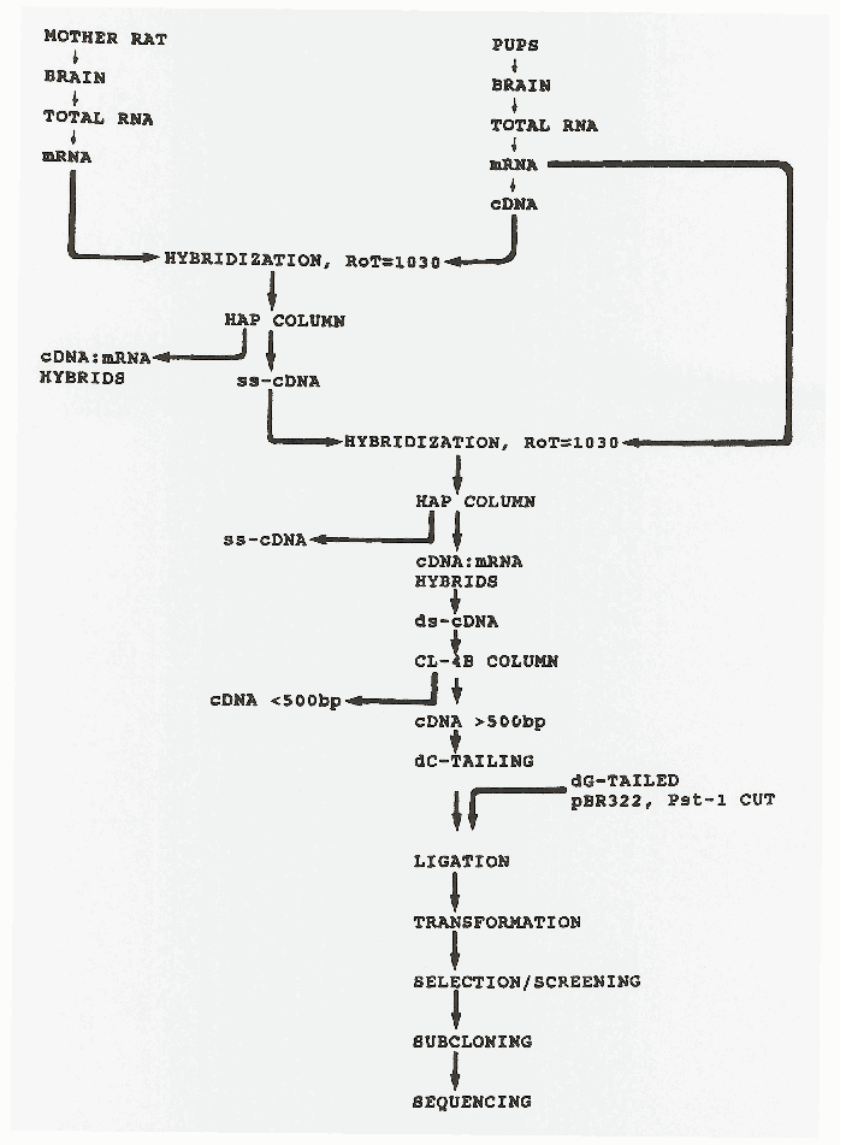


FIGURE-II-1: Preparation of the subtraction hybridization library enriched for the neonatal rat brain. mRNA was extracted from the brains of a litter of one day old rat pups, and their mother. Initially, *negative selection* was carried out by hybridizing *neonatal* cDNA and *adult* mRNA, and discarding common sequences. Single stranded-cDNA (ss-cDNA) specific for the neonatal brain was isolated using a hydroxyapatite (HAP) column. The cDNA:mRNA hybrids remained bound to the column, and were discarded. Thereafter, *positive selection* was carried out by hybridizing the ss-cDNA with an excess of *neonatal* mRNA, and discarding unbound sequences. The cDNA:mRNA hybrids, eluted from the second column, were used to prepare double stranded cDNA (ds-cDNA), and size selected using a CL-4B column. The cDNA fragments larger than 500 bp were dG-tailed, ligated into the Pst-1 site of pBR322, transformed, and selected.

