CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

Thalassemia syndrome is a group of hereditary disorders with defects in the synthesis of globin chains of hemoglobin. These defects lead to the absent or decreased synthesis of the affected globin chains. Erythrocytes of thalassemia; therefore, have a reduction in the hemoglobin content and the size. Thalassemia syndrome has high incidence and affect people living in several countries in Mediterranean, Africa, through the Middle East and South East Asia (Chong *et al*., 2000; Krishnsmurti *et al*., 1998; Weatherall and Clegg, 2001). This disease is now a serious-public health problem of these regions.

Thalassemia syndrome can be divided into two major types, β- and α-thalassemia. The α-thalassemia is usually caused by the deletion of one or more α-globin genes on chromosome 16 (Old, 2003). β-thalassemia is predominantly caused by point mutations in β-globin gene leading to the decrease of β-globin chain synthesis. The most mutations are in functionally important regions of the β-globin. β-thalassemia occurs when there is a quantitative reduction of β-globin chains that are usually structurally normal. They are caused by mutations that nearly all affect the β-globin locus and are extremely heterogeneous. Almost every possible defect affecting gene expression at transcription or post-transcriptional level, including

translation, has been identified in β-thalassemia (Thein, 2005). These genetic defects lead to a variable reduction in β-globin output ranging from a minimal deficit (mild β^+ thalassemia alleles) to complete absence (β-thalassemia). In β-thalassemia, the synthesis of normal α-globin chains from the unaffected α-globin genes continues as normal, resulting in the accumulation within the erythroid precursors of excess unmatched α-globin. The free α-globin chains are not able to form viable tetramers and instead precipitate in the red cell precursors in the bone marrow forming inclusion bodies. The excess α-globin chains are deposited in those mature red blood cells circulating in the system, damaging their membranes, leading to their destruction and adding to the severity of the anemia (Rund, 2005; Thein, 2005; Sarnaik, 2005). The process by which red cells are broken down is known as hemolysis. The modifying factors of β-thalassemia phenotype are i) type of β-globins gene mutation ii) α-globin genotype**,** variation in the amount of α-globin production iii) variation in fetal hemoglobin response. Apart from the number of α-globin genes and an inherent capacity to produce HbF, the proteolytic capacity of the erythroid precursors in catabolizing the excess α-globin chains has often been suggested as another factor, but this effect is difficult to define. Recently, the newly discovered alpha hemoglobin stabilizing protein (AHSP) has been suggested as an additional factor related to clinical severity of β-thalassemia. (Thein, 2005).

AHSP was identified as an erythroid-specific protein whose gene was induced by the essential transcription factor GATA-1. AHSP heterodimerizes with α-Hb but did not bind β-Hb or HbA (Gell *et al*., 2002; Kihm *et al.*, 2002). AHSP is an abundant, erythroid-specific protein that protects free α Hb from precipitation both in

solution and in live cells. New insights into the other potential roles of AHSP in red cells were obtained when Weiss *et al*. (2005) examined the effects of the loss of AHSP in a murine model of β-thalassemia. The anemia in these mice is much worse than that observed in either *Ahsp*$^{-/-}$ mice without β thalassemia or in mice with β-thalassemia and intact AHSP (Kong *et al*., 2004). The explanation for this observation is that AHSP normally stabilizes at least some of the excess of α-globin produced in erythroid precursors in β-thalassemia by forming α Hb-AHSP complexes, rendering at least some of the excess α-globin more soluble and stable and, therefore, causing less α-globin precipitation and limiting ineffective erythropoiesis. Normally, Apo-α-globin and α Hb are potential oxidants, especially in the absence of AHSP, generating ROS that lead to red cell membrane damage. The AHSP provides some protection against these effects of excess α-globin and α Hb. However, normal AHSP function is inadequate to stabilize the excess of α-globin in β-thalassemia and prevent the severe anemia in mice or humans with the disease. It has been reported that the altered levels and function of AHSP might account for the clinical variability observed in β-thalassemia patients. Therefore, detection of AHSP level leading to the evaluation of clinical severity in β-thalassemia patients are more interested. Since only the expression of AHSP mRNA was studied in erythroid precursors. So far, the detection of AHSP level by immunodetection assay based on monoclonal antibody (mAb) against the AHSP has not been reported.

Therefore the aim of this study is to produce monoclonal antibodies against AHSP and to characterize the generated mAbs.

