CHAPTER III

RESULTS

III.1. Isolation of DNA: DNA 112 samples were isolated from 3-5 ml blood samples. The yield was varied from 20-80 ug/ml blood. The A260/A280 ratio was 1.60-1.97.

III.2. Restriction Endonuclease Digestion: DNA was completely digested in the order of 3 ug DNA/10 units enzyme, and had a smear pattern as in Figure III.1.

III.3. Southern blotting: The time for the blotting depended on the size of the DNA fragments.If the fragments to be examined were 1.75 - 3.5 kb in size, only 1 day was required to transfer the DNA fragments from the gel to the nitrocellulose membrane. If the fragments of interest were 6.0 - 20.0 kb in size, 2-3 days were needed for the transfer.

III.4. Preparation of the DNA specific probe:

III.4.1 DNA specific probe: The plasmid DNA was digested with a specific restriction endonuclease and isolated by electrophoresis in a 1.0% agarose gel.

Alpha$_2$plasmid DNA: Digestion with Pst I gave two fragments,the 4.1 kb plasmid and the 1.5 kb alpha$_2$probe (Figure III.2).

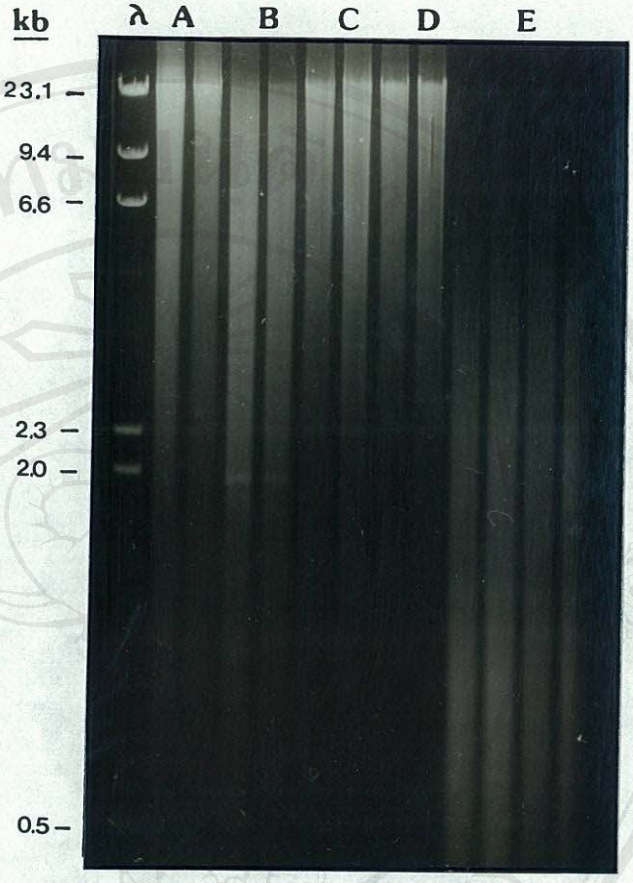

Figure III.1 The smear pattern of restriction endonuclease enzyme cleaved DNA.

A = Apa I cleaved DNA

B = Bam HI cleaved DNA

C = Bgl II cleaved DNA

D = Hind III cleaved DNA

E = Rsa I cleaved DNA

λ = Lambda Hind III marker ; given in kb.

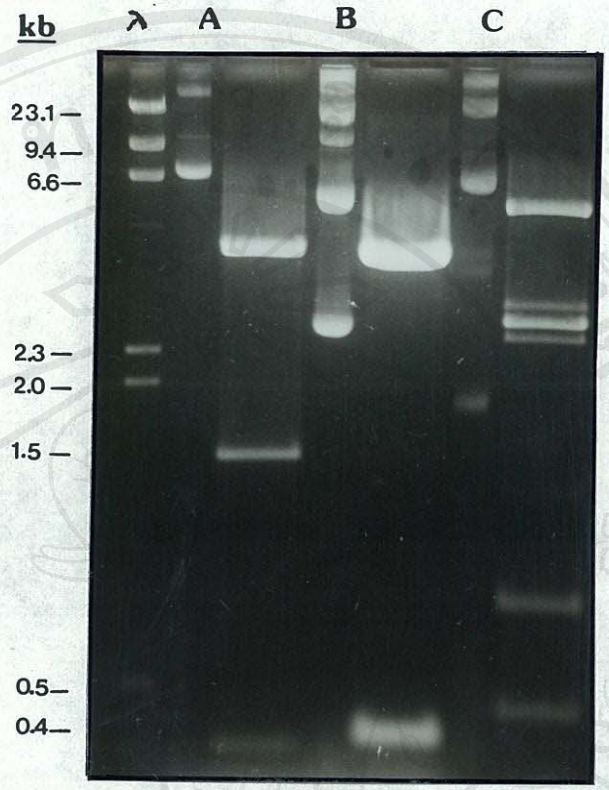

Figure III.2 Agarose gel electrophoresis for the demonstration of DNA specific probe isolation.

λ = Lambda Hind III marker ; given in kb.

A = Alpha$_2$ probe

B = Zeta probe

C = L0 probe

Zeta plasmid DNA: Digestion with Pst I and Hind III gave two fragments, the 4.0 kb plasmid and the 400 bp zeta probe (Figure III.2).

Lo plasmid DNA : Digestion with Bam HI and Eco RI gave four fragments including the Lo sequence of 410 bp (Figure III.2).

III.4.2 Oligouncleotide probe: The oligonucleotide probe for the detection of the Hb Constant Spring gene was purified by preparative polyacrylamide gel electrophoresis (2 OD/slot). The biggest band of the 19 mer oligonucleotide probe was in the range of bromphenol blue dye (Figure II.8).Both probes were eluted with 900 ul of sterile water. The concentration of the probe was determined at 260 nm and calculated by using the molar extinction coeffecient (10).

The molar extinction coefficient was calculated by summing the contribution of each nucleotide as follows: G = 12010, A = 15200, T = 8400 and C = 7050. The calculated number could be used to determine the molar concentration of the oligonucleotide solution after measurement of the solution at 260 nm.

For example: a 19 mer CS^{nml} oligonucleotide probe with 3 G, 4 A, 7 T and 5 C would have a molar extinction coefficient of 190880 at 260 nm which is equivalent to a concentration of 1 M/litre.

A260 190880 = 1 M/1

A260 1.0 = 1/190880 M/1

= 5.2 pmol/ul

Therefore, 1.9 ul of this probe with 1.0 OD/ml at 260 nm are needed for 10 pmole of the oligonucleotide probe.