The cDNA reaction included 1 mM dGTP, 1 mM dATP, 1 mM dTTP, 0.5 mM dCTP, 60 mM KC1, 7 mM $MgC1_2$, 50 mM Tris-HC1 pH 7.5, 2 mM DTT, 60 *ng/*μl Actinomycin-D, 35 ng/μl oligo-$dT_{12\text{-}18}$ (Life Technologies, Grand Island, NY), 200 ng/μl random primers (Life Technologies), 1 unit/μl RNase inhibitor (Boehringer Mannheim, Indianapolis, IN), 100 μCi ^{32}P-dCTP and 100 units/μg RNase H- MMLV-reverse transcriptase (Life Technologies). The final reaction volume was made up to 200 μl with water, and incubated at 37°C for 1 hour. The mRNA in the reaction was hydrolyzed by adding an equal volume of 0.6 N NaOH and 20 mM FDTA, and incubated at 65°C for 30 mm. ^{32}P-labelled single stranded cDNA was separated on a Sepharose G-50 column using NETS buffer containing 0. 1 M NaC1, 0. 1 mM EDTA, 10 mM Tris-HC1 pH 7.6 and 0.1% SDS. The sample was extracted with phenol:chloroformisoamyl alcohol (P:CI), extracted with CI, precipitated with sodium acetate and isopropanol, washed with 75% alcohol and suspended in 12.5 μl of hybridization buffer containing 0.12 M NaH_2PO_4 pH 6.8, 0.82 M NaCI, 1 mM EDTA, and 0.1% SDS. Low ratio hybridization subtraction was then carried out.

The first hybridization reaction between *neonatal* cDNA (tracer) and *adult* mRNA (driver) was a *negative selection. Neonatal* cDNA (25 μg RNA complexity) was mixed with *adult* mRNA (25 μg) in a total volume of 25 μl of hybridization buffer. The reaction was incubated at 65°C for 36 hours (RoT = 1030). The cDNA:mRNA hybrids, generated by this hybridization, were separated at 65°C using hydroxylapatite columns. Single-stranded cDNA was eluted from the columns with 0.12 M NaH_2PO_4 pH 6.8; under these conditions cDNA:mRNA hybrids remain bound to the column. The single-stranded cDNA isolated at this

step was specific for the pups *(neonatal* cDNA). The *neonatal* cDNA was dialyzed overnight in a cold room with a buffer containing 10 mM Tris-HC1 and 1 mM EDTA pH 8.0 to remove phosphate in the cDNA solution. The sample was concentrated to a volume of 0.5 ml using 1-butanol, precipitated with sodium acetate, washed with 75% ethanol and air dried.

Thereafter, the purified sample of single-stranded *neonatal* cDNA was used to carry out *positive selection.* For this purpose, 20 μg of *neonatal* mRNA was added in a total volume of 30 μl hybridization buffer, and incubated at 65°C for 64 hours (RoT = 1030). The preparation was again separated on a hydroxylapatite column; however, this time the cDNA:mRNA hybrids were eluted with 0.48 M NaH_2PO_4 pH 6.8, and saved. Phosphate was removed from the elute by dialysis with 10mM Tris-HCl and 1 mM EDTA, concentrated using 1-butanol, precipitated, washed, and used for the second strand cDNA synthesis reaction. The cDNA:mRNA hybrids (estimated as 0.64 μg using salmon sperm DNA to generate a standard curve with a fluorometer) were dissolved in 32 μl TE, to this was added 50 μl of 2x buffer containing 40 mM Tris-HC1 pH 7.5, 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 200 mM KCI, 100 μg/ml bovine serum albumin, 2 μl of 4 mM dNTP (all four), 18 units/ml of RNase H, and 230 units/ml of DNA polymerase. The mixture was incubated at 12°C for 1 hour, then 22°C for 1 hour, and finally, the reaction stopped with 20mM EDTA. The preparation was extracted with P:CI and CI, precipitated with ammonium acetate and isopropanol, washed with *75%* ethanol, and dissolved in 50 μl of TE.