1.2 Literature reviews

1.2.1 Structure and function of hemoglobin

Hemoglobin (Hb) is polypeptide tetramer with globular structure (Figure 1.1). Each red blood cell (RBC) contains approximately 280 million hemoglobin molecules (Sears, 1999). The main function of hemoglobin is to transport oxygen from the lungs to the tissues and then transport CO_2 back from the tissues to the lungs. One hemoglobin molecule has the ability to transport up to 4 oxygen molecules. There are two forms of hemoglobin: oxyhemoglobin, which is saturated with oxygen molecules and deoxyhemoglobin, which is desaturated with oxygen molecules (Sears, 1999). Oxyhemoglobin has a higher affinity for oxygen than deoxyhemoglobin, and deoxyhemoglobin has a higher affinity for CO_2 than oxyhemoglobin. Therefore, oxygen binds to oxyhemoglobin in the lungs and is then transported through the blood stream until it reaches the tissues. There, the oxygen is released to myoglobin, which then transports it to the mitochondria where it is used for aerobic respiration. In exchange, the deoxyhemoglobin picks up 2 protons and 2 molecules of CO_2 and returns to the lungs, where the CO_2 is released through exhalation (Perutz, 1978).

Hemoglobin is synthesized during most of the erythrocytic maturation process. This molecule is composed of two pairs of globin chains, i.e. two α-like chains, α or ζ chain, and two β-like chains, β, δ, γ, δ or ε chains (Figure 1.2) (Rifkind *et al.*, 1984). The γ globin chain has two variants, $^{A}\gamma$ and $^{G}\gamma$, which differ by a single amino acid either alanine (A) or glycine (G) at position 136. All globin chains are arranged in a series of straight stretches in the α-helical regions configuration, joined by the short nonhelical regions. The interior spaces of the hemoglobin tetramer

contain only non-polar amino acids, preserving a non-aqueous (hydrophobic) internal environment. Amino acids with polar side chains are exclusively directed at the external surface of the molecule. Each globin chain bind prosthetic heme group. The heme group containing protoporphyrin and ferrous iron (Fe^{2+}) are embedded within the hydrophobic interior space of the globin molecule. This nonaqueous environment is essential to preserve the heme in its biologically active Fe^{2+} form.

The α-like and β-like globin chains consist of 141 and 146 amino acid residues, respectively. There is some sequence homology between the two chains (64 individual amino acid residues in identical positions), and the β chain differs from the δ and γ chains by 39 and 10 residues, respectively (Clarke and Higgins, 2000). The α-globin gene and ζ-globin gene are located on the short arm of chromosome 16. For practical purposes, the two *alpha* globin genes (termed alpha1 and alpha2) are identical. Since each cell has two chromosomes 16, a total of four alpha globin genes exist in each cell. Each of the four genes produces about one-quarter of the alpha globin chains needed for hemoglobin synthesis. The mechanism of this coordination is unknown. Promoter elements exist 5' to each alpha globin gene. In addition, a powerful enhancer region called the locus control region (LCR) is required for optimal gene expression. The LCR is many kilobases upstream of the alpha globin locus. The mechanism by which DNA elements so distant from the genes control their expression is the source of intense investigation. The transiently expressed embryonic genes that substitute for alpha very early in development, designated zeta are also in the alpha globin locus.