III.5. Hybridization: For the hybridization of the oligonucleotide probe, the amount of the assay needed in the hybridization reaction was calculated as follows: The 16.0 ul assay consisted of 10 pmole oligonucleotide probe, 20 pmole of gamma ^{32}P-ATP and the specific activity = 40% of total.

Total activity = 100 mCi

= 220×10^6 dpm

40% incorporation = 88×10^6 dpm

1.0 ul of the assay = 5.5×10^6 dpm

10 ml hybridization solution = 20×10^6 dpm.

Accordingly, 3.6 ul of the assay were needed for the hybridization reaction.

III.6. Autoradiography:

DNA specific probe: specific bands on the X-ray film showed the restriction fragment length polymorphism of the studied genes:

A total of 112 DNA samples (224 chromosomes) were studied as shown in Figure III.3.

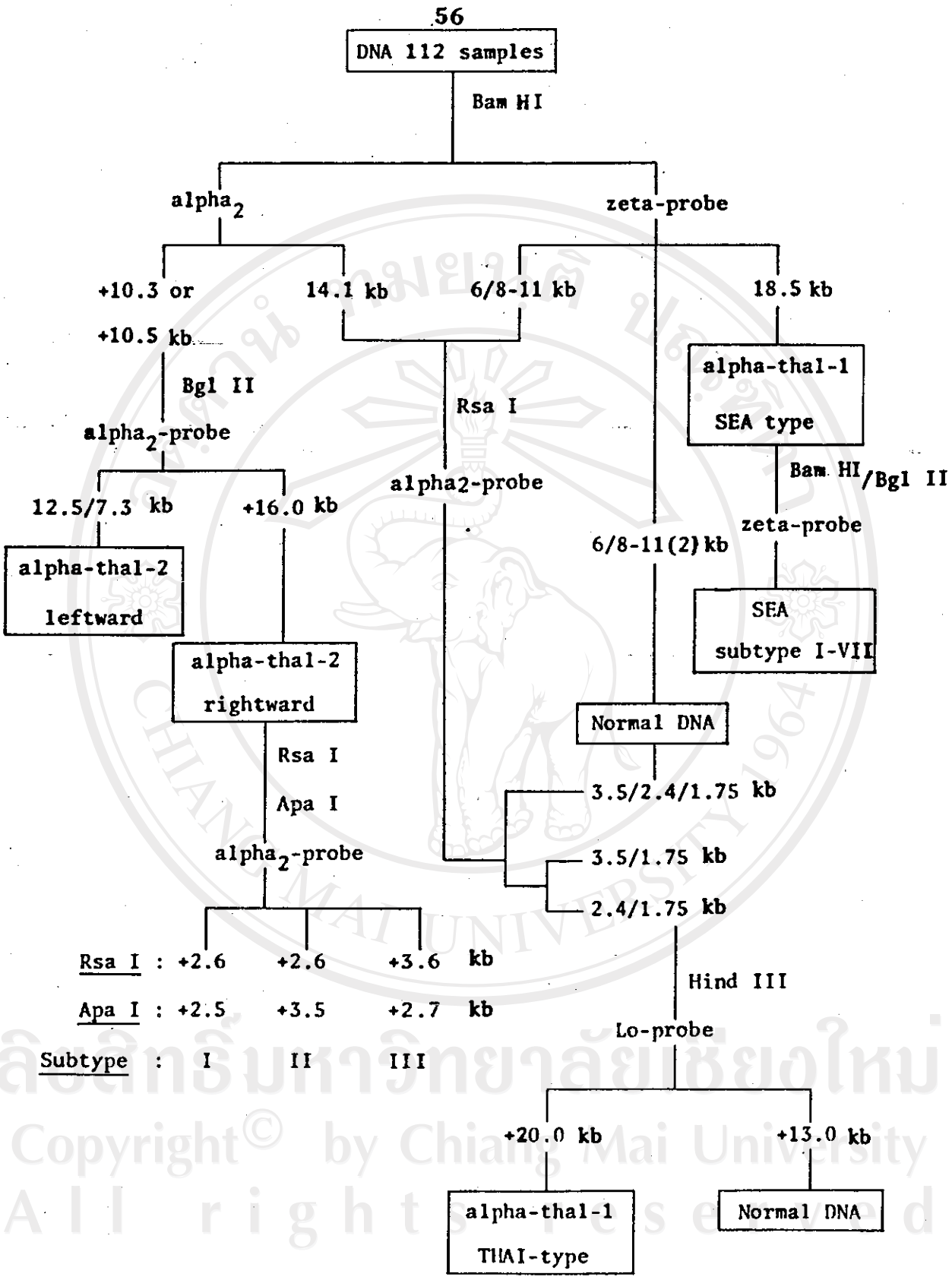

Figure III.3 Schematic of deletional alpha thalassemia detection.

Alpha-thal-1 was studied by digestion DNA with Bam HI and hybridized with a zeta probe. The normal chromosome showed two bands of 6 kb of the zeta globin gene and 8-11 kb of the pseudozeta gene. The alpha-thal-1 chromosome showed an abnormal band of 18.5 kb instead of 8-11 kb (Figure III.4, III5).

Subtyping of alpha-thal-1 was performed by digestion of the alpha-thal-1 DNA with Bam HI/Bgl II and hybridization with the zeta probe.The subtypes are determined by the length differences of the pseudozeta specific fragment 3' to the breakpoint.

subtype	I	II	III	IV	V	VI	VII
kb	10.5	11.0	9.8	9.5	9.0	8.8	8.4

Alpha-thal-2 was determined by digestion of DNA with Bam HI and hybridization with an $alpha_2$probe. The normal chromosome showed a band of 14.1 kb containing the alpha globin genes. The alpha-thal-2 chromosome showed a band of 10.5 or 10.3 kb corresponding to the deleted alpha-thalassemia chromosome (Figure III.4, III.6).