The double stranded cDNA was size selected using a Sepharose CL-4B column equilibrated with TE containing 0.1 M NaC1 pH 7.6. Aliquots #12 to 17 were pooled *(Fraction-*

1), estimated as being >500 bp; and aliquots #18 to 26 were also pooled *(Fraction-2)*, estimated <500 bp. The two fractions were precipitated, washed with 75% alcohol, subjected to 3.5% non-denaturing polyacrylamide gel electrophoresis and autoradiographed at room temperature for one hour (**FIGURE-II-2**). The large fragment fraction *(Fraction-1)* of subtracted cDNA was dC-tailed using terminal nucleotidyl transferase (Boehringer Mannheim), and ligated with dG-tailed pBR322-cut at the Pst-1 site (Life Technologies) using T4 DNA ligase. The ligation reaction was incubated overnight at 8°C.

The recombinant plasmid preparation was used to transform Max Efficiency HB101 competent cells (Life Technologies), and selected using Tetracycline (12.5 μg/ml). The colonies were screened with lifts made on nitrocellulose membranes which were hybridized with the *Fraction-1* of subtracted cDNA that was also used for cloning. Strong hybridization to transferred colonies was observed in a preparation made at high dilution indicative of successful cloning. Transformation in the control sample containing pBR322 without any cDNA inserts had a cloning efficiency of $9x10^6$ colonies/μg DNA.

SUBTRACTION LIBRARY: SIZE SELECTION

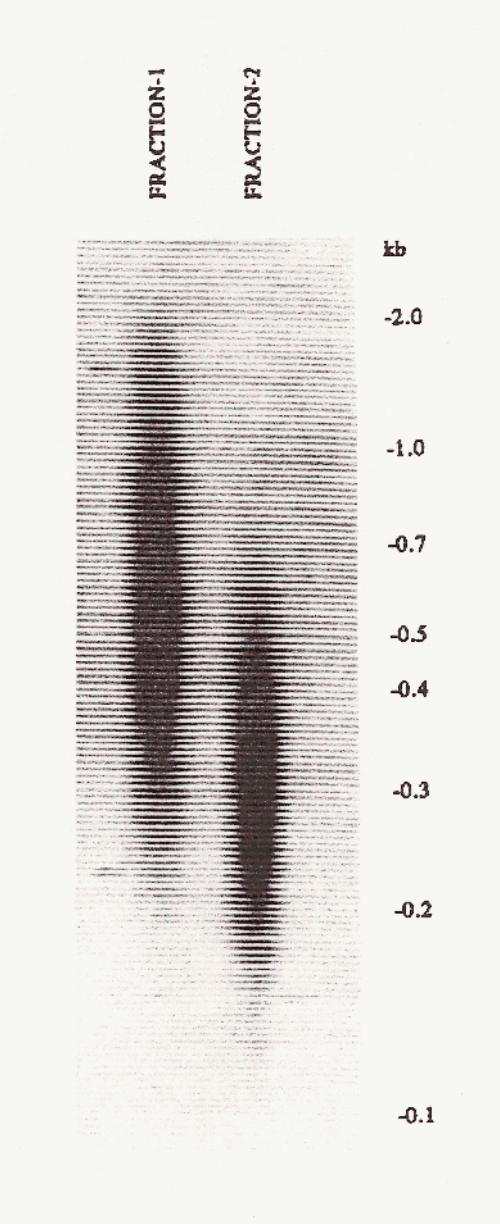


FIGURE-II-2: Size selection of the subtracted library. The aliquots of double stranded cDNA were eluted from the Sepharose CL-4B column was pooled into two separate fractions. *Fraction-1* was obtained by pooling aliquots #12 to 17, and *Fraction-2* was a mixture of aliquots #18 to 26. *Fraction-1,* being larger in size than *Fraction-2,* was used for cloning. The size markers are indicated. Kb = kilo base pairs.

