CHAPTER 2

MATERIALS AND METHODS

(Sources of materials and chemicals.)

Reagents

α^{32}P d ATP (Specific activity 1000 Ci/m mol) as the product of Amersham.

DNA polymerase I, Bam Hl and BSA (nuclease free), Bethesda Research Laboratory (BRL).

Vanadyl Ribonucleoside, Biolabs.

Lysozyme, Calbiochem.

DNase I, Miles Laboratory.

Agarose, DEAE-cellulose paper, ethidium bromide and nitrocellulose paper from Bio-Rad.

Dextran sulfate, RNA (from yeast), d NTP_s, SIGMA.

Bactoagar and yeast extract are products of Difco.

Glyoxal was purchased from Fluka.

All other reagents are of analytical grade.

Samples

Subjects were diagnosed at the division of Hematology, Department of medicine Faculty of Medicine Siriraj Hospital, Mahidol University as previously described (Wasi et al, 1969). They are 4 normal, 13 classical Hb H disease (α-$thal_1$/α-$thal_2$) and 9 Hb H with Hb Constant Spring (α-$thal_1$Hb CS).

Methods

Methodology of various experiments including reagents and materials needed are as follow:

Plasmid preparation

Materials

a) Plasmids :

Two different plasmids used in this work are listed below. Their size and antibiotic resistant properties are indicated in parenthesis.

- p α JW101 (6.1 kb Tc^R) was used as a probe for detecting the α globin messenger RNA. This plasmid was constructed by insertion of complementary α globin DNA (α c DNA) into EcoRI site of pMB 9 using A-T tailing (Wilson et al, 1978)

- p β JW102 (6.1 kb, Tc^R) was used as a probe for detecting the β globin messenger RNA. This plasmid was constructed by insertion of complementary β globin DNA (β c DNA) into EcoRI site of pMB 9 using A-T tailing (Wilson et al, 1978).

Each plasmid was transformed into Ecoli (strain C_{600}) and kept as a stock of plasmid containing E coli strain C_{600}.

b) Medium :

Luria Bertani broth (LB-broth) contains 10g tryptone, 5g yeast extract and 5g NaCl per liter.

c) Antibiotics

Tetracycline (Tc)	25 mg/ml.
Chloramphenical (Cm)	150 mg/ml.

d) Chemicals and solutions

1. Resuspending buffer : 0.05 M Glucose/0.01 M EDTA/0.025 M Tris-HCl pH 8.0/2mg/ml lysozyme. Lysozyme was added freshly before used.
2. Lysis and denaturing solution : 1.0% SDS/0.2 M NaOH.
3. 3.0 M Sodium acetate pH 4.8
4. 0.1 M Sodium acetate/0.05 M Tris-HCl pH 8.0
5. Cold absolute ethanol.
6. TE buffer : 0.01 M Tris-HCl pH 7.4/1 mM EDTA.

Method

The procedure used for plasmid preparation throughout this work was the rapid alkaline extraction method of Birnboim and Doly (1979).

To grow the bacterial cells, one loop of the plasmid containing E.coli stock culture was incubated into a 5 ml LB-broth in the presence of the specific antibiotic according to the type of plasmid inserted in the bacteria. The suspension was then incubated with shaking at 37°c for 5-12 hr until OD_{600} reached the late log phase. Chloramphenical was added to amplify the copy number of plasmids and the culture was shaked

for another 12 hr. The culture was harvested at 4°c by centrifugation in a Sorvall Refrigerated centrifuge at 4 K 4°c for 10 min.

Cell pellet obtained was washed once with cold resuspending buffer and incubated on ice for 30 min to weaken the cell wall. Then, 10 ml of NaOH/SDS was added and the cell suspension was gently swirled. To ensure the completeness of cell lysis, the lysate was maintained on ice for 5 min. Then, 7.5 ml of 3 M sodium acetate pH 4.8 was added to neutralize the solution and to precipitate macromolecules. The precipitation was allowed to complete for 1 hr and the precipitates were removed by centrifugating at 10 K 4°c for 10 min. After getting rid of some floating materials, 50 ml (2.5 volume) of absolute ethanol was added to the clear supernatant to precipitate plasmids. Precipitation was allowed to proceed for ½ hr at -20°c and the plasmid precipitate was centrifuged at 10 K for 10 min. The pellet was dissolved in 10 ml of sodium acetate/Tris pH 8.0 and reprecipitate with 2 volume of absolute ethanol at -20°c for ½ hr. After centrifugation at the same condition, the pellet was redissolved in 300-500 µl of TE buffer. Aliquots of plasmid solution was subjected to agarose gel electrophoresis. The gel was stained with ethidium bromide 2.5 µg/ml for 30 min and DNA bands were visualized under short wave UV light. Each plasmid was identified by comparing its size with the λ DNA markers generated by cutting λ DNA with enzyme Hind III (λ/Hind III). The approximate concentration of plasmid was determined by comparing the band intensity in the agarose gel with those of the λ DNA markers of known concentration in the same gel.