Subtyping of alpha-thal-2: digestion of the alpha-thal-2 DNA with BglII and hybridization with an $alpha_2$probe. The leftward deletion was characterized by two bands of 12.5 and 7.3 kb,containing the

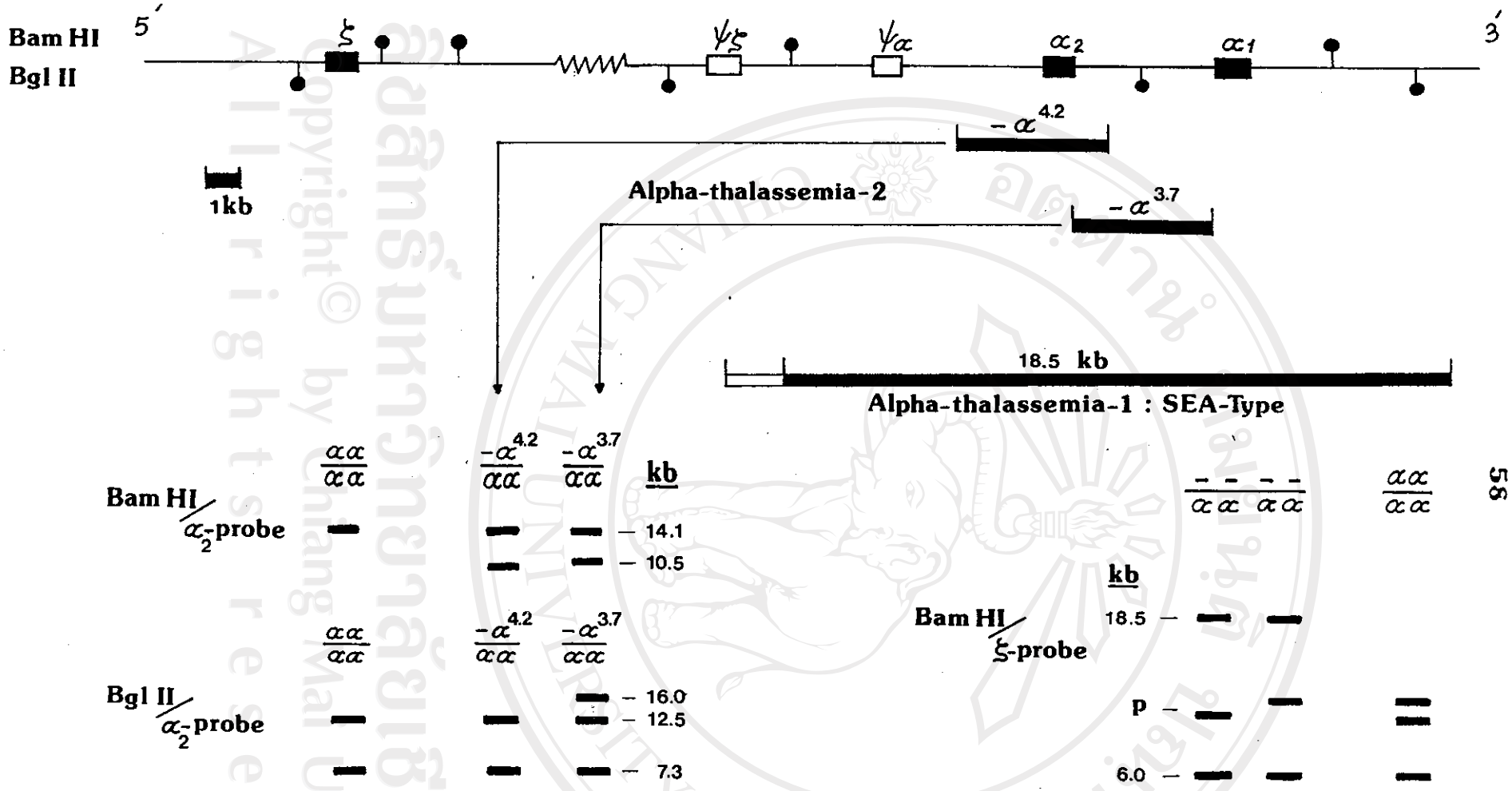

Figure III.4 Alpha thalassemia : deletional types, demonstration of Bam HI and Bgl II restriction sites.

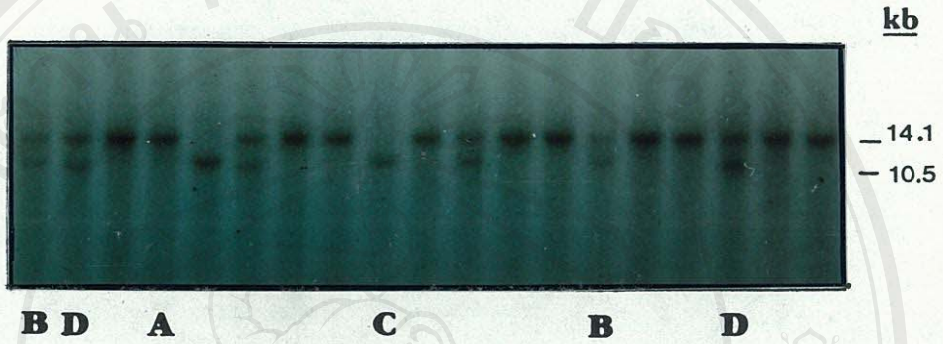

Figure III.5 Autoradiograph showing the alpha-thal-2 detection.

A = normal sample

B = heterozygous alpha-thal-2 (rightward type)

C = homozygous alpha-thal-2 (rightward type)

D = heterozygous alpha-thal-2 (leftward type)

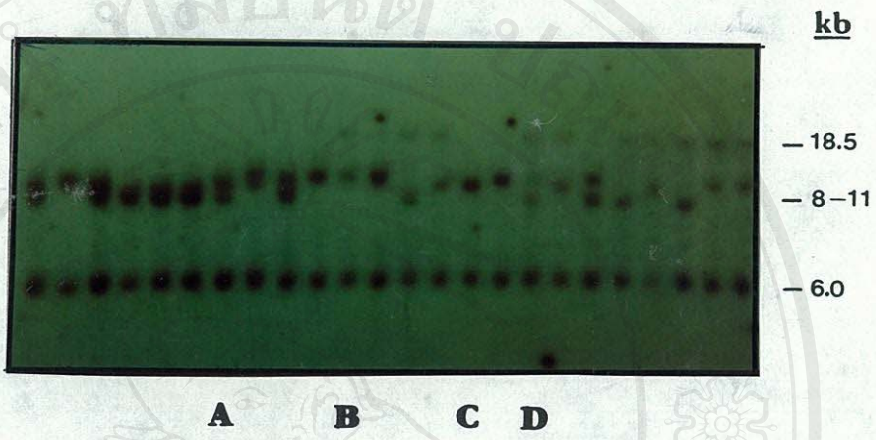

Figure III.6 Autoradiograph showing the alpha-thal-1 detection.