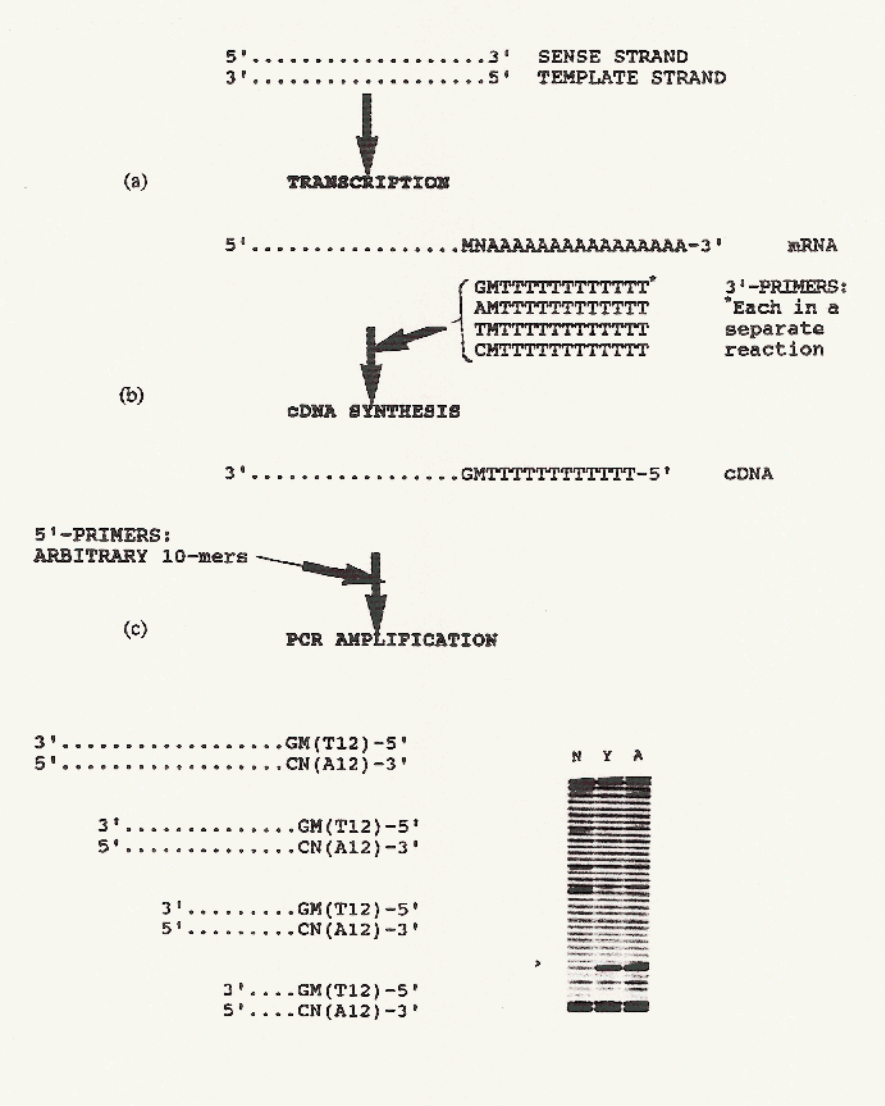
IID. DIFFERENTIAL DISPLAY

Although, the technique of differential display was described by Liang and Pardee (1992), a similar principle is used in RNA fingerprinting described by Welsh et al (1992). The basis for both techniques is the observation that poly(T) sequences with anchoring bases can function as primers (Khan et al 1991). In the differential display procedure, degenerate primers are used to reverse transcribe and amplify poly(A)-containing mRNA sequences which are then separated on polyacrylamide gels (Liang & Pardee 1992; Welsh et al 1992; Liang et al 1992, 1993; Sager et al 1993). At first, poly(T) 3'-primers with two anchored bases are used for reverse transcription. Thereafter, degenerate 10-mer sequences are added to the sample, and then the reaction amplified by PCR. The resulting products are separated on 6% acrylamide DNA sequencing gels under denaturing conditions **(FIGURE-II-3).**

