II. MATERIALS AND METHODS

II.1 MATERIALS

The chemicals, solutions and experimental instruments used in the study are listed alphabetically in Apppendix I, Appendix II and Appendix III, respectively.

II.1.1 Transformed bacterial strain

Escherichia coli strain LK 111 containing recombinant plasmid, pEMBL α_2 and pEMBL ζ, previously transformed, were used for isolation of the globin specific probes.

II.1.2 Blood samples

The venous blood samples were collected from 36 subjects with homozygous β-thalassemia who came for treatment in the thalassemia clinic at the Maharaj Nakorn Chiang Mai hospital. There were 20 samples from subjects with thalassemia major and 16 samples from subjects with thalassemia intermedia.

II.2 METHODS

II.2.1 Isolation of recombinant plasmid DNA

The two kinds of recombinant plasmids containing α_2- and ζ-globin specific fragments were amplified and isolated as below.

Ten milliliters of LG broth containing ampicillin was incubated with a single colony of Escherichia coli culture habouring the plasmid, and incubated at 37°C overnight with vigorous shaking. The overnight culture was used to inoculate 500 ml of fresh LG broth containing ampicillin and kept at 37°C until the culture reached late log phase (OD 600 nm = 0.8). Chloramphenicol was added to produce a final concentration of 200 ug/ml and incubation was continued overnight with vigorous shaking. The cell pellet was sedimented by centrifugation at 5,000 rpm for 15 min at 4°C and resuspended in 10 ml of solution I. The suspension was kept on ice for 30 min and 20 ml of a freshy prepared solution II was added. The suspension was mixed by gently inverting the tube several times, and kept on ice for 10 min before adding 15 ml of ice-cold solution III. After mixing throughly, the suspension was placed on ice again for 1 hour. Bacterial debris and chromosomal DNA were removed by centrifugation at 15,000 rpm for 30 min at 4°C. The suppernatant was transferred into another tube and 0.6 volume of isopropanol was added. After mixing, the nucleic acid was allowed to precipitate at room temperature for 15 min before centrifugation at 10,000 rpm for 15 min. The alcohol supernatant was discarded and 70% ethanol was added to wash out the salts. The precipitate was allowed to dry at room temperature and dissolved in a minimum volume of TE buffer.

II.2.2 Purification of plasmid DNA by Sephacryl S-1000 (47,48)

Nucleic acid solution was treated with RNase A at a concentration of 50 ug/ml solution and incubated at 37°C for 30 min. Then the SDS solution was added to a 0.5% final concentration followed by the proteinase K at a concentration of 50 ug/ml solution. The mixture was incubated at 37°C for 15 min. The salt concentration was adjusted to 1 M sodium chloride and the mixture was extracted with an equal volume of chloroform-isoamyl alcohol. After centrifugation, the aqeous layer was transferred to another tube and 0.2 volume of 5x loading dye was added before applying to the Sephacryl S-1000 gel filtration column.

Sephacryl S-1000 gel was packed in a siliconized glass column (1.5 x 45 cm) and equilibrated with TE buffer. The prepared sample was applied onto the column, eluted with the same buffer with a flow rate of 30 ml/hour and 2 ml fractions were collected. The elution was monitored at 254 nm in a flow-cell and the peak fractions were examined by 0.8% agarose gel electrophoresis. The plasmid fractions were pooled and concentrated by ethanol precipitation. The pellet was washed twice with 70% ethanol, let dry at room temperature and redissolved in a minimum volume of TE buffer.

II.2.3 Preparation of α_2- and β - specific fragments

The characterization of the pEMBLα_2 and pEMBLβ are shown in Figure II.1 and Figure II.2 respectively.

