IV. DISCUSSION

The application of globin gene mapping for studying of the α-thalassemias greatly advanced our knowledge of α globin gene structure and organization in these syndromes. In particular, the technique of gene mapping was used to look at questions concerning α globin gene deletions that could not be answered by globin gene analysis using quantitation by molecular hybridization assays in solution. These included (1) identification of individuals with the deletion of only one out of four α globin genes; (2) analysis of the extent and endpoints of α globin gene deletions ; (3) differentiation of α globin gene deletions in trans from those in cis ; and (4) reliable identification and characterization of nondeletion types of α-thalassemia (Bunn et al., 1986).

α-Thalassemia is common throughout Southeast Asia (Wasi P., 1983). In Thailand, the incidence is in the range of 15-30%. It could be estimated that about 1,250 fetuses will be born with Hb Bart's hydrops fetalis and about 7,000 fetuses with Hb H disease per year based on a total birth rate of about 2 million per year (Winichagoon et al., 1992). Many previous studies have shown that the most frequently encountered defects in Thailand are the --SEA and $-\alpha^{3.7}$ determinant (Hb Bart's hydrops fetalis --SEA/--SEA and Hb H disease --SEA/$-\alpha^{3.7}$) (Fischel-Ghodsian et al., 1988). The 5' start of the --SEA determinant may exist within the third exon of the ψζ1 gene and the 3' end of this deletion terminates close to the hypervariable region (HVR). located at the 3' end of the α globin gene complex, removing about 17.5 kb of DNA from the α-globin gene cluster. The point of

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crossover giving rise to the $-\alpha^{3.7}$ defects in Thais is located within the homologous region 5' to the third exon of α-globin gene in the Z segments, of which the homology is more than 99 %. This is called the rightward α-thal 2, subtype I (Higgs et al., 1984).

Hb H disease is a genetic disease which is a heterogeneous form. The spectrum of molecular defects underlying Hb H disease ranges from the purely deletional(--/-α) genes to those where no gross deletion can be found[$\alpha\alpha^T$ /$(\alpha\alpha)^T$]. A number of compound forms have also been reported , e.g. -- / $(\alpha\alpha)^T$ and $-\alpha^T/-\alpha$ (Galanello et al., 1983; Olivieri et al., 1987). Previous attempts to correlate the clinical phenotype with the molecular pathology in Hb H disease agree in considering that nondeletional forms have a more severe clinical course as compare to the deletional ones (Kattamis et al.,1988;Aru et al.,1989). α-Thal 1/α-thal 2 and α-thal 1/ HbCS are the most common genotypes responsible for Hb H disease in Thailand. The manifestation of Hb H disease in the two genotypes is identical. However, Hb H disease with α-thal 1/Hb CS is more severe than that with α-thal 1/α-thal 2, the former has higher levels of Hb H and more red cells containing inclusion bodies(Adirojnanon et al.,1980). Enlargement of the liver and the spleen are also observed more frequently and are more pronouced in Hb H disease with Hb CS (Fucharoen et al., 1988). Quantification of a mRNA also demonstrated a more significant loss of α-globin mRNA synthesis from the $\alpha^{CS}\alpha$ chromosome than from the $-\alpha^{3.7}$ chromosome (Winichagoon et al., 1988 a,b). Furthermore, homozygosity for Hb CS ($\alpha^{CS}\alpha$ /$\alpha^{CS}\alpha$) is symptomatic with mild

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anemia, jaundice and spleenomegaly (Pootrakul et al., 1981), whereas homozygous α-thalassemia 2 with the $-\alpha^{3.7}$ deletion type($-\alpha^{3.7}/-\alpha^{3.7}$) is asymptomatic. This observation is consistent with the finding that there is a 1.8 - fold compensatory increase in the expression of the single α-gene in the $-\alpha^{3.7}$ deletion type (Liebhaber et al., 1985).

In this study, the restriction endonuclease *Eco* R I was used to differentiate α-thalassemia 1 from α-thalassemia 2 upon hybridization with the ζ-globin specific probe. The α-globin specific probe was not employed because it merely fails to hybridize with DNA of α-thalassemia 1 determinant. The α - thalassemia 1 was not further characterized here but it is expected to be the --SEA type because of its high frequency observed in several previous studies (Fischel - Ghodsian et al., 1988; Winichagoon et al., 1984). The α-thalassemia 2 was characterized whether it is $-\alpha^{3.7}$ or $-\alpha^{4.2}$ determinant by the use of restriction endonuclease *Bgl* II and hybridization with the α- globin specific probe. Twenty-eight patients with Hb H disease all possessed α-thalassemia 1 (--/) determinant. Among these, 18 cases possessed $\alpha^{T}\alpha$, 7 cases possessed $-\alpha^{3.7}$, and 3 cases possessed $-\alpha^{4.2}$ determinant. The frequency of $--/\alpha^{T}\alpha$ is much more often observed than the $--/-\alpha^{3.7}$ and $--/-\alpha^{4.2}$; 64%, 25%, 11%, respectively. However, the nondeletional α-thalassemia determinant was not characterized here, but it is expected to be Hb CS in almost all cases according to the studies by which Laig et al.(1990) reported that the incidence of Hb CS is as high as 6-11 % in the Northern and Northeastern Thailand. Additional experiment involving hybridization