The differential display technique allows the simultaneous visualization and comparison of cDNA bands in different samples. Therefore, cDNA was prepared from *neonate, young adult* and *aged* rat brain, and compared on polyacrylamide gels. The inclusion of the *aged* brain sample was based on the reasoning that there may exist differences in gene expression between the *adult* and *aged* brain as well. Total RNA samples from *neonatal* (3-days old), *young adult* (3-months) and *aged* (33-months) male Fischer 344 rat brain were prepared and processed for differential display (Joseph et al 1994) (**FIGURE-I-9**).

Fifty μg of total RNA from each age group was treated with DNase-I to remove

DIFFERENTIAL DISPLAY



FJGURE-II-3: The technique of differential display. The mRNA transcribed from the template strand (a), was used for cDNA synthesis with 3'-primers consisting of dT-oligonucleotides with two anchored bases (b). Four 3'-primers were used, their penultimate bases being G, A, T or C. The last base, M, was a mixture of G +A + C. After cDNA synthesis, arbitrary 10-mer sequences were added to the mixture, and PCR amplified (c). The cDNA fragments were separated on 6% polyacrylamide gels.

contaminating genomic DNA in a mixture containing 20 u of RNase-free DNase-I, 80 u human placental RNase Inhibitor, 10 mM Tris HC1, 1.5 mM $MgC1_2$ and 50 mM KCI. The mixture was incubated at 37°C for 30 min, phenol:chloroform extracted and ethanol precipitated. One μg aliquots of DNase-I treated total RNA from *neonatal, young adult* and *aged* rat brain were reverse transcribed in separate reactions containing 2.5 μM T_{12}MG as the 3'-primer (M=equal mixture of G+A+C), 20 μM dNTP (all four), 300 u MMLV-Superscript-II and lx MMLV buffer. The preparation was incubated at 35°C for 60 mm, and the enzyme inactivated at 95°C for 5 min. In parallel reactions, reverse transcription was also carried out using the remaining three 3'-primers, T_{12}MA, T_{12}MT and T_{12}MC. Each reverse transcription reaction was PCR amplified with five degenerate 10-mer sequences (5'-primers, Operon Technologies, Alameda, CA), in separate reactions. The concentration of each 5'-primer was 0.5 μM. There were a total of 20 separate reactions, containing combinations of the four anchored 3'-primers and the five 10-mer 5'-primers.

The sequences of the primers used (**FIGURE-II-4**), and the 20 primer combinations used are shown (**FIGURE-II-5**). Included in each PCR reaction, in a total volume of 20 μl, were ^{35}S-αdATP (1386 Ci/mmol, 0.5μM) and *2.5* u Taq DNA polymerase. The preparation was denatured at 94°C for *5* min, and cycled 40 times. Each cycle consisted of denaturation at 94°C for 30 secs, annealing at 40°C for 2 min, and extension at 72°C for 30 secs. Thereafter, the samples were extended at 72°C for *5* min, and kept at 4°C. Loading buffer was added to the samples and electrophoresed on 6% polyacrylamide gels.

DIFFERENTIAL DISPLAY: PRIMERS

3'-PRIMERS*

T_{12}MG: 5' -TTTTTTTTTTTTMG-3'

T_{12}MA: 5' -TTTTTTTTTTTTMA-3'

T_{12}MT: 5' -TTTTTTTTTTTTMT-3'

T_{12}MC: 5' -TTTTTTTTTTTTMC-3'

*

Mixture of G+A+C

5'-PRIMERS**

OPA-1: 5'-CAGGCCCTTC-3'

OPA-2: 5' -TGCCGAGCTO-3'

OPA-3: 5' -AGTCAGCCAC-3'

OPA-4: 5' -AATCGGGCTG-3'

OPA-5: 5' -AGGGGTCTTG-3'

**

Obtained from Operon Technologies Inc., Alameda, CA.