Agarose gel electrophoresis

Materials

a) Equipments

1) Horizontal gel electrophoretic chamber.

2) Power supply (pharmacia or L K B)

b) Chemicals

1) Agarose gel

2) Running buffer : (1x) Tris-acetate buffer containing 40 mM Tris-OH, 20 mM Na Acetate 3 H_2O, 0.2 mM EDTA Na_2 adjust pH 8.3 by acetic acid.

3) Tracking-dye : 0.1% Bromphenol blue/5 mM EDTA in 40% Na_2 EDTA (pH 8.0)/3% Ficoll.

4) Ethidium bromide 0.5-2.5 µg/ml

Method

Agarose gel electrophoresis was performed in a horizontal gel apparatus with the gel bed completely submerged in the Tris-acetate buffer 0.7% Agarose gel was used throughout this work. After melting the gel in Tris-acetate buffer, poured the gel in the chamber which preformed slot by a comb. DNA or RNA samples were loaded into slots by mixing with tracking dye in 3:1 ratio and delivering with autopipettes. Running conditions was from cathode (-) to anode (+) in fixed voltage gradient. After finishing the electrophoresis, the gel was stained

with 0.5 µg/ml ethidium bromide for ½ hr then destained in an excess amount of water. The nucleic acids could be visualized as fluoresed bands when irradiated with UV light.

Purification of plasmid DNA from agarose gel electrophoresis

Materials

1. DEAE - cellulose paper cut to a desired size (approximately 1.0 x 8.0 cm)
2. Sterile Eppendorf tubes punched at the bottom and plugged with siliconized glasswool.
3. Sterile coated test tubes and petridishes.

Chemicals

1. 2.5 M NaCl.
2. 1 mM EDTA.
3. Elution buffer : 1 m Nacl/50 mM Arginine pH 8.5
4. Absolute ethanol.
5. TE buffer.

Methods

The method was based on the technique described by Dretzen. et al (1981) and Kemp et al (1983).

A) Treatment of DEAE - paper

DEAE - cellulose paper was cut to an appropriate size according to the thickness of the gel. The DEAE-strips then were soaked in 2.5 M NaCl in a sterile petridish for a few hours, rinsed several

times in sterile distilled water and kept in 1 mM EDTA at 4°c until use.

B) Separation and trapping of supercoil and relaxed plasmid

Since many form of plasmids were obtained including chromosomal DNA and ribosomal RNA, 0.7% agarose gel electrophoresis was used to purify these plasmids. After 2 hr of electrophoresis, the part of the agarose bed run with size markers was cut out and stained with 2.5 µg ethidium bromide for 15 min. When the position of the supercoil and relax form of plasmid were located, the gel was cut with a sterile surgical blade both below and above the plasmid DNA band to be purified. The treated DEAE - papers were inserted into both sides of the band, the gel was then squeezed together and sealed with a few drops of melted agarose. Electrophoresis was continued until the plasmid band moved to the paper which would take about 30-45 minute according to the distance between the band and DEAE - cellulose strip.

C) Elution of DNA from DEAE - cellulose paper

When electrophoresis was finished, the paper was removed from the gel, rinsed with cold distilled water, drained and blotted dry with filter paper. The strip was cut into small pieces and put into a coated glass tube. Elution buffer was added for 300 µl/50 mm^2 of paper and mixed by vortexing. After 2 hr of incubation at 37°c, the mixture was transfered to an Eppendorf tube prepared previously. The tube was placed on top of a sterile 13 x 100 mm coated tube and contrifuged at 1600xg for 5 min, the solution would be filtered through the glasswool

into the new tube. The purified plasmid DNA in elution buffer was then precipitated overnight with 2 volumes of cold absolute ethanol at -20°c. The precipitate was recovered by centrifugation at 10 K for 10 min in an Eppendorf centrifuge. The pellet was washed with cold 70% ethanol centrifuged again and finally dissolved in an appropriate volume of TE buffer.

