III. MATERIALS AND METHODS

1. Blood samples

Twenty-eight patients with Hb H disease were diagnosed at thalassemia clinics, Maharaj Nakorn Chiang Mai hospital. The diagnosis was based on the presence of Hb H in the Hb electrophoresis and inclusion body found by the brilliant cresyl blue stain. Some hematological data of these patients was shown in Table 2.

2. Transformed bacterial strain

Escherichia coli strain LK 111 containing recombinant plasmid, pEMBL α_2 and pEMBL ζ, previously transformed were generous gifts of Professor Hiroshi Takei, Department of Biochemistry II, Faculty of Medicine, University of the Ryukyus, Japan.

2.1 The α_2-globin specific fragment, 1.5 kb, was cloned into the *Pst* I site and ligated with the pEMBL vector(See Figure 4).

2.2 The ζ- globin specific fragment, 400 bp, was cloned into the *Pst* I / *Hin* d III site and ligated with the pEMBL vector(See Figure 5).

Table 2. Some hematological data of Hb H patients according to out-patients department (OPD) cards.

Patient No	Age	Hb(g/dl)	Inclusion bodies test	Hb typing	Frequency of blood transfusion (years ago)	Spleenectomy
#1	13	6.0	2+	A,H,Bart	3	-
# 2	13	7.1	-	A,H,F	6	✓
# 3	10	5.0	2+	A,H,Bart	1	-
# 4	15	7.2	2+	A,H,Bart	2	-
# 5	10	6.4	2+	A,H	-	-
# 6	11	6.7	2+	A,H	-	-
# 7	13	8.6	2+	A,H	-	-
# 8	15	6.8	2+	A,H,Bart	6	-
# 9	10	7.3	2+	A,H,CS	5	-
# 10	10	8.1	2+	A,H	-	-
# 12	10	8.9	2+	A,H,Bart	6	-
# 13	15	10.0	2+	A,H	-	-
# 14	11	4.5	1+	A,H,Bart	6	-
# 15	13	9.2	2+	A,H,Bart	4	-
# 16	15	7.9	2+	A,H	-	-
# 17	13	10.2	2+	A,H	-	-
# 18	9	11.2	2+	A,H	2	-
# 19	2	5.5	2+	A,H,Bart	2	-
# 20	10	9.8	2+	A,H,Bart	2	-
# 21	8	5.8	-	A,H,Bart	0.4	-
# 22	6	9.0	2+	A,H,Bart	-	-
# 23	6	7.3	1+	A,H	-	-
# 24	9	6.9	2+	A,H,Bart	3	-
# 25	6	7.3	2+	A,H,Bart	6	-
# 26	11	5.5	2+	A,H,Bart	4	-
# 27	15	9.8	3+	A,H,Bart	-	-
# 28	8	6.3	2+	A,H	-	-

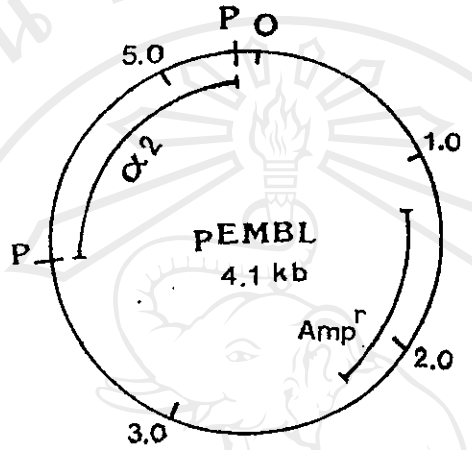

Figure 4. The recombinant plasmid containing α_2 probe.
The α_2-globin specific fragment(1.5 kb) was cloned into the *Pst* I cleavage site of pEMBL vector.