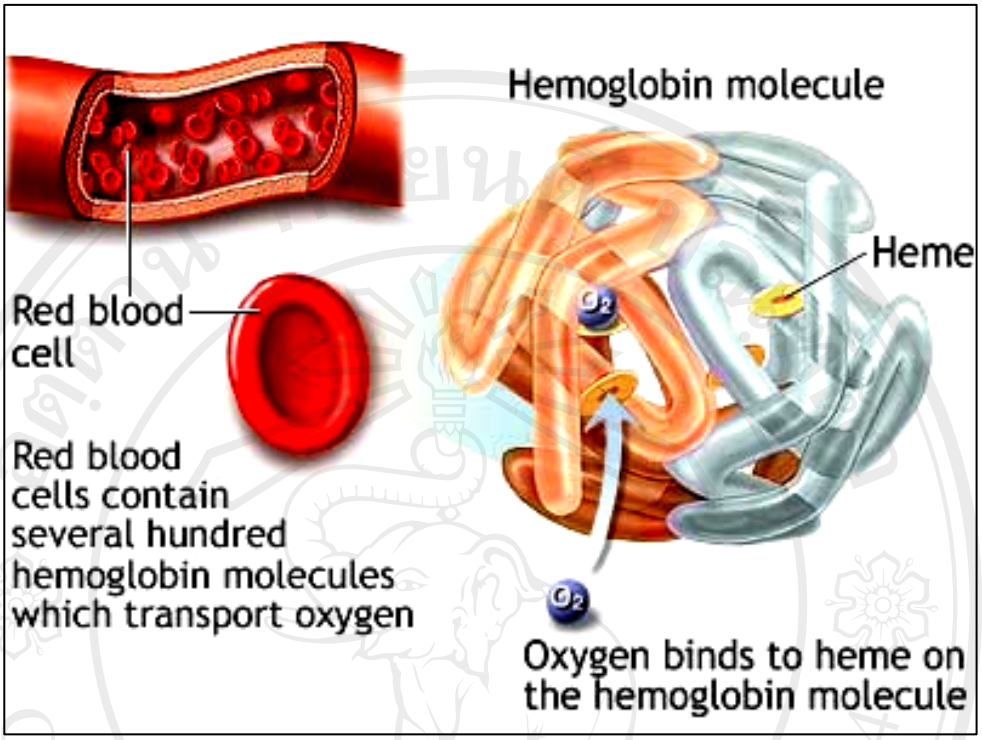


Figure 1.1 Molecular structure of hemoglobin. The molecule of Hb composes of a porphyrin ring with a central iron atom (heme) and two pairs of unlike globin chains, which form a shell around a central cavity containing four-oxygen-binding heme groups each covalently linked to globin chain.

(http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/19510.jpg)

The non-α-globin genes including β, δ, γ and ε genes are located as a cluster on the short arm of chromosome 11. The genes in the beta globin locus are arranged sequentially from 5' to 3' beginning with the gene expressed in embryonic development (the first 12 weeks after conception; called epsilon). The beta globin locus ends with the adult beta globin gene. The sequence of the genes is: epsilon, gamma, delta, and beta. There are two copies of the gamma gene on each chromosome 11. The others are present in single copies. Therefore, each cell has two beta globin genes, one on each of the two chromosomes 11 in the cell. These two beta globin genes express their globin protein in a quantity that precisely matches that of the four alpha globin genes (Bunn,1986). Different types of hemoglobin result from matching the different chains and different types of hemoglobin produced at each stage of life.

