CHAPTER 2

MATERIALS AND METHODS

ANIMALS

Cohorts of Wistar strain rats in-bred over generations and maintained in our animal house were used. The three age groups studied were 5 days postnatal, 6 months and >2 years. We designated these three age groups as 'Young', 'Adult', and 'Old' respectively. Rats were maintained in a pathogen free environment with a 12h light-dark cycle. Food and water were provided ad libitum.

CHEMICALS

Highly Polymerized calf thymus DNA, Bovine serum albumin, Adenosine Triphosphate (ATP), Leupeptin, Pepstatin, Phenylmethyl sulfonyl Flouride (PMSF), Dithiothreitol (DTT), Sephadex G-50, Trypsin Type V-S from bovine pancreas, Trypsin inhibitor type II from soyabean, Sigmacote, were purchased from Sigma chemical Co., St.Louis, MO, USA. Unlabeled nucleotides, 2'-deoxyadenosine 5'- triphosphate (dATP), 2'-deoxycytidine 5'- triphosphate(dCTP), and 2' -deoxyguanosine 5'- triphosphate (dGTP) and thymidine 5'- triphosphate(TTP) were purchased from pharmacia Fine chemicals, Uppsala, Sweden. Poly (dA). Oligo (dT) $_{12\text{-}18}$ and Poly (dA.dT) were purchased from Midland Certified Reagent Co. (Midland, TX, U.S.A.). Monoclonal antibody, SJK 132-20 against human DNA Polymerase a was obtained from PL-Biochemicals, Wisconsin, U.S.A. Dimethyl sulphoxide (DMSO) was from Sisco Research Laboratory (Bombay, India). ddTTP was from Boehringer Mannheim, Germany. The nucleotide analogs, N2- (p-n-butylphenyl)-2'-deoxyguanosine-5'-triphosphate (BuPdGTP) and N2- (p-n-butylanilino)-2'-deoxyadenosine-5'-triphosphate (BuAdATP), were generous gifts from Dr George Wright (Dept. of Pharmacology, University of Massachusetts Medical School, Worcester, MA, U.S.A.). (H)-TTP (specific activity 78 Ci/mmol) was purchased from New England Nuclear, USA. Radiolabeled (^{32}P –α-dCTP; ^{32}-P -γ -ATP) were purchased from BARC (Bombay, India),

E.Coli DNA Polymerase I (Pol 1), Ficoll 400 were purchased from Amersham Pharmacia Biotech, Uppasala, Sweden. Calf thymus terminal transferase was purchased from Roche Applied Science. Restriction endonuclease HinP1 was purchased from New England Biolabs. 2,5-Diphenyl-1,3-Oxazole (PPO) and 2,2'-p–Phenylene-bis[5-henyloxazole] (POPOP) were purchased from Beckman instruments Inc., Fullerton, CA, USA. GF/C filters were purchased from Schleicher and Schuell, Dassel, Germany. Nitex nylon screens of definite pore sizes were purchased from Small parts Inc., Miami, Florida,USA. PAGE purified synthetic deoxyoligonucleotides were supplied by BangaloreGenei, Bangalore, India. All other chemicals used were of analytical grade.

Isolation of Neuronal and astroglial enriched fractions from Young, Adult and Old rat brains

Reagents

1) Isolation medium: 8% glucose (w/v), 5% fructose (w/v) and 2% Ficoll in 10 mM KH_2PO_4 -NaOH buffer, pH 6.0.
2) 0.1% (w/v) Trypsin in isolation medium.
3) 0.1% (w/v) Trypsin inhibitor in isolation medium.
4) 7% (w/v) ficoll in isolation medium.
5) 10% (w/v) ficoll in isolation medium.
6) 22% (w/v) ficoll in isolation medium.
7) 28% (w/v) ficoll in isolation medium.

Neuronal and astroglial cell enriched fractions from rat cerebral cortex of different ages were prepared essentially as standardized in this laboratory (Usha rani et al., 1983). The rats were decapitated, brain removed and taken in isolation medium in ice. The entire cerebral hemispheres were removed. Grey and white matter were separated from cerebral

cortex and grey matter was sliced into very small pieces and incubated at 37°C for one hour in the 0.1% trypsin. Grey matter from young was incubated in isolation medium at 37°C for 30minutes. After the incubation trypsin containing medium was carefully removed and an equal amount of 0.1% soyabean trypsin inhibitor in isolation medium was added and chilled on ice for 5 minute. The remaining procedure was carried out at 0-4°C.The medium containing trypsin inhibitor was discarded and the tissue was washed with ice cold isolation medium and passed through nylon membranes of pore sizes 105μm, 80μm, 48μm. The tissue was placed on 105uM nylon mesh stretched over a porcelain Hirsch funnel, and gently stirred by using a glass rod to aid the screening process. During this process the tissue was kept moist by addition of ice cold isolation medium. The cell suspension obtained after passage through the 105μM mesh was then passed through 80μM nylon mesh and finally through 48μM nylon mesh three times each.