Labelling of DNA by Nick Translation

Materials

1) 10X nick translation buffer (0.5 M Tris-HCl pH 7.5/0.1 M $MgCl_2$/10 mM DTT/500 µg/ml BSA (nuclease frees).
2) d ATP, d CTP, d GTP and d TTP.
3) α-^{32}P ATP (specific activity 7000 Ci/m mol).
4) DNA Polymerase I of E. coli (2 units/µl).
5) DN ase I (1 mg/ml as stock solution).
6) DNA substrate to be labelled: JW 101 or JW 102 plasmid.
7) Sephadex G-50 column packed in disposable plastic pipette preruned with TE buffer.
8) 500 µl and 1.5 ml microtubes (sterile).
9) 0.25 M EDTA pH 7.4

Method

A normal reaction mixture comprised of 10 µM of each nonredio active d NTPs, 2 µM of the radioactive d ATP, 250 ng of DNA substrate, about 20 pg of DNaseI diluted just before use, 1 µl of pol in (1x) 0.05 M Tris HCl pH 7.5/10 mM $MgCl_2$/1 mM DTT/50 µg/ml BSA of 25 µl in total volume. The reaction was started by the addition of Pol I and incubate was at 15°c. Kinetics of the reaction was followed by sampling 1 µl from the reaction mixture at given points and precipitated on TCA-soaked GF/A filter. After washing the filter with 5% TCA and

95% ethanol, they were put in 10 ml water and counted in a LS-100 Beckman liquid Scintillation counter. When the reaction reached maximum incorporation, it was stopped by addition of 20 µl of 0.25 M EDTA and heated at 70°c for 5 minute. The mixture was then passed through a Sephadex G-50 column. The labelled DNA could be separated from unincorporated by TE buffer. The aliquotes of 250-500 µl were collected in sterile Eppendorf tubes. The labelled DNA was pooled and 5 µl was counted. The total radioactivity was then calculated.

Extraction of total cellular RNA from whole blood

Subjects

Subjects were diagnosed at the division of Hematology, Department of medicine Faculty of Medicine Siriraj Hospital, Mahidol University as previously described (Wasi et al, 1969). They are 4 normal, 13 classical Hb H disease (α-thal$_1$/α-thal$_2$) (3.7 kb) and 9 Hb H with Hb Constant Spring (α-thal$_1$/Hb CS).

Materials

1) Redistilled Phenol saturated with NETS buffer.
2) 4% Isoamylalcohol in $CHCL_3$
3) Phenol/chloroform mixture : 1 vol of Redistilled Phenol and 1 vol of 4% Isoamyl alcohol in $CHCL_3$
4) NETS buffer : 0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.2%/SDS.
5) Absolute ethanol.
6) 1 mM $MgCl_2$
7) 4 M NaCl.

Method

20 ML of heparinised blood was taken from each subject. Reticulocyte-enriched portion was obtained by removing plasma and buffy coat from the centrifuged whole blood. The cell suspension was washed three times with cold normal saline to remove serum proteins. Two volume of cold 1 mM $MgCl_2$/10 mM Vanadyl ribonucleoside was added to the washed

packed red cells obtained after centrifugation at 2500 rpm to lyse the red blood cells. The mixture was then centrifuged at 10k 4°c for 15 min to obtain the cell pellet. Supernatant was tipped off and $\frac{1}{20}$ vol of 10% SDS was added to denature proteins and release RNA from ribonuclear proteins. The SDS lysate was extracted with equal volume of Phenol/ Chloroform mixture at 37°c (for cold phenol extraction) or 62°c (hot phenol extraction). After centrifugation at 10k 4°c for 10 min the aqueous phase (upper layer) was reextracted with equal volume of phenol/ chloroform mixture and the phenol phase (lower layer) was reextracted with equal volume of NETS for 15 min at 37°c. The aqueous phase were pooled and reextracted once with equal volume of phenol/chloroform mixture for 15 min. The aqueous phase was removed again. $\frac{1}{20}$ Volume of 4 M NaCl and 2 volume of absolute ethanol was added and the mixture was kept at 4°c overnight to precipitate the RNA. After centrifugation the RNA precipitate was dried in vaccuum and dissolved in 1.5 ml of double distilled water. RNA concentration was determined by reading its optical density (OD) at 260 nm.

Quantitation of the amount of total cellular RNA in the blood cells by Pentose analysis.