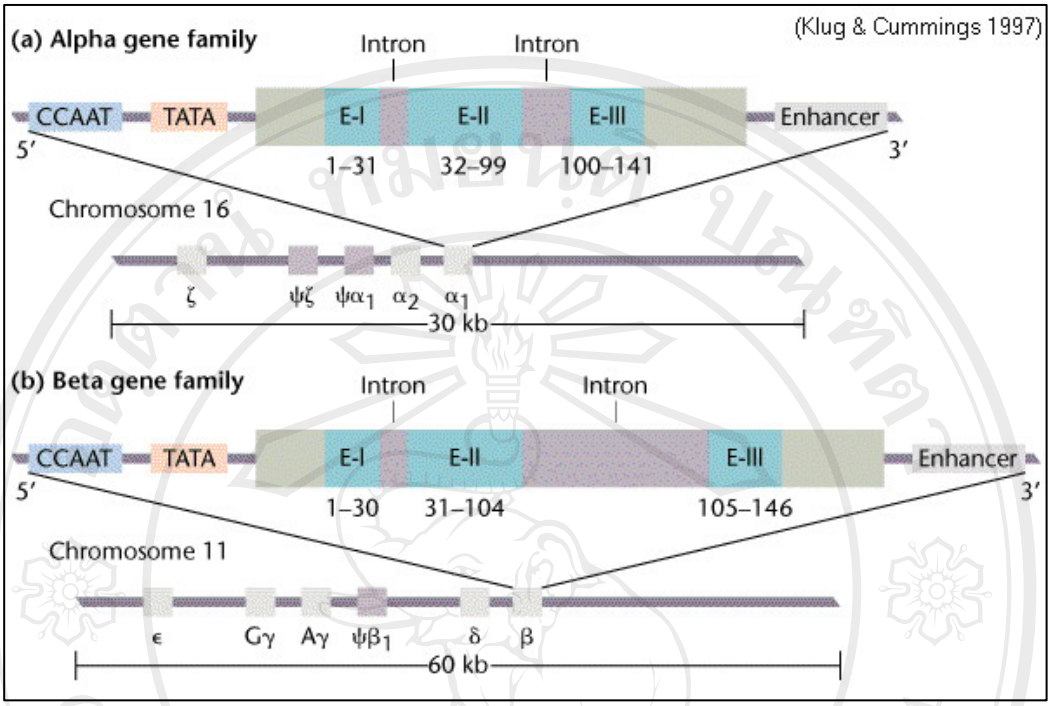


Figure 1.2 The chromosome of α and β-globin genes family. (a) The α-globin gene family including α and ζ are located on chromosome 16. (b) The β-globin gene family including β, δ, $^{A}\gamma$, $^{G}\gamma$ and ε are located on chromosome 11.

(http://www.mun.ca/biology/scarr/Fg17_19.gif accessed 15 July 2006)

The ability of hemoglobin to take up oxygen molecules in the lungs and then release them in the tissues is regulated by several factors both within the hemoglobin molecule itself and through external chemical factors. One of the biggest regulators of the oxygen affinity of the hemoglobin is the presence of oxygen itself. In the lungs where the oxygen levels are high, the hemoglobin has a higher affinity for oxygen and this affinity increases disproportionately with the number of molecules it already has bound to it. In other words, after the oxyhemoglobin binds one molecule of oxygen its affinity for oxygen increases until the hemoglobin is fully saturated. In the same way, the deoxyhemoglobin has a lower affinity for oxygen and this affinity decreases disproportionately with the number of molecules it already has bound. Thus, the loss of one oxygen molecule from the deoxyhemoglobin lowers the affinity for the remaining oxygen. This regulation is known as cooperativity and is essential to the functioning of the hemoglobin because it allows the oxyhemoglobin to carry the maximum amount of oxygen to the tissues and then allows the deoxyhemoglobin to release the maximum amount of oxygen into the tissues (Sears, 1999).

When fully saturated, each gram of hemoglobin binds 1.34 ml of oxygen. The degree of saturation is related to the oxygen tension (pO_2), which normally ranges from 100 mmHg in arterial blood to about 35 mmHg in veins. The relation between oxygen tension and hemoglobin oxygen saturation is described by the oxygen-dissociation curve of hemoglobin. The characteristics of this curve are related in part to properties of hemoglobin itself and in part to the environment within the erythrocyte, including pH, temperature, ionic strength, and concentration of phosphorylated compounds, especially 2,3-diphosphoglycerate (2,3-DPG). Oxygen affinity of hemoglobin is generally expressed in terms of the oxygen tension at which

50% saturation occurs. When measured in whole erythrocytes, this value averages 27.1 mmHg in normal, nonsmoking males and 27.5 mmHg in normal, nonsmoking females. When oxygen affinity is increased, the dissociation curve is shifted leftward, and the value is reduced. Conversely, with decreased oxygen affinity, the curve is shifted to the right. The oxygen-dissociation curve of hemoglobin is distinctly sigmoid; the steepest part of its slope occurs at levels of oxygen tension corresponding to those found in tissues. The change in oxygen affinity with pH is known as the Bohr effect. Hemoglobin oxygen affinity is reduced as the acidity increases. Since the tissues are relatively rich in carbon dioxide, the pH is lower than in arterial blood; therefore, the Bohr effect facilitates transfer of oxygen. The Bohr effect is a manifestation of the acid-base equilibrium of hemoglobin (Figure 1.3).