P = *Pst* I cleavage site
Amp^r = Ampicillin resistance sequences

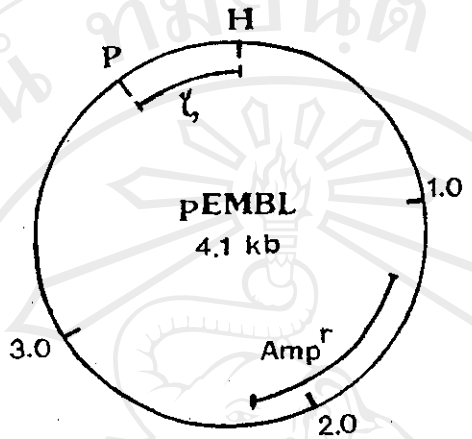

Figure 5. The recombinant plasmid containing ζ probe.
The ζ-globin specific fragment (400 bp) was cloned into the *Pst* I/ *Hin* d III cleavage site of the pEMBL vector.

P = *Pst* I cleavage site
H = *Hin* d III cleavage site
Amp^r = Ampicillin resistance sequences

3. Preparation of genomic DNA

Genomic DNA was prepared by the method described by Miller et al.(1989). Ten ml of blood was collected using 1 ml of acid citrate dextrose (ACD) as an anticoagulant. The whole blood was centrifuged at 3,000 rpm for 20 min, the resulting packed cells was added with red cell lysis buffer (RCL) to a final volume of 50 ml. The red cells lysate was removed by centrifugation at 3,000 rpm for 20 min. This step was repeated again with 15 ml of RCL to complete red cells lysis. The white blood cells obtained were then resuspended in 3 ml of nucleic lysis buffer (NCL), and 200 µl of 10%SDS and 600 µl of pronase K solution (10 mg/ml) were added. The solution was vigorously shaken at 55 ^{o}C for 18 h followed by the addition of 1 ml of 6 M NaCl into 4 ml of the solution. The solution was left at 4^{o}C for 20 min to precipitate the proteins completely. The DNA solution was then collected by centrifugation at 5,000 rpm for 25 min. The DNA was recovered by precipitating with an equal volume of isopropanol. After washing twice with 70%EtOH, the DNA pellet was allowed to dry at room temperature and then redissolved in Tris-EDTA(TE) buffer. The concentration and purity of DNA was estimated by measuring optical density(OD) at 260 and 280 nm, assuming that 1 OD260 equals the concentration of 50 µg/ml and the purity is indicated by the ratio of OD260/OD280 between 1.70-1.95 .

4. Restriction endonuclease digestion of genomic DNA

The reaction mixture, composed of 10 µg of genomic DNA solution, 6 µl of 10x reaction buffer specific for each restriction enzyme , 30-40 units of restriction enzyme either *Eco* R I or *Bgl* II and sterile distilled water to a total volume of 60 µl, was incubated at 37°c for 5 h. After that 12 µl of gel loading buffer was added, then samples were ready for application into the agarose gel.

5. Agarose gel electrophoresis

The digested genomic DNA was fractionated by electrophoresis through a 0.7% agarose gel in 0.5x Tris/borate/EDTA(TBE) buffer at 1.3 volts.cm^{-1} for 18 h along with λ DNA cut with *Hin* d III as a DNA marker. The gel was stained with ethidium bromide(0.5 µg/ml) for 30 min, destained and visualized under a short wavelength ultraviolet light source. The λ/*Hin* d III marker correspond to DNA sizes of 23.1, 9.5, 6.6, 4.4, 2.3, 2.0 and 0.5 kb were measured. The distances that DNA migrates were plotted against the sizes of DNA marker on semilog graph paper, and the sizes of DNA fragments on the gel can be estimated from this curve.

6. Southern blotting

The transfer of DNA from an agarose gel to a solid support; nylon

membrane was performed by standard technique described by Southern E.M,(1975).