The resulting crude cell suspension was centrifuged at 760 x g for 15 minutes. The supernatant th obtained was discarded and the crude cell rich pellet which consisted of both neurons and astrocytes was suspended in 20ml(10ml per gram of the tissue) 7% ficoll in isolation medium and centrifuged at 270xg for 10 minutes and the pellet obtained is mostly composed of neurons. The supernatant composed mostly of astrocytes.

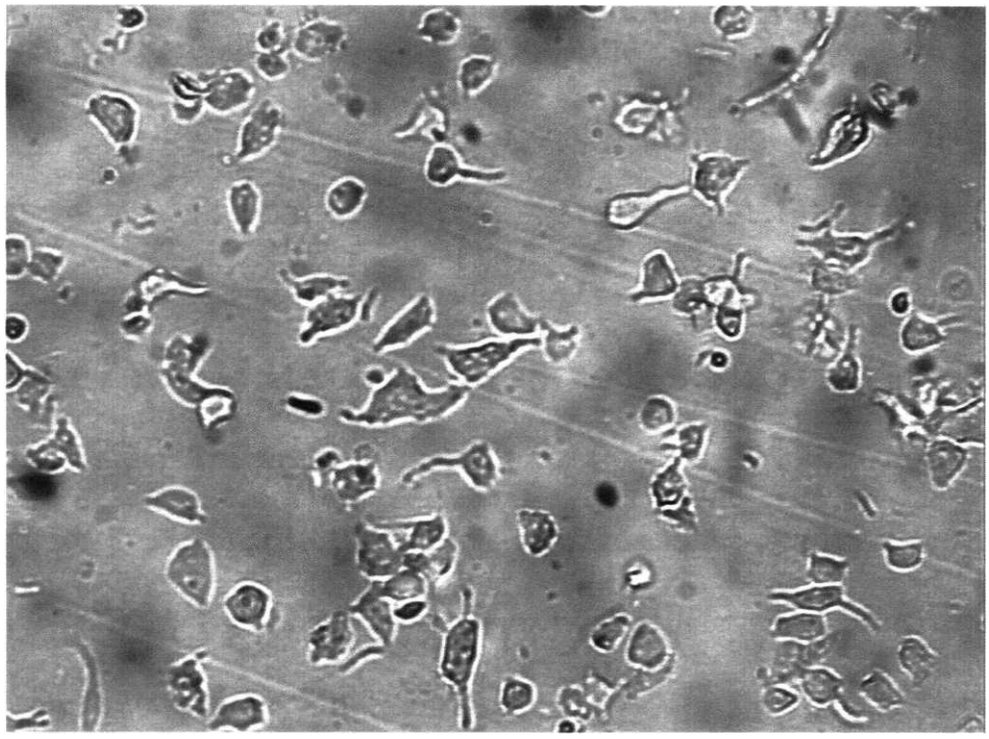
The crude neuronal pellet was suspended in 5 ml of isolation medium and was loaded onto discontinuous Ficoll gradients for further purification. The supernatant was diluted in a ratio of 1:1.125 with isolation medium and centrifuged at 1100 x g for 10 minutes. Supernatant was discarded and the astrocyte rich pellet obtained was suspended in 5ml of isolation medium and was loaded onto discontinuous ficoll gradients for further purification. Ficoll gradients were prepared in 50ml Polycarbonate tubes from the bottom

up, of 5ml each of 28%, 22%, 10% ficoll (w/v) in the medium. The neuronal and astroglial cell suspension was loaded onto the 10% ficoll and centrifiiged at 7800 x g for 20 minutes in swinging bucket rotor. The layers at each interface were removed carefully with a Pasteur pipette. Neurons were obtained as a pellet in 28% ficoll gradient. Astrocytes were obtained as a layer in 22% ficoll gradient. The interface between 22% and 10% consisted of broken processes and debris and was discarded. Cells, both neurons and astrocytes were collected from gradient and then washed with 5ml of medium without ficoll three times at 1500 x g for 10 minutes and then in 5ml of phosphate buffered saline (1X PBS pH 7.4) thrice at 1500 x g for 10 minutes. Counting of the cells was done and viability of the cells was determined by Trypan blue exclusion and was found to be > 85%. The cells were routinely examined for their characteristic morphology (Figure 2).