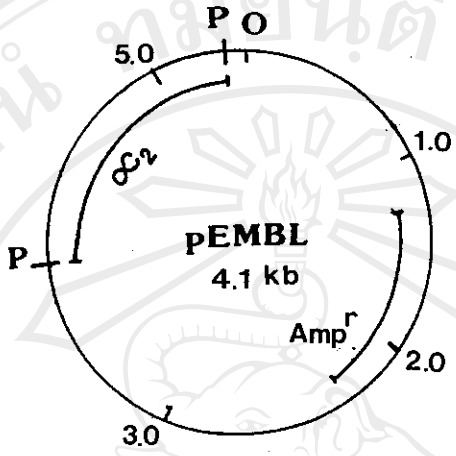

Figure II.1 The recombinant plasmid containing α_2 probe.
The α_2 - globin specific fragment, 1.5 kb was cloned into the PstI site and ligated with the pEMBL vector.
P = Pst I cleavage site
Amp = Ampicillin resistance sequences.

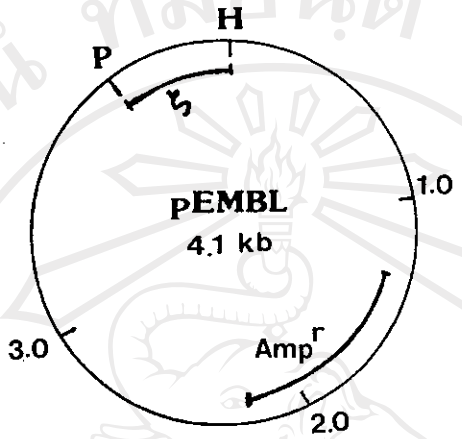

Figure II.2 The recombinant plasmid containing ζ probe.

The ζ - globin specific fragment., 400 bp, was cloned into the Pst I/Hind III sites and ligated with the pEMBL vector.

P = Pst I cleavage site

H = Hind III cleavage site

Amp = Ampicillin resistance sequences

The method can be divided into two steps.

A. Digestion of plasmids, pEMBL α_2 and pEMBLζ, by restriction endonuclease enzymes.

The reaction mixture was composed of 50 ug of pEMBL α_2 or pEMBLζ, 10 ul of 10x reaction buffer specific for the enzyme PstI and Hind III, 100 units of restriction enzymes, either Pst I or Hind III and sterile distilled water in 100 ul total volume. The mixture was incubated at 37°C for 2-4 hours.

B. Isolation and extraction of the DNA specific fragments.

Digested plasmid DNA was eletrophoresed on 0.7% low melting point agarose gel with λ Hind III markers. The gel was stained with ethidium bromide and the desired band was detected by using a long wavelength UV light source. The desired band was cut out of the gel and transferred to the Eppendorf tube. The gel slice was melted at 65-68°C and then extracted twice with one volume of saturated phenol and 0.1 M final concentration of sodium chloride. The clear aqueous layer was removed and extracted twice with butanol saturated in water to remove ethidium bromide. After spinning, the butanol layer (upper layer) was discarded. The DNA was recovered by precipitation with 0.1 volume of 3M sodium acetate and 2 volume of absolute ethanol at -20°C for at least 1 hour. The precipitated DNA was dissolved in an appropiate volume of TE buffer. The amount of the DNA specific fragment was estimated by comparing the intensity of DNA fragment over

the UV light with the standard λ DNA markers.

II.2.4 Human DNA preparation

Five ml of venous blood was collected in the EDTA containing tube. The packed cells were obtained by centrifugation at 3,000 rpm for 10 min. After removing the plasma, the packed cells were washed with sterile normal saline to remove plasma proteins. This packed cell sample could be kept at -20°C for a few weeks.

Ice-cold lysis buffer (45 ml) was added to the fresh packed cells or thawed frozen sample to lyse the red cell membranes. After inversion mixing, the suspension was allowed to stand on ice for 10 min. The nuclei were pelleted by centrifugation at 4,000 rpm for 20 min at 4°C. The hemolysate supernatant was discarded as much as possible before adding 9.4 ml of STE buffer. The suspension was vigorously vortexed until small pieces of nuclei were obtained. Then 0.5 ml of 10% SDS was added and gently swirled before adding the 0.1 ml of proteinase K solution (10 mg/ml). The suspension was incubated at 55°C overnight. At the end of incubation, the suspension should become a clear and viscous solution.