A = normal sample

B = heterozygous alpha-thal-1 (SEA type)

C = undifferentiated sample

D = heterozygous alpha-thal-1 (SEA type) with a triplicated zeta globin gene chromosome.

alpha$_2$globin gene and the alpha$_1$globin gene. The rightward deletion resulted in a band of 16 kb (Figure III.4).

Subtyping of rightward alpha-thal-2 was done by digestion of the rightward alpha-thal-2 DNA with Rsa I and Apa I and hybridization with an alpha$_2$probe. Each subtype showed characteristic bands as follows:

	Rsa I	Apa I
subtype I	2.6*	2.5
II	2.6*	3.5
III	3.6*	2.7

* = this fragment was 1.1 kb shorter if the chromosome had the Rsa I polymorphic site (Figure III.7).

Recognition of the total deletion of the zeta alpha gene cluster. Digestion of the DNA (which showed homozygosity for the pseudozeta gene fragments and the Rsa I polymorphic site) with Hind III and hybridization with the Lo probe.The normal chromosome showed a band of 13kb of the alpha gene complex,the ($--^{THAI}$) chromosome showed a band of 20 kb corresponding to the deleted chromosome (Figure III.8).

The Rsa I polymorphic site was studied by the digestion of DNA with Rsa I and hybridization with an alpha$_2$probe.This site is present at the 5'end of the Z_2 block. In the absence of the Rsa I polymorphic site, the size of the specific fragment of the alpha$_2$globin gene was

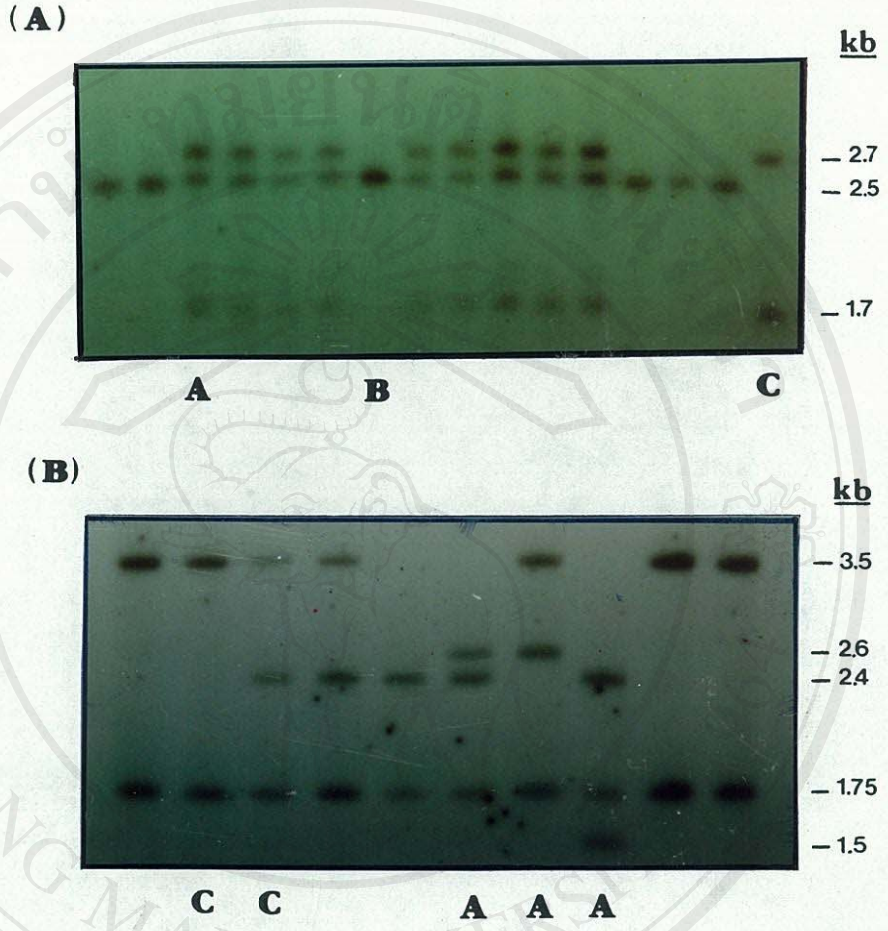

Figure III.7 Autoradiograph from subtyping of the rightward alpha-thal-2 in comparison with the normal samples.

(A) = Apa I cleaved DNA hybridized with alpha$_2$ probe.

(B) = Rsa I cleaved DNA hybridized with alpha$_2$ probe.

A = heterozygous rightward alpha-thal-2

B = homozygous rightward alpha-thal-2

C = normal sample

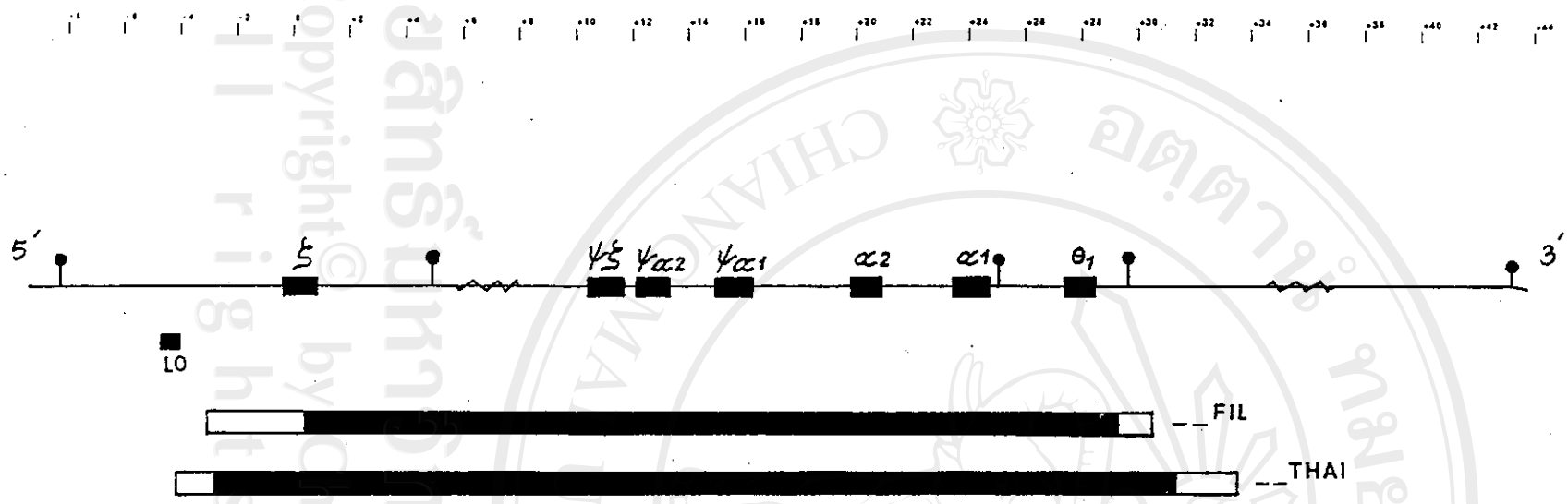

Figure III.8 Hind III enzyme mapping around the human alpha globin gene cluster.