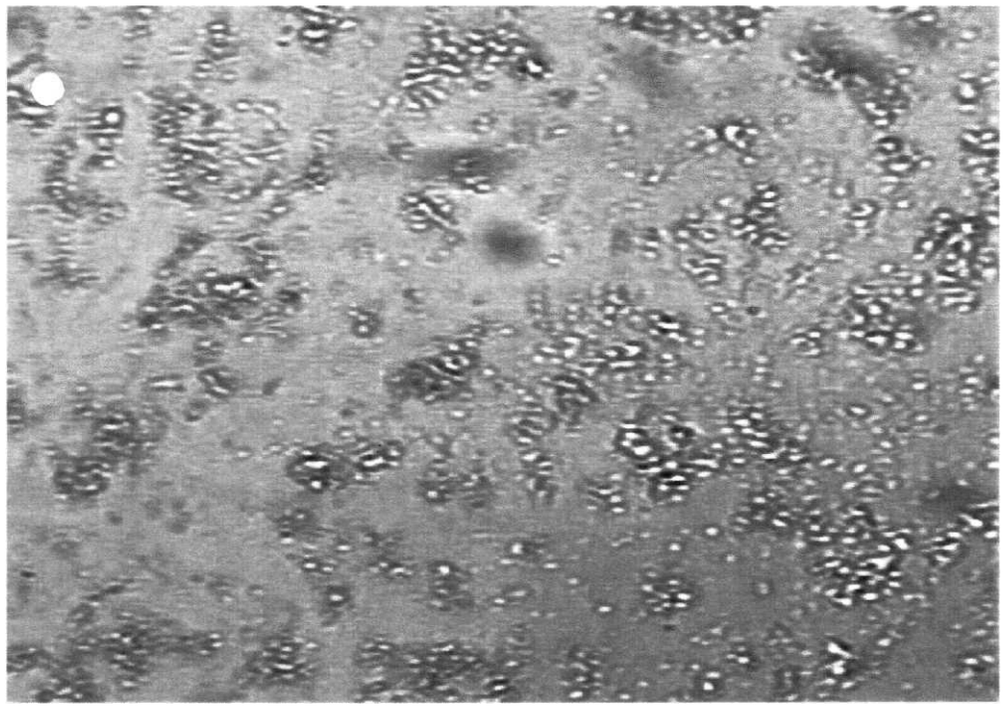
Figure 2

Morphology of the Neurons and Astrocytes isolated from 'Young' (5 days postnatal) rat brain cerebral cortex by Ficoll gradient (28% ficoll). Image taken at 40X magnification from an inverted microscope

NEURONS



ASTROCYTES



Preparation of DNA **Polymerase enzyme extract**

The final preparation of the cells was suspended at a concentration of 10 million cells/ml in extraction medium. The extraction medium consists of 20 mM Tris pH 7.5, 0.1 mM dithiothreitol, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 0.1 mM PMSF (just before use), 5 mM P-mercaptoethanol, 1 mM $MgCl_2$, 1 μg/ml Leupeptin and 1 μg/ml Pepstatin A (both just before use) and 0.5 M KCl and sonicated for 5 seconds, 3 times with the setting at 5 in a Branson sonifler. The suspension was kept at 0-4°C for 30 min and then centrifuged at 100,000 xg for 1 hour in a Beckman Ultracentrifuge and the clear supernatant was used as the source for DNA Polymerases and exonuclease activity. Protein concentration was estimated by the method of Bradford (1976)

DNA Polymerase assay

The activity of total DNA Polymerase was assayed according to the procedure of Prapurna and Rao (1996).