The DNA was partially depurinated by soaking the gel in 20 gel volumes of 0.25 M HCl for exactly 13 min. Immediately the DNA was denatured by soaking the gel in 20 gel volumes of 1.5 M NaCl, 0.5 M NaOH for 20 min at room temperature and agitated gently. This denaturing step was repeated with fresh solution for a further 25 min. The gel was then neutralized by soaking in 20 gel volumes of 1.0 M Tris (pH 7.5), 1.5 M NaCl for 20 min at room temperature and agitated gently. This neutralizing step was repeated with fresh solution until the pH of the gel was below 7.8. A piece of PhotoGene^{TM} nylon membrane was cut precisely to the same size as the gel. It was marked by cutting at the right corner, wet in deionized water and soaked in 20x SSC for 15 min prior to use. A blot apparatus was partially filled with 20x SSC and the platform was covered with a wick of filter paper saturated with 20x SSC. The gel was then placed on the saturated filter paper, making certain no air bubbles were trapped between the gel and the filter paper. Subsequently, the wet nylon membrane was placed on top of the gel, avoiding air bubbles trapped between the gel and the nylon membrane. Filter papers saturated with 20xSSC were placed on the nylon membrane. A stack of absorbent papers was placed on top of filter papers, then a glass or plastic plate was placed on top of absorbent papers, and a 500 g weight was placed on top of the plate. The DNA was transferred overnight. After that the nylon membrane was removed

and washed in 5x SSC for 5 min at room temperature with gentle agitation. The membrane was placed on a piece of clean filter paper and air dried for 30 min. Later the membrane was dried in an oven at 80°C for 2 h.

7. Large - scale preparation of recombinant plasmid DNA

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

A stock of Escherichia coli culture haboring the recombinant plasmid was inoculated for growing in 5 ml of Luria-Bertani(LB) broth containing ampicillin at 37°C overnight, then E. coli was streaked on LB plate containing ampicillin to obtain a single colony. A single colony was picked up and inoculated into 30 ml of LB broth containing ampicillin and incubated at 37°C overnight with vigorous shaking. The culture was then inoculated into 500 ml of LB broth containing ampicillin and shaken at 37°C until it reached late log phase(OD 600 nm = 0.8).Chloramphenicol was added to produce the final concentration of 170 µg/ml and the culture was incubated for a further 12-16 h at 37°C with vigorous shaking. The bacterial pellet was sedimented by centrifugation at 5,000 rpm for 5 min at 4°C. After draining all of the supernatant, the pellet was resuspended well in 7.5 ml of sucrose buffer on ice, then 2.25 ml of lysozyme (5 mg/ml) was added to the suspension, and the suspension was kept on ice for 10 min with occasional shaking. Subsequently, 6 ml of triton-x solution was added and vortexed

immediately, incubated at 37°C for 15 min. After that it was centrifuged at 15,000 rpm for 45 min and then the supernatant was poured off into a sterile bottle. A half volume of 30% polyethylene glycol in 1.5 M NaCl was added, leaving on ice for 15 min. The mixture was centrifuged at 3,000 rpm for 5 min, the pellet was resuspended in CsCl - ethidium bromide solution. (9.5 ml TE buffer + 9.5 g CsCl + 925µl ethidium bromide)

8. Purification of recombinant plasmid DNA by equilibrium ultracentrifugation in CsCl-ethidium bromide gradients

The recombinant plasmid DNA in CsCl-ethidium bromide solution was transferred to ultracentrifuge tubes using a pasteur pipette. The tubes were balanced and sealed. The bands of DNA were obtained by ultracentrifugation at 40,000 rpm for 48 h at 25°C(Beckman model L-60,Quick seal centrifuge tube 16*76 mm). Two bands of DNA located in the center of the gradients should be visible in UV light. The upper band which usually contains less material, consists of linear bacterial (chromosomal)DNA, nicked circular plasmid DNA. The lower band consists of closed circular recombinant plasmid DNA. The deep red pellet on the bottom of the tube consists of ethidium bromide / RNA complexes. The desired band of closed circular recombinant plasmid DNA was collected by inserting a 21-gauge hypodermic needle into the top of the tube to allow air to enter, then a second hypodermic needle was inserted just below the band of closed circular recombinant plasmid

DNA to collect it into a plastic tube. After that an equal volume of butanol and isopropanol saturated with CsCl was added. Ethidium bromide was extracted into the butanol and isopropanol and then removed. The extraction was repeated four to six times until all the pink color disappeared from both the aqueous phase and the organic phase. The CsCl was removed from the recombinant plasmid DNA solution by dialysis for 24-48 h against several changes of TE buffer(pH 8.0). The final solution of recombinant plasmid DNA was measured at OD 260 and the concentration was calculated from the formula:

concentration of plasmid DNA(μg/ml) =OD260 x 50 x dilution factor

This plasmid DNA was divided into aliquots and stored at -20° C for further purification.