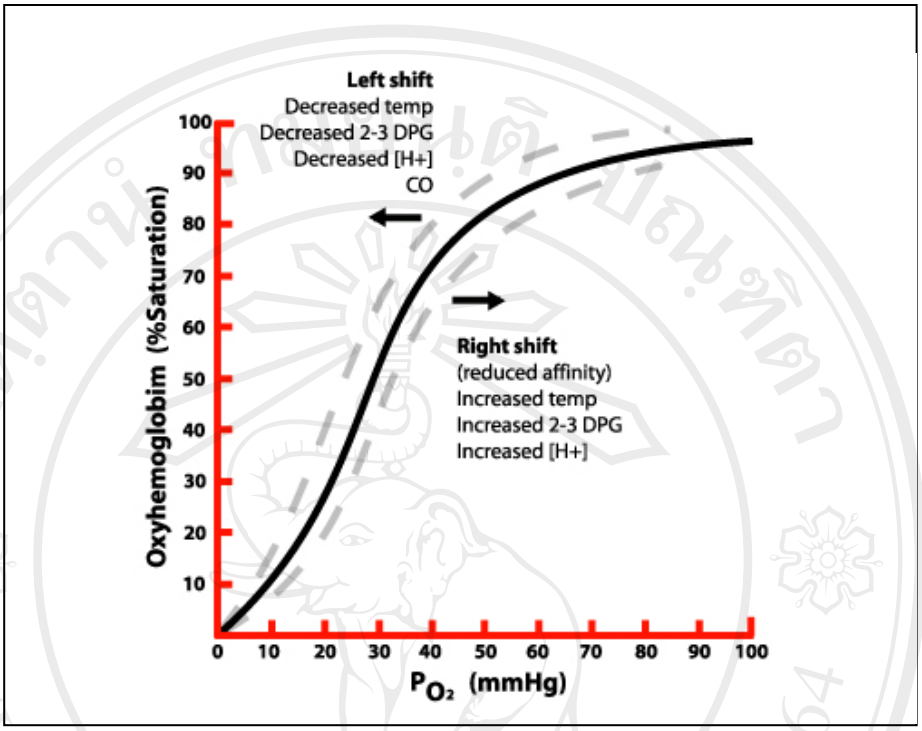


Figure 1.3 Hemoglobin oxygen dissociation curve. The change in pH, temperature and red cell 2,3-DPG level are effect on oxygen affinity of Hb.

(http://www.anaesthesiauk.com/images/ODC_3.jpg)

1.2.2 Hemoglobin synthesis

Sixty-five percent of hemoglobin is synthesized in the erythroblast and 35% at the reticulocyte stage. Heme is a ring structure synthesized in a complex series of steps involving enzymes in the mitochondrion and in the cytosol of the cell (Figure 1.4). The first step in heme synthesis takes place in the mitochondrion, with the condensation of succinyl CoA and glycine by ALA synthase to form 5-aminolevulic acid (ALA). This molecule is transported to the cytosol where a series of reactions produce a ring structure called coproporphyrinogen III. This molecule returns to the mitochondrion where an addition reaction produces protoporhyrin IX. The enzyme ferrochelatase inserts iron into the ring structure of protoporphyrin IX to produce heme. Each molecule of heme is combined with a globin chain. A tetramer of four globin chains with its own heme group in a 'pocket' is then formed to make up a hemoglobin molecule (Figure 1.5). The normal Hb molecule possesses a cyclic affinity for oxygen bound to iron. When the first iron atom binds to oxygen, three remaining atoms of iron exhibit an increased affinity as more oxygen is bound. This phenomenon is known as the heme-heme interaction (Simmons and Fimls, 1989).

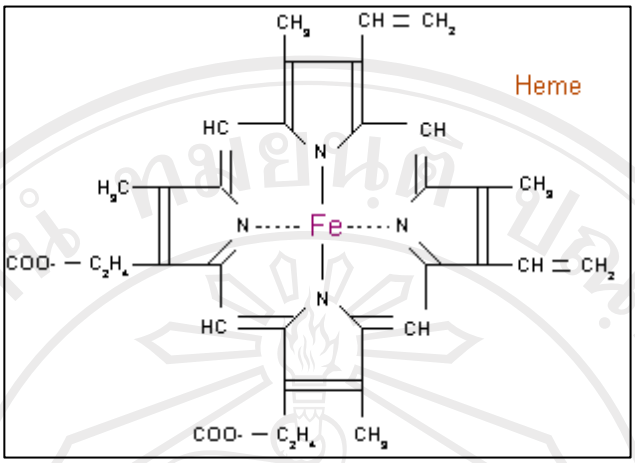


Figure 1.4 Heme molecule. Porphorin ring with iron atom ligand bound inside. *(http://altmed.creighton.edu/wheatgrass/structure.htm)*