Material

1. 10% TCA
2. 95% Ethanol
3. Orcinol reagent : 1 gm of purefied orcinol is dissolved immediately in 100 ml HCl conc which was mixed with 0.5 gm $FeCl_3$

Methods

Removal of Acid-Soluble Compound.

1 ml washed packed red cell was mixed with 2.5 ml cold 10% TCA for 5 minute and the supernatant was remained after centrifugation with 2.5 ml cold 10% TCA. Acid soluble compound will removed after this treatment.

Removal of Lipoidal Compound

The final sediment remaining after removal of the acid soluble compounds was extracted twice with 5 ml of 95% ethanol and recovered by centrifugation. This procedure is sufficient to remove the phosphorous-containing lipids.

Removal of RNA

The lipid-free cells residue was suspended in 1.3 ml distilled water and 1.3 ml 10% TCA. The mixture was then heated at 90°c for 15 minute with occasional stirring. RNA will be solubilized in the solution. After separating the RNA solution to the new test tube, the insoluble residue was washed again with 2.5 ml 5% TCA. This will remove more RNA from the tissue protein.

Estimation of RNA

A standard curve using yeast RNA was prepared relating color intensity to mg of RNA with purified yeast RNA serving as the standard. Yeast RNA and sample of RNA was dissolve in water and heated with orcinol reagent for 20 minute in boiling water. The intensity of the green color is read at 660 nm.

Denaturation and transfer of total cellular RNA to nitrocellulose paper

Materials

1. 1M Glyoxal
2. 10 mM sodium phosphate buffer pH 7.0
3. 20 x SSC : 3M NaCl, 0.3M Na_2 citrate
4. 20 mM Tris-HCl
5. nitrocellulose pretreated with H_2O and equilibrated with 20 x SSC

Method

20 µg total cellular RNA was incubated in 1 M glyoxal/10 mM sodium phosphate buffer pH 7.0 in a final volume of 16 µl at 50°c for 1½ hr. The glyoxalated RNA was spotted directly on to the nitrocellulose which was previously treated. The concentration of each glyoxalated RNA sample on nitrocellulose was 1,3,6,10 µg. The nitrocellulose was then dired under a lamp and baked in a vacuum oven at 80°c for 2 hr. Baking was required for retention of the RNA on the nitrocellulose. After baking, the RNA blots were treated with 20 mM Tris pH 80 at 100°c for five minutes. This can be accomplished by immersing the blots in about 200 ml of 20 mM Tris buffer, pH 8.0 at 100°c and allowing the blots to cool to room temperature in the buffer. The RNAs have been retained on the filter during this treatment, this procedure removed the residual of glyoxal and increased the sensitivity of hybridization.

Hybridization on nitrocellulose papers

Materials

1) Prehybridization solution (PHB) : 50% Formamide/5x SSC/2 x Denhardt solution/50 mM sodium phosphate pH 6.5
2) Hybridization solution (HB) : 50% Formamide/5x SSC/2x Denhardt solution/50 mM sodium phosphate pH 6.5/10% Dextran sulfate.
3) 2x SSC/0.2% SDS.
4) 0.1x SSC/0.1% SDS.
5) ^{32}P-Probe(s) in TE (denatured by heating for 5-10 minute in boiling water and quick cool)
6) Baked nitrocellulose paper with RNAs.
7) Denhardt solution (25x) 0.5% Ficoll/0.5% PVP/0.5% BSA in 3x SSC treated with 0.5% DPC at 37°c for 1 hr.

Method

The nitrocellulose paper was put into plastic bag and 4 ml PHB solution was added. The bag was then sealed and incubated at 42°c for 8-24 hr. After prehybridization, the bag was cut at one edge to remove the PHB and 4 ml HB solution containing 8×10^6 cpm of probe previously performed by nick-translation was substituted. The bag was then released and hybridization was carried out at 42°c for 48 hr.

After hybridization the bag was cut at one corner to dispense the hybridization mixture. The solution was poured into a container for ^{32}P-waste. The nitrocellulose was removed to the plastic box containing 100 ml.

The filter was washed at room temperature with four changes of this solution: 2x SSC/0.1% SDS for 5 minute each and then washed twice at 50°c for 15 min each with 0.1x SSC/0.1% SDS. The nitrocellulose filter was air dried and cut at the appropriate RNA spot. Each piece of the nitrocellulose containing RNA was put in 10 ml of Bray's scintillant cocktail and the radioactivity was counted by LS-100 Beckman Liquid scintillation counter.