9. Preparation of α_2- and ζ -globin specific fragments

The method was divided into two steps.

9.1Digestion of the recombinant plasmid, pEMBL α_2 and pEMBL ζ by restriction endonuclease.

The reaction mixture was composed of 50 μg of pEMBL α_2 or pEMBL ζ , 10 μl of 10 x reaction buffer specific for the restriction enzyme *Pst* I and *Hin* d III, 100 units of restriction enzymes either *Pst* I or *Hin* d III and sterile distilled water to a total volume of 100 μl. The

mixture was mixed well and incubated at 37°C for 5 h, followed by adding 20 µl of gel loading buffer.

9.2 Isolation and extraction of the DNA specific fragments using DNA trapping cell TM(Daiichi Pure Chemicals)

Digested recombinant plasmid DNA was electrophoresed on 0.65% agarose gel along with λ/*Hin* d III markers at 50 volts for 3 h. After that the gel was stained in ethidium bromide solution (0.05 µg/ml) for 30 min, the DNA bands were detected by using a short wavelength UV light source. Then the desired band (1.5 kb for α_2 - globin specific fragment, 400 bp for ζ– globin specific fragment) was cut out and transferred to the DNA trapping cell™(see Figure 6). The DNA moved from cathode site to a room of DNA trapping cell™ which was on the anode site under a voltage of 50 at 4°C until all DNA has already been in the room, then the electric pole was reversed for 1 min. The DNA was collected into a plastic tube and extracted 3 times with saturated butanol to remove ethidium bromide. After that it was precipitated by adding 1/10 volume of 5 M sodium chloride and 2.5 volumes of ice-cold absolute ethanol,leaving at -20°C overnight. The precipitated DNA probe was collected by centrifugation at 10,000 rpm for 30 min and washed twice with 70% ethanol. Subsequently it was allowed to dry at room temperature, redissolved in 50 µl of TE, the concentration was calculated by absorbance value, and the DNA was stored at -20°C.

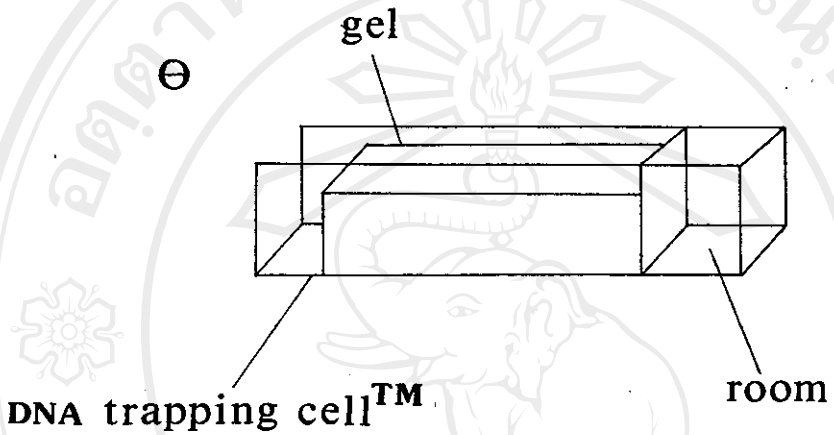

Figure 6. Isolation of the DNA specific fragments from the gel by using DNA trapping cell™

10. Probe biotinylation with BioNick Labelling System™

The DNA specific fragment (α_2- probe or ζ-probe) was labelled with biotin according to the instructions of BioNick Labelling System™ (Bethesda Reseach Laboratory). The process was based on nick translation (see Figure 7).