The solution was extracted by adding 1/2 volume of saturated phenol and 1/2 volume of chloroform-isoamyl alcohol. The mixture was agitated gently for 5 min before centrifugation at 3,000 rpm for 10 min. The organic layer at the bottom was removed slowly

with a Pasteur pipette and a second extraction was performed. After removing the organic phase, 1 volume of chloroform-isoamyl alcohol was added. The mixture was extracted again to remove the residual traces of phenol. After centrifugation, the highly viscous, aqeous layer was carefully transfered to another tube using a wide mouthed pipette.

The DNA was precipitated by adding 1/10 volume of 3M sodium acetate (pH 6.0) and 2 volumes of ice-cold absolute ethanol. By inversion mixing at room temperature, the DNA was precipitated while the RNA remained in fine suspension. The precipitated DNA was transferred to an Eppendorf tube. After washing with 70% ethanol, the DNA pellet was allowed to dry for at least 30 min at room temperature and finally dissolved in an appropiate volume of TE buffer. The DNA solutions were kept at 4°C while they were in use.

The concentration of DNA was estimated by determination of its absorbance at 260 nm. assuming that its A1cm, 260 was 200 (that is, 1 g/100 ml DNA solution in a 1 cm light path has an absorbance at 260 nm of 200). Thus a 50 ug/ml solution gives a reading of 1 OD. For example an aliquot of 10 ul DNA solution was made up to 1 ml (1:100 dilution) and the OD. at 260 is 0.10000. The concentration of this DNA solution was 50 x 0.100 x 100 = 500 ug/ml or 0.50 ug/ul.

II.2.5 Restriction endonuclease enzyme digestion

The reaction was performed in 50 ul composed of 10 ug of DNA solution, 5 ul of 10 x reaction buffer specific for the enzyme

EcoRI and BglII, 30-40 units of restriction enzymes either EcoRI or Bgl II and sterile distilled water was added for adjusting to a 50 ul total volume, then incubated at 37°C for 3-5 hours, or prolonged to overnight until digestion was completed. The digested DNA was precipitated by ethanol and redissolved in 16 ul of TE buffer. The solution was left to dissolve at 4°C overnight. The following day, 4 ul of 5x loading dye was added into the sample ready for application into the agarose gel.

II.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a horizontal direction and run in the submarine fashion to overcome the problem of the sample wells drying out during the electrophoresis.

A clean, dried tray was sealed with tape to make a mold. The comb was placed 1.0 mm over the bottom of the mold. The 0.8% agarose in TBE buffer was cooled to 50°C before pouring into the mold. The gel was allowed to set at room temperature. After removing the comb and the sealing tape, the gel was placed in the electrophoresis tank. The samples and λ Hind III marker were applied into the wells and the gel was electrophoresed at 40 V for 24 hours at room temperature.

After electrophoresis, the gel was immersed in the ethidium bromide solution (0.5 ug/ml) for 10 min and placed in the short wavelength UV light source. The distances between the origin and

each band of λ Hind III marker which has fragment sizes of approximately 23.1, 9.5, 6.7, 4.3, 2.3, 2.0 and 0.6 kb were marked and measured. A curve for the measurement of the sizes (kb or bp) of DNA fragments were made on semilog graph paper by plotting the sizes (kb or bp) of known DNA markers against the distances of each band from origin (cm.) The sizes of unknown DNA was thus estimated from this curve.

II.2.7 Southern transfer

The technique was modified from the original method described by **Southern E.M.** (49).