■ = LO probe

$--^{THAI}$; $--^{FIL}$ = The two deletions are presented by black bars with white boxs at the ends.

3.5 kb for the normal chromosome and 2.6 kb for alpha-thal-2 (rightward).The fragment was 1.1kb shorter when the Rsa I polymorphic site was present (1.5 kb for alpha-thal-2 and 2.4 kb for the normal chromosome). In the leftward form, the Rsa I polymorphic site was included in the deleted 4.2 kb fragment and cannot be demonstrated (Figure III.9, III.10).

Hb Constant Spring: the frequency of this mutant was studied by digesting DNA with RsaI and hybridization with two specific oligonucleotide probes:

CS^{nml} = CTCCAGCTTA*ACGGTATTT (homologous to the normal sequence)

CS^{mut} = AAATACCGTC*AAGCTGGAG (homologous to the mutant sequence)

The normal chromosome showed bands of alpha globin genes with only the CS^{nml} probe, but the mutant chromosome showed a band of the $alpha_2$globin gene with only a CS^{mut} probe. (Figure III.11).

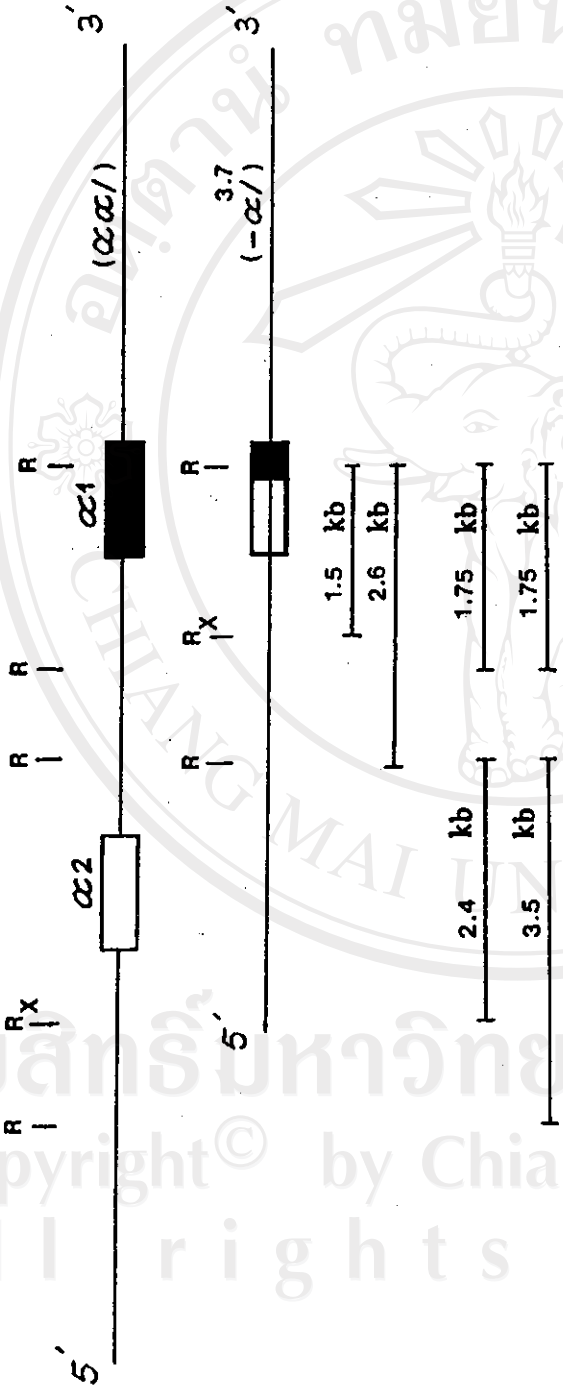

Figure III.9 Details of Rsa I restriction sites on the alpha globin gene cluster.

Rx = Rsa I polymorphic site.

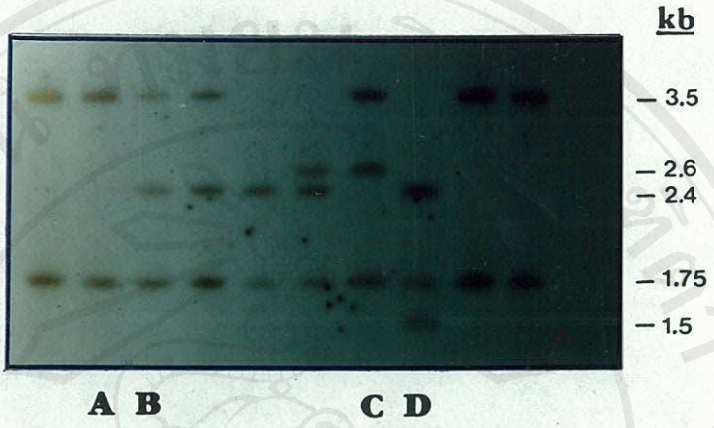

Figure III.10 Autoradiograph showing the Rsa I polymorphic site detection.