The reaction mixture was composed of 5 µl of 10x dNTP mix, 1 µg of α_2 - or ζ- probe, steriled distilled water to a volume of 45 µl and 5 µl of 10x enzyme mix. This was mixed well and centrifuged briefly, then it was incubated at 16°C for 1 h followed by adding 5 µl of stop buffer. Unincorporated nucleotide was separated from the labelled DNA probe by ethanol precipitation : 1/10 volume of 3 M sodium acetate and 2 volumes of cold absolute ethanol were added to the reaction tube, and mixed by inversion. Then it was kept at -70°C for 15 min or at -20°C for 2 h. After that it was centrifuged at 15,000 rpm for 30 min to remove the supernatant containing unincorporated nucleotides; the pellet was allowed to dry, resuspended in 50 µl of TE buffer and stored at -20°C.

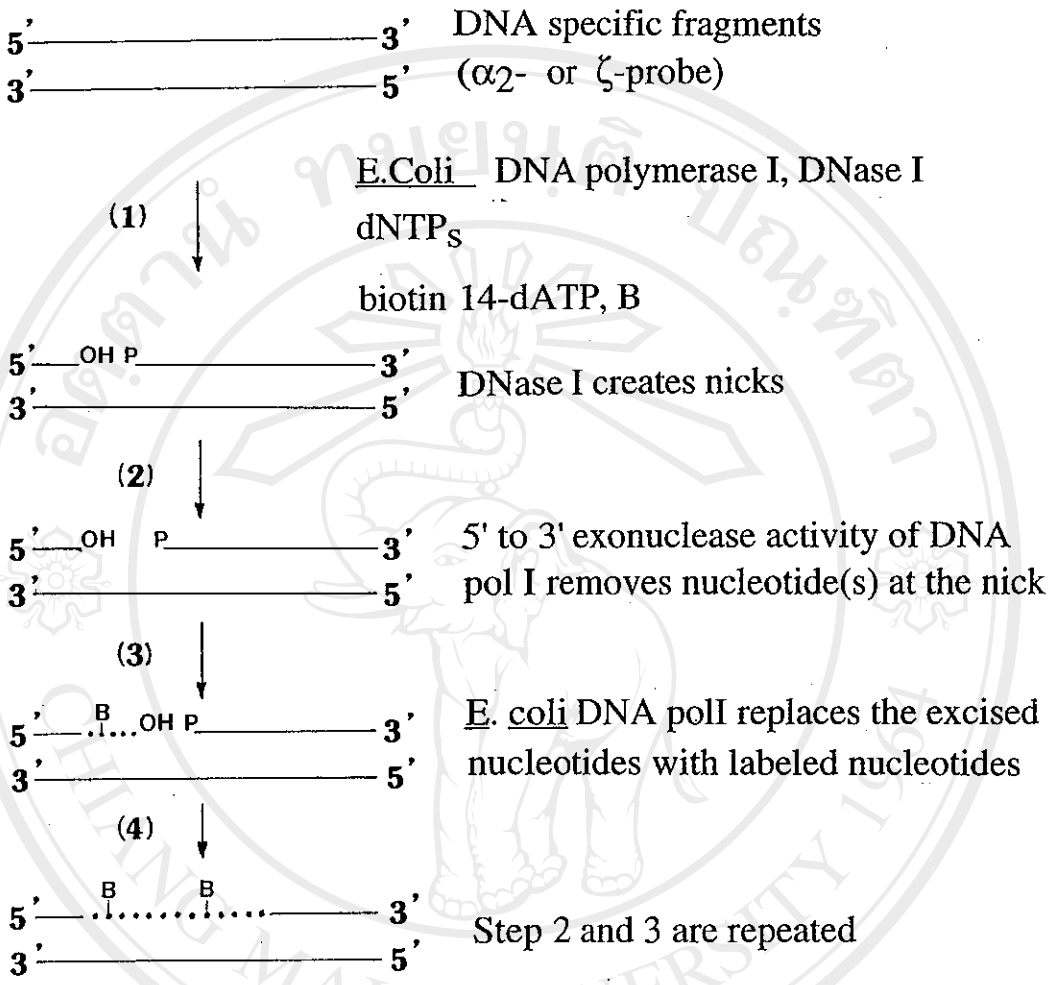

Figure 7. Principle of BioNick Labelling System™

11. Hybridization

The nylon membrane was placed in plastic hybridization bags and prehybridized in 250 µl of prehybridization solution per membrane surface area(cm^2) at 42°C for 2 to 4 h. The amount of probe needed for hybridization was calculated as follows:

$$\text{ng probe} = \text{area of membrane in } cm^2 \times 50 \text{ ng probe/ml} \times 0.1 \text{ ml/}cm^2 \text{ membrane}$$

The denatured probe solution was prepared by mixing equal amount of 2x hybridization buffer, 20% dextran sulfate in formamid and the calculated amount of probe together, then boiling for 5 min and chilling it immediately. After 2 to 4 h passed, the prehybridization solution was removed from the hybridization bag and the denatured probe solution was replaced, and incubation was continued at 42°C overnight.

12. Washing

To achieve the most intensity of signal and the least background from nonspecific binding of the probe, it is beneficial to optimize time and temperature of washing as follows.

The membrane was washed twice with 2 ml/$cm^2$of 5x SSC,0.5% (w/v) SDS at 56°C for 15 min each, followed by washing twice with

2 ml/cm^2 of 0.1x SSC, 1%(w/v) SDS at 56^{o}C for 30 min each and finally washed once with 2 ml/cm^2 2x SSC for 5 min at room temperature.

13. Detection

The hybridized membrane was washed for 1 min with 1 ml/cm^2 of TBS-Tween 20 along with the DNA control strip*. Non specific protein-binding sites on the membrane were blocked by incubation of the membrane in 0.75 ml/cm^2 of blocking solution (recommended by the manufacturer) at 65^{o}C for 1 h. Streptavidin-alkaline phosphatase (SA-AP) conjugate solution was prepared by microcentrifugation of the tube of SA-AP at 10,000 rpm for 4 min at room temperature, 7 µl removed from the supernatant solution used for each 100 cm^2 of membrane area was diluted 1:1000 in TBS-Tween 20. The membrane was incubated with this SA-AP dilution for 10 min at room temperature with gentle argitation. It is essential that the membranes are completely covered by the SA-AP solution. Subsequently, the membranes were washed twice with 1 ml/cm^2 TBS-Tween 20 for 15 min each at room temperature and washed with 1ml/cm ^{2} 1x final wash buffer for 90 min at room temperature while agitating gently. After that the membrane were blotted on 3 MM filter paper to remove excess buffer, then placed in a Photogene^{TM}development folder. The detection reagent (0.01 ml/cm^2) was used to cover the membranes, immediately the membranes were covered with the development folder while a 10-ml pipet was

rolling over the plastic sheet to spread the detection reagent and to remove any air bubble over membranes. The edges of the development folder were sealed with heat sealer, and the membranes were stored inthe dark for 3 h. At that time the membranes were first exposed to X-ray film in an intensifying cassette for 15 min. Subsequent exposure time can be adjusted to increase or decreasethe signal intensity. Exposed films were developed and fixed with a developer - fixer kit (G150-G350)(Agfa, Belgium) according to the manufacturer's instruction.

.....

*DNA control strip: Biotinylated DNA is provided as a positive control for testing the activity of PhotoGene^{TM} detection reagents. An aliquot of biotinylated DNA is diluted in DNA dilution buffer, and small aliquots are spotted on the nylon membrane.