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with an oligonucleotide specific probe to HbCS, gene cloning and nucleotide sequencing may be solve this problem. The other nondeletional α-thalassemia such as Hb Mahidol (Q) and Hb Suan Dok can be associated among these patients but both are uncommon. In contrast to Sardinia, where Hb H disease is common [occuring in 1:1,250 live births (Galanello et al., 1984)], the majority (78%) of the cases are due to gene deletion (--/-α). Among those due to a combination of deletion and nondeletion defects ($\alpha^T\alpha$), the most prevalent nondeletion lesion was the initiation codon (AUG-->ACG) mutation of the α2 gene (Paglietti et al.,1986). In Greece, 14 to 21 patients with Hb H disease had a --/-α genotype(usually --MED/ $-\alpha^{3.7}$) while five had --/$\alpha^T\alpha$ and two were nondeletion homozygotes ($\alpha^T\alpha/\alpha^T\alpha$) (Kattamis et al.,1988). In Southeast Asia, 40% of individuals with Hb H disease carry the α^{CS} mutation (--/$\alpha^{CS}\alpha$)(Wasi et al., 1974).

In general, each population has a different group of mutations, consisting of a few that are very common and a variable number of rare ones (Chan et al., 1987). Recent molecular studies of the α thalassemia led to similar conclusions (Flint et al., 1986; Higgs et al., 1983; Hill et al., 1986). The deletions that cause α thalassemia 1 in the Mediterranean and Southeast Asia are different (Pressley et al.,1980). Furthermore, detailed analysis of the RFLP haplotypes in the α-globin gene cluster, together with studies of the crossover events that produce the α thalassemia 2, have shown that the common form of α thalassemia 2 due to a 3.7-kb deletion (type I), has occurred on many

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restriction enzyme digestion protocol was developed using *Bgl* 11 and *Asp* 718 with a 3 's-globin probe to distinguish the most common α-thalassemia deletion (Lebo et al.,1990). The other one is a non-radioactive technique, Gap-PCR, which used DNA amplification to produce a 1.66 kb amplification fragment only when the 3.7 kb single α-gene deletion was present. The primers were chosen such that they were separated by a 5.48 kb stretch too far apart for amplification to take place in normal DNA without the 3.7 kb deletion (Chee et al.,1991).

Although the advanced technology, PCR, has been applied in the research field of thalassemia and other genetic diseases, the Southern hybridization still is required for accurate genotype of these disease other than those describe above. A number of different systems have been employed for the nonradioactive detection of nucleic acids sequences immobilized on a membrane. For example, the α-probe was located with digoxigenin-11 dUTP. After hybridization to the target DNA on the membrane, the detection of the hybrids was by an enzyme immunoassay using an antibody conjugate (antidigoxigenin alkaline phosphatase conjugate). Color detection was obtained with 5' -bromo-4'-chloroindoxyl phosphate and nitro blue tetrazolium salt Mulgia et al.,1993). The other one is ECL(enhanced chemiluminescence) gene detection system (Amersham). The system involves directly labelling probe with the enzyme horseradish peroxidase. The detection step is reduction of hydrogen peroxide by the enzyme coupled to the light producing reaction by the detection reagent containing luminol, which on oxidation produces blue light. Nevertheless, it is important to

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take care that the enzyme activity is not lost and therefore, the temperature of hybridization and wash never exceed 42°C. Moreover, the sensitivity will be severely reduced if there is a delay of more than 5 min during the detection step.

In this study, the PhotoGene™ Detection System was chosen instead of radioisotope ^{32}P radiation because of its greater advantages compared to radioisotope and other methods such as short exposure times, have time to optimize signal detection, label with convenient, stable biotinylated probes, and offer the same versality as radioactive hybridization. However, many steps should be adjusted to achieve the desired level of hybridization stringency. Agarose gel electrophoresis of a pair of digested genomic DNA (normal & sample) and Southern blotting were performed under the same conditions to produce multiple sets of nucleic acids-immobilized membranes. These membranes were used to vary the hybridization and washing condition to obtain either the better signal or the least background.

Several points have been noticed as follows.

1) The salmon sperm DNA is required to shear completely.

2) The amount of probe needed for hybridization should be increased to double of the amount calculated from the manufacturer's instruction.

3) After hybridization, the membrane was washed to remove any probe not specifically hybridized to complementary nucleic acid sequences. The optimal conditions of washing are :

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- washing the membrane twice with 2 ml/cm^2 5 x SSC, 0.5% (w/v) SDS at 56 ^{o}C, 15 min each wash.

- washing the membrane twice with 2 ml/cm^2 0.1 x SSC, 1 % (w/v) SDS at 56^{o}C, 30 min each wash.

4) After the step of binding the streptavidin-alkaline phosphatase conjugate, the time of final wash should be increased from 60 min to 90 min.

From this study,the introduction of non-radioactive Southern hybridization adapted to detect Hb H disease provide a good way for practical use in the laboratories for producing knowledge of the characteristic of Hb H disease at the molecular level and may be useful in genetic counselling, prevention and treatment.

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