Total DNA Polymerase assay

The reaction mixture contained in a final volume of 50μl, 40 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 1mM P-mercaptoethanol, 4 mM ATP, 100μM each dATP, dGTP, dTTP, 25μM dCTP, 5 ug `Activated' calf thymus DNA, 1μCi α-^{32}P-dCTP (4000Ci/mmol) and cell extract as enzyme source (10 μg protein). The incubation was carried out at 37 C for 20 minutes. 200 ug of Calf thymus DNA and BSA were added as carriers and the reaction was stopped with 1M perchloric acid: 10 mM tetra sodium pyrophosphate. The samples were kept on ice for 5 minutes and centrifuged at 3000 x g for 10 min. The supernatant was aspirated carefully and the precipitate was dissolved in 0.5 ml of 0.2 M NaOH. A 2 ml volume of stop solution was again added and centrifuged after 10 min. The alkali

solubilization step and reprecipitation with stop solution was repeated. The whole solution along with the precipitate was transferred to 2.5 cm glass fiber filters (Schleicher & Schuell) and washed three times with ice-cold stop solution and twice with 95% ethanol. The washed filters were dried by keeping in oven at 40° C for 20 min or keeping in a hood overnight. The dried filters were then taken in toluene-based scintillation fluid containing 5g PPO and 0.5g POPOP per litre having 0.1% Triton-X- 100 and the radioactivity was counted in a Wallac 1409 counter. The specific activity is expressed as picomoles of dCMP incorporated into acid insoluble fraction /mg protein/ hour.

DNA Polymerase assay with Poly (dA). Oligo (dT) $_{12-18}$ or Poly (dA.dT) as **template primer**

The reaction mixture with Poly (dA). Oligo (dT) $_{12-18}$ or Poly (dA.dT) as template primer contained in a final volume of 50 µl, 40 mM Tris-HCl pH 7.5, 0.8 mM $MgCl_2$, 5 µg of bovine serum albumin, 2% glycerol, 2 mM dithiothreitol, 50 uM dATP (when Poly dA.dT was the substrate), 25 uM TTP, 1 µCi α-^{32}P-TTP (4000Ci/mmol) and enzyme (20 ug protein). The incubation was carried out at 37^0C for 20 minutes. The rest of the procedure is carried out as that of the total DNA Polymerase assay. The specific activity was expressed as picomole of TMP incorporated into acid insoluble fraction/mg protein/hour.

Different DNA Polymerase levels in Neuronal and Astroglial fractions

The reaction mixture contained either 'Activated DNA' or Poly (dA). oligo $(dT)_{12-18}$ as substrates and the components of the reaction mixture are same as that of total DNA Polymerase assay with 'Activated DNA' or with Poly (dA). oligo (dT) $_{12-18}$. When 'Activated' DNA is the substrate inhibitors were used at two different concentrations in polymerase assay and the inhibitors are BuAdGTP, BuAdATP, ddTTP and Pol **a** specific monoclonal antibody-SJK-132-20. BuAdGTP is used at two concentrations of 1 and

200μM, for BuAdATP 1 and 100μM, for ddTTP 50uM and 1mM and Pol a specific monoclonal antibody-SJK-132-20 is used at a concentration of 1μg.

With Poly (dA). Oligo (dT) $_{12\text{-}18}$ the same procedure is followed except that the concentration of BuAdGTP used were 10μM and 100μM and those for BuAdATP were 15 and 200μM respectively.

Concentration of the enzyme taken was 20μg. The extracts were preincubated with the inhibitor for 10 min on ice prior to the start of the reaction. After the preincubation the reaction was carried out at 37^0C for 20 minutes. The rest of the procedure is carried out as that of the total DNA Polymerase assay. The specific activity was expressed as picomole of dCMP/TMP incorporated into acid insoluble fraction/mg protein/hour. Statistical analysis of the data was done using Sigma Plot 2000 software.

Primer extension assays

A model substrate to measure the Pol β activity in neuronal extracts of rats of different ages is designed as follows.

5' - c g c g a t c g g t a g c G –3	(14 mer-oligo 1)
3 - g c g c t a g c c a t c g C g t t a c c g - 5	(21 mer-C- oligo 2)
5 - c g c g a t c g g t a g c G - 3	(14 mer- oligo 1)
3 - g c g c t a g c c a t c g T g t t a c c g - 5	(21 mer -T- oligo 3)
5 - c g c g a t c g g t a g c G - 3	(14 mer- oligo 1)
3 - g c g c t a g c c a t c g A g t t a c c g – 5'	(21 mer -A - oligo 4)
5 - c g c g a t c g g t a g c G - 3	(14 mer- oligo 1)
3 ' - g c g c t a g c c a t c g G g t t a c c g - 5	(21 mer –G- oligo 5)

Oligo Duplexes Used for the Gap repair assays

1 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c - a g a t t t t t g c g g t g c c - 3' (17 mer)
3'-gc t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

4 Gap Oligo duplex:

(14 mer) ^{32}P 5'- c g a g c c a t g g c c g c ——— t t t t t g c g g t g c c - 3' (14 mer)
3'- g c t c g g t a c c g g c g g t c t a a a a a a c g c c a c g g - 5' (32 mer)