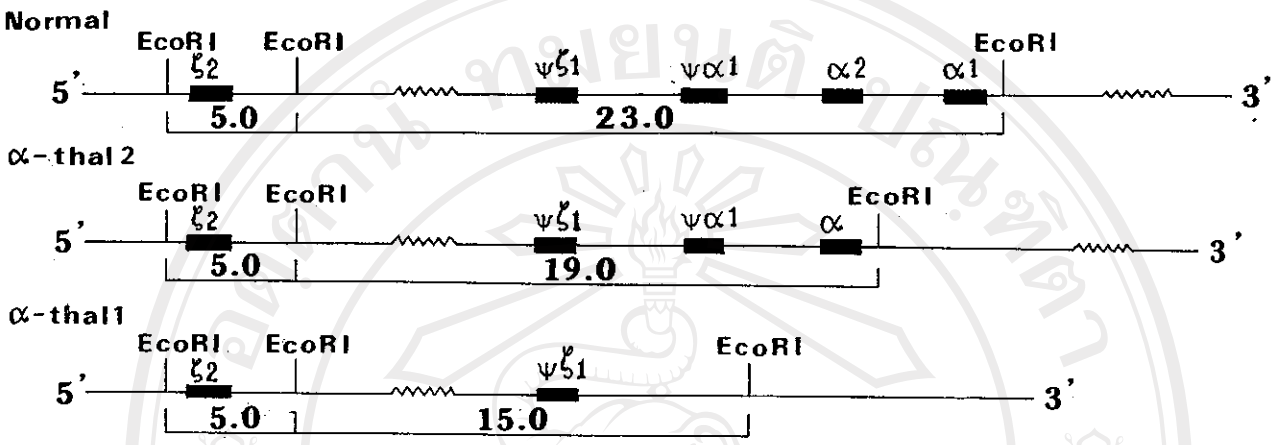

Figure 8 . *Eco* R I restriction endonuclease maps of the area around the α-globin gene cluster in normal,α-thalassemia 2 and α-thalassemia 1. (according to Thonglairuam et al., 1989)

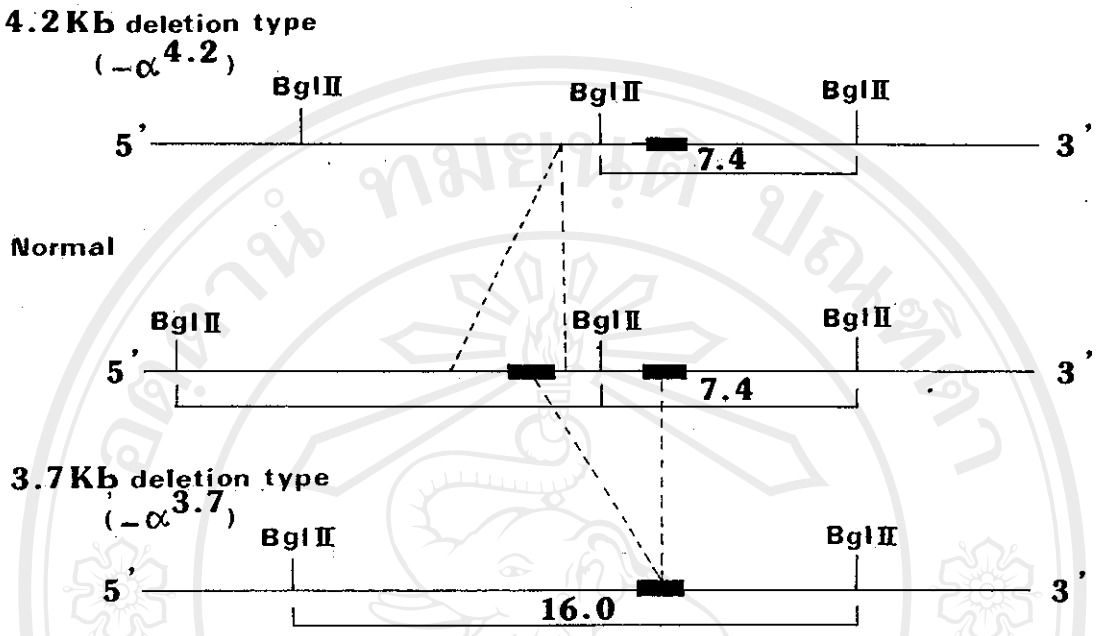

Figure 9. *Bgl* II restriction endonuclease maps of the area around the α-globin gene cluster in normal an both α-thalassemia 2 genotype. (according to Thonglairuam et al., 1989)