FIGURE-II-4: The primers used for differential display. The four 3'-primers were T_{12}MG, T_{12}MA, T_{12}MT and T_{12}MC, and the five 5'-primers were OPA-1 to OPA-5.

DIFFERENTIAL DISPLAY: PRIMER COMBINATIONS

GEL-1	GEL-3*	GEL-4	GEL-5
N: T_{12}MG:OPA1	N: T_{12}MA:OPA1	N: T_{12}MT:OPA1	N: T_{12}MC:OPA1
Y: T_{12}MG:OPA1	Y: T_{12}MA:OPA1	Y: T_{12}MT:OPA1	Y: T_{12}MC:OPA1
A: T_{12}MG:OPA1	A: T_{12}MA:OPA1	A: T_{12}MT:OPA1	A: T_{12}MC:OPA1
N: T_{12}MG:OPA2	N: T_{12}MA:OPA2	N: T_{12}MT:OPA2	N: T_{12}MC:OPA2
Y: T_{12}MG:OPA2	Y: T_{12}MA:OPA2	Y: T_{12}MT:OPA2	Y: T_{12}MC:OPA2
A: T_{12}MG:OPA2	A: T_{12}MA:OPA2	A: T_{12}MT:OPA2	A: T_{12}MC:OPA2
N: T_{12}MG:OPA3	N: T_{12}MA:OPA3	N: T_{12}MT:OPA3	N: T_{12}MC:OPA3
Y: T_{12}MG:OPA3	Y: T_{12}MA:OPA3	Y: T_{12}MT:OPA3	Y: T_{12}MC:OPA3
A: T_{12}MG:OPA3	A: T_{12}MA:OPA3	A: T_{12}MT:OPA3	A: T_{12}MC:OPA3
N: T_{12}MG:OPA4	N: T_{12}MA:OPA4	N: T_{12}MT:OPA4	N: T_{12}MC:OPA4
Y: T_{12}MG:OPA4	Y: T_{12}MA:OPA4	Y: T_{12}MT:OPA4	Y: T_{12}MC:OPA4
A: T_{12}MG:OPA4	A: T_{12}MA:OPA4	A: T_{12}MT:OPA4	A: T_{12}MC:OPA4
N: T_{12}MG:OPA5	N: T_{12}MA:OPA5	N: T_{12}MT:OPA5	N: T_{12}MC:OPA5
Y: T_{12}MG:OPA5	Y: T_{12}MA:OPA5	Y: T_{12}MT:OPA5	Y: T_{12}MC:OPA5
A: T_{12}MG:OPA5	A: T_{12}MA:OPA5	A: T_{12}MT:OPA5	A: T_{12}MC:OPA5

N = Neonate, Postnatal Rat Pup (3 Days)
Y = Young, Young Adult Rat (3 Months)
A = Aged, Aged Rat (33 Months)

*

Gel-2 was a repeat of Gel-1.

FIGURE-II-5: The primer combinations used for differential display. A total of 20 primer combinations were used. With each primer combination, differential display was carried out using neonatal (N), young adult (Y), and aged (A) rat brain. In Gel-1, T_{12}MG was the 3'-primer, used in combination with five separate 5'-primers, OPA- 1, OPA-2, OPA-3, OPA-4 and OPA-5. The 3'-primer in Gel-3 was T_{12}MA, Gel-4 was T_{12}MT, and in Gel-5 T_{12}MC.