5'-End labeling of 14-mer with T4 Polynucleotide Kinase (T4PNK)

5'- end of the 14 mer (Primer) is radioactively labeled with γ-^{32}P-ATP. In a final reaction volume of 5µl, 2 picomoles of the 14-mer (oligo-1) was 5'-kinased using equimolar γ-^{32}P-ATP (specific activity, 5000 Ci/mmol) and T4-Polynucleotide kinase (2.5 units/ picomole of substrate). The reaction was carried out for 40 minutes at 37° C in the buffer to a final concentration (70 mM Tris Hcl, pH 7.6, 10 mM $MgCl_2$ and 5 mM dithiothreitol). The reaction is stopped by heating at 70°C for lOminutes. Each µl of the substrate contains 400 femtomoles of the 5'end labeled 14-mer.

5'-End phosphorylation of Oliogodeoxynucleotide with unlabeled ATP

5'- end of the 14 mer (Primer) is phophorylated with unlabeled ATP. In a final reaction volume of 5 µl, 1 picomoles of the 14-mer (oligo-1) was 5'-kinased using 1mM ATP and T4-Polynucleotide kinase (2.5 units / picomole of substrate). The reaction was carried at 37°C for 10 min and the reaction is stopped by heating at 70°C for 10 min. Each µl of the substrate contains 400 femtomoles of the 5'end phosphorylated 14-mer.

3'-End labeling of 14-mer with Terminal transferase (TdT)

The 3' end of the 14 mer is radioactively labeled using α-^{32}P-dCTP and Terminal transferase enzyme. The final reaction volume of 5µl contained 1 picomoles of the deoxyoligonucleotide, 1 picomoles of a- P-dCTP, 1X reaction buffer (200mM potassium cocadylate, 25mM Tris-Hcl, Bovine serum albumin 0.25mg/ml pH 6.6 at 25°C), 1 mM $CoCl_2$ and 1 unit of terminal transferase. Incubation was carried at 37^0C for 10 min and the reaction is stopped by heating at 70°C for 10 min. Each µl of the substrate contains 400 femtomoles of the 3'end labeled 14-mer.

Annealing of Oligodeoxynucleotides

The 5'-^{32}P-kinased or 3'- end labeled 14-mer was hybridized with each of the four different 21-mers (oligos 2 to 5) in equimolar concentrations of 14mer and 21-mer in the reaction mixture containing 50 mM NaCl and 5 mM $MgCl_2$. The annealing reaction was carried out at 70°C for 10 minutes and gradually cooling to room temperature.

For annealin£ he substrates in the gap repair experiments, 32 mer (Template) was annealed to the 5'-end labeled 14 mer and either 17mer or 14 mer as the downstream primer. Equimolar concentrations of the oligos were annealed in the reaction mixture containing 50 mM NaCl and 5 mM $MgCl_2$. The annealing reaction was carried out at 70°C for 10 minutes and gradually cooling to room temperature.

DNA Polymerase assay with synthetic oligodeoxy duplexes

The procedure for the DNA Polymerase assay using synthetic oligodeoxyduplexes is essentially similar to that of DNA Polymerase assay except that the substrate used is 400 nm of Oligoduplex template primer (unlabeled 14-mer hybridized to four different 21-mers) but with one of the four dNTPS labeled (usually dCTP). Specific activity was expressed as the

femtomoles of the radioactive nucleotide incorporated into acid insoluble fraction/mg protein/hour.

Assay of the mismatch removal and primer Extension (exo-extension) activity of neuronal extracts.

Primer Extension assays was carried as follows. The final reaction volume of 30ul contained 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, O.lmg/ml bovine serum albumin, 2% glycerol and 20 uM of all the four dNTPS. 400 femtomoles of the hybridized oligo duplex either with correctly matched (G-C) or with mismatched base pair (G-A, G-G, G-T) or single stranded 5'-end labeled 14-mer/21-mer were incubated with 5μg of neuronal protein extracts at 37^0C for 20minutes or at different time intervals of 1,3,5,10,20 minutes and the reaction is stopped by heating at 70°C for 10 minutes.

Single step reaction

Single step reaction essentially consists of incubating the 400 femtomoles of 5'- P-kinased primer (14mer) hybridized to four different 21 mers in the reaction buffer containing 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, O.lmg/ml Bovine serum albumin, 2% glycerol and 20 uM of all the four dNTPS in a final reaction volume of 30μl with 5 μg of Young, adult and old neuronal extracts alone or supplemented with pure recombinant rat liver DNA Polymerase p or pure calf thymus α-Polymerase or E.coli Polymerase I.The reaction was carried at 37°C for 20minutes and stopped by heating at 70°C for 10 minutes.