A = normal sample without Rsa I polymorphic site (R-)

B = normal sample with Rsa I polymorphic site (R+)

C = rightward alpha-thal-2 without Rsa I polymorphic site (R-)

D = rightward alpha-thal-2 with Rsa I polymorphic site (R+)

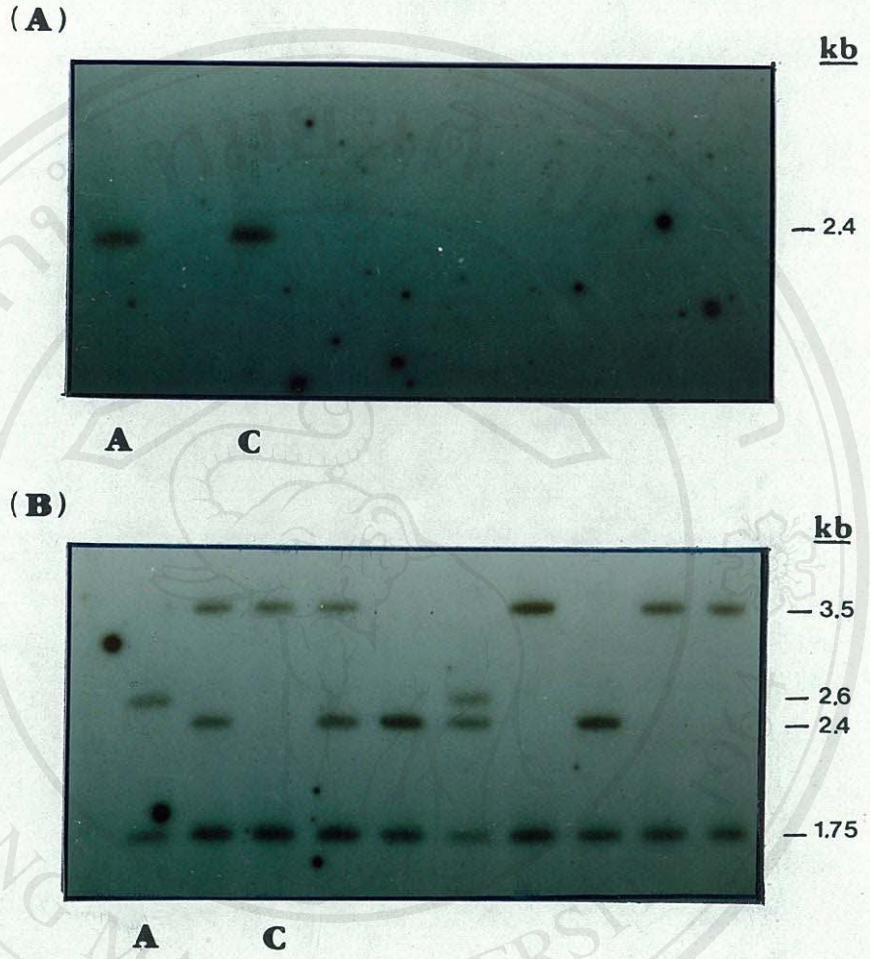

Figure III.11 Autoradiograph showing the Hb CS detection.

(A) = hybridization with the CS^{mut} probe : sample A and C showed the 2.4 kb fragment of mutant $alpha_2$ gene.

(B) = hybridization with the CS^{nml} probe : sample A and C showed the absent of 2.4 kb fragment.

The results of the RFLP study were shown in Tables III.1-III.5. The following abbreviations were used:

The restriction enzyme and the probe used are indicated as follows:

Bam HI/α_2 = digestion of the DNA with Bam HI and hybridization with an alpha$_2$probe. In the tables the size of DNA fragments (bands) in kb was shown. The following symbols indicated the district of origin of the probands:

J = samples from Jomtong.

S = samples from Smeang.

MT = samples from Mae Tang.

P = samples from Prou.

Interpretation of the alpha globin gene status:

= normal status (with 4 alpha globin gene loci).

= heterozygous alpha-thal-2 rightward, subtype I.

= homozygous alpha-thal-2 rightward, subtype I.

= heterozygous alpha-thal-2 leftward.

= heterozygous alpha-thal-1.

Table III.1. The RFLP of DNA samples when hybridization the Bam HI digested DNA with the alpha$_2$ probe or the zeta probe.

Sample	Bam HI/α_2	Bam HI/ζ	Typing
J1	14.1/10.5	6/8-11*	α-thal-2
J2	14.1	6/8-11	?
J3	14.1	6/8-11*	αα/αα
J4	14.1	6/8-11	?
J5	14.1	6/8-11*	αα/αα
J6	14.1	6/8-11*	αα/αα
J7	14.1/10.5	6/8-11*	α-thal-2
J8	14.1/10.5	6/8-11*	α-thal-2
J9	14.1	6/8-11*	αα/αα
J10	14.1	6/8-11	?
J11	14.1	6/8-11/18.5	α-thal-1
J12	14.1	6/8-11	?
J13	14.1	6/8-11/18.5	α-thal-1
J14	14.1	6/8-11/18.5	α-thal-1
J15	14.1	6/8-11	?
J16	14.1	6/8-11	?
J17	14.1	6/8-11*/18.5	α-thal-1*
J18	14.1	6/8-11/18.5	α-thal-1
J19	14.1	6/8-11*	αα/αα

* showed two bands of 8-11 kb.

α-thal-1* = triplicated zeta globin gene sample.

Sample	Bam HI/α_2	Bam HI/ζ	Typing
S1	14.1	6/8-11/18.5	α-thal-1
S2	14.1/10.5	6/8-11*	α-thal-2
S3	14.1/10.5	6/8-11	α-thal-2
S4	14.1	6/8-11	?
S5	14.1/10.5	6/8-11	α-thal-2
S6	14.1/10.5	6/8-11	α-thal-2
S7	14.1	6/8-11	?
S8	10.5	6/8-11	α-thal-2*
S9	14.1	6/8-11	?
S10	14.1	6/8-11*	$\alpha\alpha/\alpha\alpha$
S11	14.1	6/8-11	?
S12	14.1/10.5	6/8-11	α-thal-2
S13	14.1	6/8-11*	$\alpha\alpha/\alpha\alpha$
S14	14.1	6/8-11*	$\alpha\alpha/\alpha\alpha$
S15	14.1/10.5	6/8-11	α-thal-2
S16	10.5	6/8-11	α-thal-2*
S17	14.1	6/8-11	?
S18	14.1	6/8-11*/18.5	α-thal-1*
S19	14.1	6/8-11	?
S20	14.1	6/8-11	?
S21	14.1	6/8-11	?
S22	14.1	6/8-11	?
S23	14.1	6/8-11	?