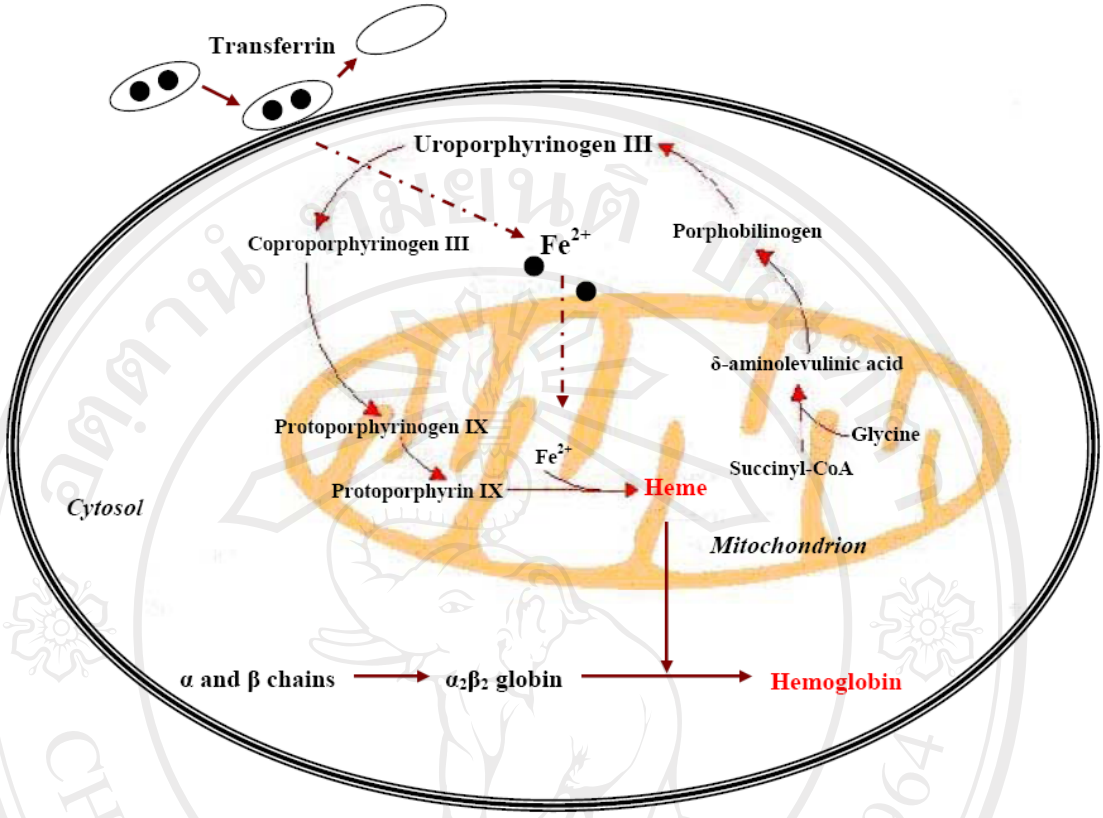
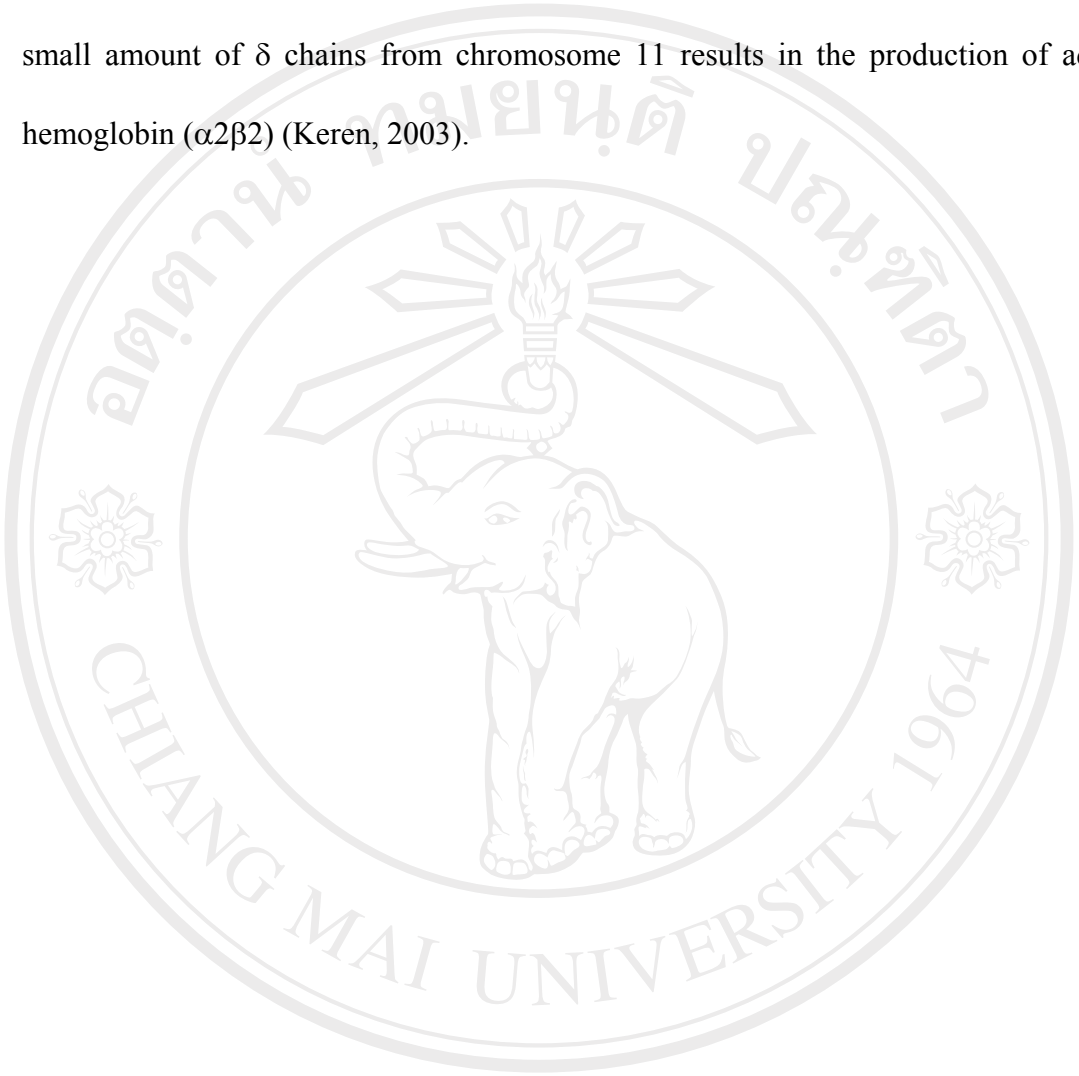