Two step reaction

The two step reaction is divided into two steps. The first step consisted of final reaction volume of 10μl and the reaction buffer to the final concentration of 20mM HEPES pH 7.5, 1mM $MgCl_2$, 0.1 mM DTT, O.lmg/ml Bovine serum albumin, 2% glycerol without

dNTPS. 400 femtomoles of the hybridized oligo duplex was incubated with 5 μg of the neuronal protein extracts at 37^0C for 10minutes and then stopped by heating at 70°C for 10 minutes. The second step consisted of final reaction volume of 30μl and the reaction buffer to the final concentration of 20mM HEPES pH 7.5, 1mM $MgCl_2$, 0.1 mM DTT, O.lmg/ml bovine serum albumin, 2% glycerol and dNTPS were added to a final concentration of 20μM. Pure recombinant rat liver β-Pol (2.5 units)was added and incubated further for 20 minutes at 37^0C and then the reaction was stopped by heating at 70°C for 10 minutes. Two step reactions with Mncl2 were essentially carried out as outlined above with lmM $MnCl_2$ instead of 1mM $MgCl_2$. lmM $MnCl_2$ is used in the second step of the two step reaction only.

Three step reaction

The final reaction volume of the three step reaction is 40 ul. Half the reaction product of the two step reaction as outlined above (15μl) was taken and the restriction digestion w. carried out in a reaction volume of 40 ul with lOunits of restriction endonuclease HinPI in the 1x reaction buffer (5 mM NaCl, 1 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol (pH 7.9 at 25°C). The incubation was carried for 6 hours at 37°C and then stopped by addition of 1ul of 0.5M EDTA.

3'-5' exonuclease assay with neuronal extracts

Exonuclease assays was carried exactly similar to that of the primer extension assays. The final reaction volume of 30μl contained 20mM HEPES pH 7.5, lmM $MgCl_2$, 0.1 mM DTT, 0.1mg/ml Bovine serum albumin, 2% glycerol and with or without 20 uM of dATP, dGTP, dTTP, dCTP. 400 femtomole of the hybridized oligo duplex either with 573' ^{32}P end labeled correctly matched (G-C) or with mismatched base pair(G-T) or single

stranded 5'/3'-end labeled 14-mer/21-mer was incubated with 5ug of neuronal protein extracts at 37^0C for 10minutes or at different time intervals of 1,3,5,10,20 minutes. The reaction was stopped by heating at 70°C for 10 minutes.

Sephadex G-50 Column purification of labeled Oligoduplex

Sephadex G-50 columns were prepared in 1ml tips by clogging the tip ends with glass wool. The columns were packed by slowly pouring the slurry of the Sephadex G-50 presoaked in water overnight. The columns were washed twice with water before loading the radiolabeled exoextended products.

Sephadex G-50 columns were used to separate out the excess and unbound p -y-ATP from the labeled substrate. Before loading on to the columns the samples were made upto 100ul with double distilled water and eluted with double distilled water. The first 300ul void volume is discarded and the fractions from 301-900 ul (600µl) were pooled and freeze dried under vacuum. The dried samples were reconstituted with 6ul of DDW and 6X loading dye ٦.002gms of bromophenol blue/ml formamide) in the ratio of DDW to dye of 2:1 ratio. Samples were denatured at 85°C for 5minutes and then immediately cooled on ice.

Assessing the Chain length on 20% PAGE-UREA (7M Urea) sequencing gel

20% Polyacrylamide denaturing Urea gel was poured between glass plates (35 x 45mm). 14.25 gms Acrylamide, 0.750 gms Bisacrylamide, 31.5gms Urea were dissolved in 15ml of 1X TBE (90mM Tris-borate EDTA buffer, pH 8.3) and made upto 75ml.The solution was filtered through cotton and 140ul 10% APS, 14µl of TEMED were added and slowly poured between the glass plates with the help of a syringe. The gel was pre run at 3000v in 1X TBE for 30 minutes by loading the 6X loading dye (0.02gms bromophenol blue in lml formamide).

The denatured samples were loaded onto the gel along with markers and electrophoresed in 1X TBE, at 3000v for 5 hours. The gel was then exposed to the X-ray film for autoradiography. Under these conditions the oligos could be separated on a single base difference.