A. Gel treatment

The electrophoresed gel was soaked in several volumes of 1.5 M sodium chloride and 0.5 M sodium hydroxide for 45 min at room temperature with constant shaking to denature DNA. After rinsing the gel briefly with distilled water, the gel was neutralized by soaking in several volumes of 1 M Tris.Cl (pH 8.0) and 1.5 M sodium chloride for 1 hour at room temperature with constant shaking. After treatment, the gel was rinsed briefly with 2xSSC.

B. Membrane preparation

The nitrocellulose membrane was cut the same size as the gel and soaked throughly in distilled water for 10 min and then soaked in 2xSSC for 10 min before use.

The Southern blotting technique was set up as shown in Figure II.3. A large tray was filled with 20xSSC, and a sheet of Whatman paper, presoaked with 20xSSC, was placed over the small supporter in the tray to be a wick, and this was followed by a sheet of Whatman paper which was about 2 mm. larger than the gel. The gel was placed on an exposed paper without any air bubbles between the gel and the paper, and then the four sides around the supporter were covered with polyethylene sheets to prevent evaporation of the water. A sheet of nitrocellulose membrane previously soaked was placed on the gel, and the orientation was marked by cutting the same corner of the gel and the nitrocellulose filter. Special care was taken not to allow any bubbles to be present. Four sheets of Whatman paper, previously soaked in 2xSSC, were then placed on the nitrocellulose membrane. To make sure that no air bubbles were trapped underneath, the pipette was rotated on the stack of Whatman paper briefly. This was followed by a stack of paper towels (5-8 cm height), a glass plate and a weight of about 500 g on the top.

The blotting set was left for 48-72 hours with several changes of paper towels and addition of more buffer in the tray. After blotting, the filter was soaked in 2xSSC for 20 min with gently shaking and dried at room temperature. After labelling, the blotted membrane was placed between two sheets of Whatman paper and wrapped with aluminum foil before baking at 80°C in an oven for at least 2 hours. The baked membrane could be kept at room temperature until ready for hybridization.