Sample	Bam HI/α_2	Bam HI/ζ	Typing
S24	14.1	6/8-11	?
S25	14.1	6/8-11*	αα/αα
S26	14.1	6/8-11*	αα/αα
S27	14.1	6/8-11	?
S28	14.1/10.5	6/8-11	α-thal-2
S29	14.1	6/8-11*	αα/αα
S30	10.5	6/8-11*	α-thal-2*
S31	14.1/10.5	6/8-11*	α-thal-2
S32	14.1/10.5	6/8-11*	α-thal-2
S33	14.1	6/8-11	?
S34	14.1	6/8-11*	αα/αα
S35	10.5	6/8-11	α-thal-2*
S36	14.1/10.5	6/8-11	α-thal-2
S37	14.1	6/8-11	?
S38	14.1	6/8-11*	αα/αα
S39	10.5	6/8-11*	α-thal-2*
S40	14.1	6/8-11	?
S41	14.1/10.5	6/8-11*	α-thal-2
S42	14.1	6/8-11	?
S43	14.1	6/8-11*	αα/αα
S44	14.1/10.5	6/8-11	α-thal-2
S45	14.1	6/8-11	?
S46	14.1	6/8-11*	αα/αα

Sample	Bam HI/α_2	Bam HI/ζ	Typing
S47	14.1/10.5	6/8-11*	α-thal-2
S48	14.1	6/8-11	?
S49	14.1	6/8-11	?
MT1	14.1	6/7.5*/8-11	?
MT2	14.1	6/8-11	?
MT3	14.1	6/8-11*	αα/αα
MT4	14.1/10.5	6/8-11	α-thal-2
MT5	14.1	6/8-11*	αα/αα
MT6	14.1	6/8-11-18.5	α-thal-1
MT7	14.1	6/8-11	?
MT8	14.1/10.5	6/8-11*	α-thal-2
MT9	10.5	6/8-11*	α-thal-2*
MT10	14.1	6/8-11/18.5	α-thal-1
MT11	14.1	6/8-11*	αα/αα
MT12	14.1/10.5	6/8-11	α-thal-2
MT13	14.1	6/8-11	?
MT14	14.1	6/8-11*	αα/αα
MT15	14.1	6/8-11*	αα/αα
MT16	14.1	6/8-11	?
MT17	14.1/10.5	6/8-11	α-thal-2
MT18	14.1	6/8-11*	αα/αα
MT19	14.1	6/8-11	?

Sample	Bam HI/α_2	Bam HI/ζ	Typing
P1	14.1	6/8-11*	αα/αα
P2	14.1	6/8-11	?
P3	14.1	6/8-11	?
P4	14.1	6/8-11*	?
P5	14.1	6/8-11*	αα/αα
P6	14.1	6/8-11*	αα/αα
P7	14.1	6/8-11/18.5	α-thal-1
P8	14.1/10.5	6/8-11	α-thal-2
P9	14.1/10.5	6/8-11	α-thal-2
P10	14.1/10.5	6/8-11	α-thal-2
P11	14.1	6/8-11	?
P12	14.1	6/8-11*	αα/αα
P13	14.1	6/8-11	?
P14	10.5	6/8-11	α-thal-2*
P15	14.1	6/8-11*	αα/αα
P16	14.1	6/8-11*	αα/αα
P17	14.1/10.5	6/8-11*	α-thal-2
P18	14.1	6/8-11*	αα/αα
P19	14.1	6/8-11*	αα/αα
P20	14.1	6/8-11/18.5	α-thal-1
P21	14.1	6/8-11/18.5	α-thal-1
P22	10.5	6/8-11/18.5	Hb H disease
P23	14.1	6/8-11	?

Sample	Bam HI/α_2	Bam HI/ζ	Typing
P24	10.5	6/8-11*	α-thal-2*
P25	14.1	6/8-11	7

α-thal-2* : homozygous deletion ($-\alpha/-\alpha$)

* : abnormal band (MT1)

7 : undifferentiated sample

Out of 112 samples, 33 showed anomalous bands characteristic of alpha-thal-2 (10.5 kb) with the alpha$_2$probe, and 8 of these were homozygotes. Thirteen samples showed the alpha-thal-1 bands with the zeta probe (18.5 kb), two other cases had triplicated zeta globin genes (J17,S18),one with an abnormal zeta globin gene fragment (MT1) (Figure III.12) and one within Hb H disease (P22).Twenty-nine of the remainder were typed as normal on account of the presence of two pseudozeta globin gene fragments.

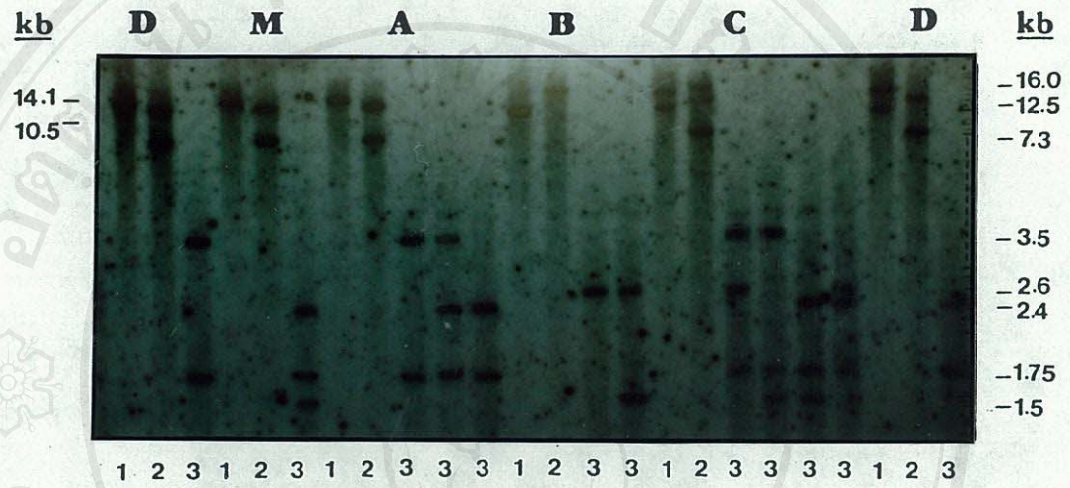

Figure III.12 Autoradiograph showing the RFLP of mutant sample (MT1) in comparison with the normal sample , the rightward and the leftward alpha-thal-2 samples.

A = normal sample

B = homozygous rightward alpha-thal-2

C = heterozygous rightward alpha-thal-2

D = heterozygous leftward alpha-thal-2

M = MT1 sample

lane 1 = Bam HI / $alpha_2$ probe

lane 2 = Bgl II / $alpha_2$ probe

lane 3 = Rsa I / $alpha_2$ probe

Table III.2 The RFLP of 33 alpha-thal-2 samples when digestion with Bgl II, Rsa I, or Apa I and hybridization with alpha$_2$probe.