The gels (GEL#-l, 3, 4, 5) were dried (80°C for *45* min) and exposed at -70°C to film (X-OMAT-AR, Eastman Kodak, Rochester, NY) for 16 hours. GEL-2 was a duplicate of GEL-1; both GEL-1 and GEL-2 were similar in appearance. Differentially expressed cDNA fragments seen to be strongly expressed in *neonatal*, *adult* or *aged* brain were cut out of the gel by overlaying the autoradiogram. The cDNA fragment in the cut piece of gel was extracted by boiling with 500 μl of 1x TE for 15 min, phenol-chloroform extracted, ethanol precipitated, and PCR amplified. Amplification was carried out using the same set of primers and conditions as that employed in the original differential display reaction. The sizes of the amplified PCR products were confirmed on 1.5% agarose gels.

IIE. RAT BRAIN cDNA LIBRARY

A cDNA library was constructed using a combination of mRNA from *neonatal, young adult* and *aged Fischer 344* rat brain adapting established procedures (Jendrisak et al 1987). One mg of total RNA was prepared by the single step acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987), contaminating genomic DNA was removed by digestion with RNase-free DNase, and poly(A) RNA isolated by oligo-dT cellulose chromatography (Pharmacia). Four μg of poly(A) RNA was reverse transcribed using 200 u Superscript-II MMLV reverse transcriptase, 0.5 μg of oligo-$dT_{12\text{-}18}$, in 80 μl of 50 mM Tris, pH 8.3. The resulting RNA-DNA hybrids were converted to ds-cDNA with 4 u RNase-H, 46 u DNA polymerase in 100 μl of 20 mM Tris pH 7.5. The ds-cDNA was blunt ended using mung bean nuclease (43 u/200, μl of reaction mixture), 30mM sodium acetate pH 5.2 and 1 mM Zn^{2+} as an activator. For high efficiency cloning of blunt ended ds-cDNA, EcoRI adapters with a nested NotI site (Life Technologies) were ligated to the ends of the cDNA using 20 u T4 Ligase, 66 mM Tris pH *7.5* in a 50 μl reaction volume.

Excess adapters and smaller cDNA fragments (< 500 bp) were removed using a cDNA fractionation column (Life Technologies). The ds-cDNA with ligated EcoRI(NotI)-adapters was phosphorylated using 40 u T4 nucleotide kinase, 66 mM Tris pH 7.5 and 1 mM ATP, and cloned into the unique EcoRI site located at the 3'-end of LacZ gene in λgt11. Recombinant λgt11 phage was packaged using X Packaging System (Life Technologies), and used to infect E.coli Y1090($R^-M^-\Delta$LacU169). The efficiency was about 3.5 x 10^5 pfu/μg cDNA. More than

90% of the plaques were detected by blue/white selection to contain recombinant phage.

IIF. CELL CULTURE

The cell lines used were PC12 (rat pheochromocytoma) (Greene & Tischler 1976), NCB2O, a hybrid cell line (Chinese hamster cortical neuron x rat neuroblastoma) (Klee & Nirenberg 1974) and 9L (rat gliablastoma) (Kim et al 1990).

PC12 cells are a well studied model of neuronal growth and differentiation. Treatment of undifferentiated PC12 cells with *Nerve Growth Factor (NGF)* results in the cessation of cell proliferation and transformation into a neuronal phenotype, including the extrusion of neurites, typical of the differentiated adult neuron. PC12 cells (#CRL-1721) were obtained from American Type Culture Collection, Bethesda, MD.

NCB2O cells were generously provided by Dr. M. Nirenberg, National Institutes of Health, Bethesda, MD, and 9L cells were provided by Dr. S. Gautam, Henry Ford Hospital, Detroit, MI. In addition, Dr. Gautam also provided a primary rat glial cell line. PC12 cells were grown on collagen-coated dishes in a medium containing 85% DMEM with high glucose, 10% horse serum and 5% fetal calf serum. NCB2O, 9L and the primary glial lines were grown in 90% DMEM with high glucose and 10% fetal calf serum. All cells were grown at 37°C in a humidified incubator with 5% CO_2 (Joseph & Han 1992).