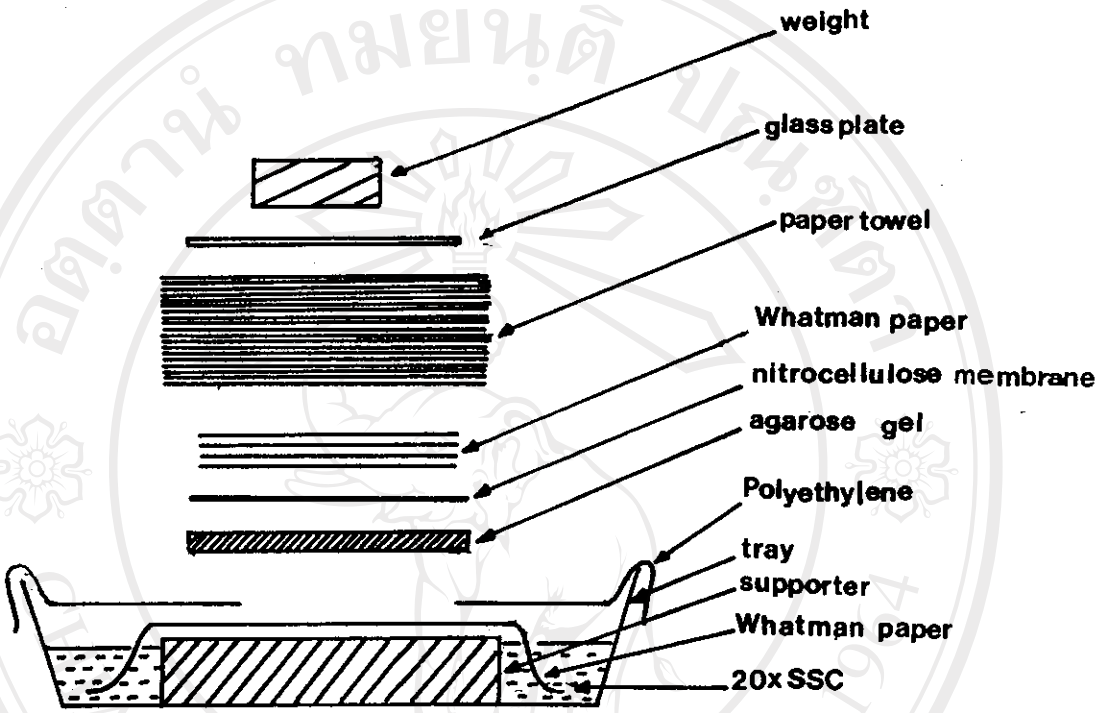

Figure II.3 The Southern blot apparatus for the transfer of DNA from an agarose slab gel to a nitrocellulose membrane (51).

II.2.8 Preparation of α-^{32}P labelled DNA probe

The DNA specific fragment was labelled by using the Multiprimer DNA labelling kit in a total volume of 20 ul. DNA specific fragment (100-150 ng) was dissolved in adjustable volume of distilled water, then heated in boiling water for 5 min to seperate the duplex DNA to be single stranded and placed on ice immediately. After heat shocked, 4 ul of the reaction buffer was added and followed by 2 ul of hexaprimer, 1 ul of Klenow polymerase enzyme and finally 2 ul of ^{32}P dCTP (3,000 Ci/mmol). The mixture was mixed gently before incubation at 20°C for at least 3 hours.

The incorporation of α-^{32}P dCTP into DNA probe was determined by Sephadex G-75 chromatography column. One microliter of reaction mixture was removed and added to 19 ul of blue dextran and phenol red mixed dye, then applied onto the Sephadex G-75 column. After eluting with TE buffer, three fractions were collected; first blue fraction of incorporated DNA probe, followed by the colorless which was the connecting range and the last is a red fraction of unincorporated nucleotides and free radioactives. Activities of these three fractions were monitored with a hand-held, end window geiger counter and the percentage of incorporation was calculated. The labelling reaction was stopped by adding 5 ul of 0.5 M EDTA, and 50 ul of denatured salmon sperm DNA (10 mg/ml) was added as the competitive DNA. The labelled probe was denatured by boiling for 5 min and immediately chilled in ice bath before hybridization.

II.2.9 Hybridization

The hybridization was performed in a siliconized cylindrical tube (4 cm. diameter, 25 cm. long). The tube was filled with 2xSSC. Then the baked nitrocellulose membrane was rolled and dropped into the solution in the tube, allowing the back side of the membrane to be exposed to the inner surface of the tube. After removing 2xSSC, about 20 ml was left. The membrane was placed in the 42°C incubator rotator for 1 hour. The 2xSSC was discarded and replaced with 20 ml of prehybridization solution. The incubation was performed in the same temperature (42°C) for an additional 4 hours. After the end of incubation, the prehybridization solution was removed and replaced again with the hybridization solution containing the denatured probe. The hybridization was allowed to proceed at 42°C with rolling for 12-16 hours.

II.2.10 Washing

At the end of hybridization period, the membrane was removed from the tube and washed twice with 300 ml of solution I (2xSSC, 0.1% SDS) at room temperature with shaking for 10 min each. The membrane was then washed twice with solution II (2xSSC, 0.1% SDS, 1x Denhardt's solution) at 65°C with shaking for 20 min each and once with solution III (0.1 xSSC, 0.1% SDS) at 65°C for 30 min in the waterbath shaker. Finally, the membrane was rinsed in 2xSSC and left

to dry thoroughly at room temperature. The dried membrane was put into the polyethylene bag to make ready for autoradiography.

II.2.11 Autoradiography

The membrane was placed between two sheets of X-ray films in the cassette fitted with the intensifying screens. The cassette was kept in a -70°C freezer. The first film was developed after 1-3 days exposure and another was exposured for an appropiate time to obtain more intensities.