Samples	Bgl II/α_2	Rsa I/α_2	Apa I/α_2
S2, S12, S15, S28 S31, S41, S44, MT4 P9, P17	16/12.5/7.3	3.5/2.6/1.75	2.7/2.5/1.7
J1, J7, J8, S3, S6 MT12,MT17, P8	16/12.5/7.3	2.6/2.4/1.75	2.7/2.5/1.7
MT8	16/12.5/7.3	3.5/1.75/1.5	2.7/2.5/1.7
P10	16/12.5/7.3	2.4/1.75/1.5	2.7/2.5/1.7
S8, S16, S35 S39, MT9, P14 P22*, P24	16.0	2.6	2.5
S30	16.0	2.6/1.5	2.5
S5, S32, S36, S47	12.5/7.3	3.5/2.4/1.75	2.7/1.7

P22* = Hb H disease.

Of 33 samples with α-thal-2 deletion, 29 were diagnosed as the rightward type because of the presence of the abnormal 16.0 kb band after digestion with Bgl II and hybridization with the $alpha_2$probe. The other four cases were typed as the leftward deletion. All the rightward deletions showed the specific band of the subtype I (2.4 or 1.5 kb).

Table III.3 The RFLP of alpha-thal-1 samples when hybridization the consecutive Bam HI/Bgl II digested DNA with the zeta probe.

Samples	Bam HI/Bgl II/ζ	Subtyping
J11, J13, J14, J17,J18 P22, S1, MT6, MT10,P7, P21.	10.5	SEA(I)
S18*	10.5/11.0	SEA(I,II)
P24	11.0	SEA (II)

J17* ,S18* = triplicated zeta genes.

Of 13 samples with the alpha-thal-1 deletion, 12 cases showed the specific band of the subtypeI (10.5 kb)and two cases of subtypeII (11.0 kb).

Table III.4 The RFLP of undifferentiated DNA digested with Rsa I and hybridized with an alpha$_2$probe.

Samples	Rsa I/α_2	Typing
J: 10,12,16 S: 7,9,11,19,22,23,27	3.5/1.75	
S: 37,42,49, MT: 2,7 P: 2,3,4,11		?
J: 15 S: 20,48 MT: 16,19	2.4/1.75	?
J: 2,4 S: 4,17,21,24,33,40,45 MT: 13 P: 13,15,23,25	3.5/2.4/1.75	

? = undifferentiated samples

Of 38 undifferentiated samples (Table III.1), 14 were typed as normal based on the heterozygosity for the Rsa I polymorphic site.The remaining 24 undifferentiated samples were then digested with Hind III and hybridized with the Lo probe to detect the ($--^{THAI}$) deletion. Every sample showed the normal band of the zeta globin gene fragment of 13 kb. This indicates that none of these samples had a large deletion of the entire zeta alpha globin gene cluster and that four alpha globin gene sequences were present.

The result of the oligonucleotide hybridization :

CS^{nml} probe: every sample showed bands with this probe.

CS^{mut} probe: 4 samples showed bands with this probe (S36, S48, MT12 and MT14) (Table III.5)

Table III.5 The RFLP of four Hb CS samples when hybridization the Rsa I digested DNA with the $alpha_2$ probe or the oligonucleotide CS probe.

Sample	Rsa I/α_2	Rsa I/CS^{nml}	Rsa I/CS^{mut}
S36	2.4/1.75	1.75	2.4
S48	2.4/1.75	2.4/1.75	2.4
MT12	2.6/2.4/1.75	2.6/1.75	2.4
MT14	3.5/2.4/1.75	3.5/1.75	2.4

All four samples showed heterozygosity for the Hb CS mutation. S48 and MT14 did not show a deletion. S36 was a leftward alpha-thal-2 deletion and MT12 was a rightward alpha-thal-2 deletion. Accordingly, the genotypes of these samples were :

S36 = Hb CS / leftward alpha-thal-2 ($\alpha\alpha^{CS}/-\alpha^{4.2}$)

MT12 = Hb CS / rightward alpha-thal-2 ($\alpha\alpha^{CS}/-\alpha^{3.7}$)

S48, MT14 = Hb CS / normal ($\alpha\alpha/\alpha\alpha^{CS}$)

From Tables III.1 and III.2, the frequency of the alpha globin haplotypes was determined (Table III.6)

Table III.6 The prevalence of the alpha globin haplotypes in the total population sample.

Alpha globin haplotype	Number observed	Frequency
$\alpha\alpha/$	166	0.741
$-\alpha^{3.7}$	37	0.165
$-\alpha^{4.2}$	4	0.018
$--^{SEA}$	13	0.058
$\alpha\alpha^{CS}$	4	0.018
Total	224	1.000

The observed and expected frequencies of the alpha globin genotypes were compared in Table III.7.

Table III.7 The prevalence of the alpha globin genotypes.

Alpha globin genotype	Number of subjects		Percent observed
	Observed	Expected**	
$\alpha\alpha/\alpha\alpha$	65	61.5	58.0
$\alpha\alpha/-\alpha^{3.7}$	19	27.4*	17.0
$\alpha\alpha/-\alpha^{4.2}$	3	3.0	2.7
$\alpha\alpha/--^{SEA}$	12	9.6	10.7
$-\alpha^{3.7}/-\alpha^{3.7}$	8	3.0*	7.1
$-\alpha^{3.7}/--^{SEA}$	1	2.1	0.9
$\alpha\alpha/\alpha\alpha^{CS}$	2	3.0	1.8
$-\alpha^{3.7}/\alpha\alpha^{CS}$	1	0.7	0.9
$-\alpha^{4.2}/\alpha\alpha^{CS}$	1	0.1	0.9
Others	0	1.6	0.0
	112	112.0	100.0

**: calculated according to the Hardy-Weinberg equilibrium.

The observed distribution agreed not with the Hardy-Weinberg equilibrium, $X^2 = 22.4$, $p < 0.05$

* The critically different values

Table III.8 Linkage of the Rsa I polymorphism and alpha globin haplotypes.

Alpha globin haplotype	Number observed	Percent observed (A)	Percent observed (B)
* : Rsa I (+)	54	31.7	26.1
: Rsa I (-)	116	68.3	55.5
-** : Rsa I (+)	3	8.1	1.4
: Rsa I (-)	34	91.9	17.0
Total	207		100.0

(A) = Percent observed in each group.

(B) = Percent observed of total samples.

* both $\alpha\alpha/$ and $\alpha\alpha^{CS}/$ samples.

-** only $-\alpha^{3.7}$ samples.