Figure 1.5 Hemoglobin synthesis in the developing red cell. The mitochondria is the main site of heme synthesis, iron is supplied from circulating transferrin and globin chains are synthesized in the cytosol. Four globin chains contained heme group are combine with each other to form the hemoglobin tetramer in cytosol of the red cell. *(http://edoc.hu-berlin.de/dissertationen/xie-jing-2003-12-15/HTML/ xie_html_ 3a64873a.gif)*

1.2.3 Developmental change in hemoglobin

Human hemoglobin is heterogeneous at all stages of development, beginning with the youngest embryos that have been studied their continuing throughout adult life (Figure 1.6). In embryos, hemoglobin synthesis is confined in the yolk sac, where Hbs Gower1 ($\zeta_2\varepsilon_2$), Gower2 ($\alpha_2\varepsilon_2$), and Portland ($\zeta_2\gamma_2$) are produced. Synthesis of β chain becomes detectable at about 6 weeks, when it comprises ~ 1.5% of the non-α chains, increasing to 5% at 7 weeks. At around 7-8 weeks of gestation, the liver becomes the major site of erythropoiesis, producing large nucleated cells. Throughout most of fetal life, Hb F production is predominates with a small amount (< 10%) of Hb A. The different γ chains are produced in a ratio of $^{G}\gamma$ and $^{A}\gamma$ of 3:1, which remains constant until late of gestation. At mid-term, the bone marrow being to take over as the major site of red cell production, through erythropoiesis are found in the spleen, as well as in other tissue. At birth, the cord blood normally contains ~ 70% of Hb F and this will decline to ~ 20% and less than 2% by the age of 1 year. At the same time, there is a differential decline in $^{G}\gamma$ and $^{A}\gamma$ chain production. Both fetal and adult hemoglobin are produced in the same cell until the switching period, a gradual increase in the proportion of cells containing predominantly of Hb A. The proportion of Hb F continues declining throughout childhood and probably adult life. At this time the small amount of Hb F are detectable only 3-5% of red cells, known as F cells (Weatherall and Clegg, 2001). For α chain synthesis, ζ and ε chains are synthesized resulting in the embryonic hemoglobins Gower1, Gower 2, and Portland during the embryonic life. After the first two months of gestation, the production of ζ chains are turn off and start to produce of α chains. Similarly, production of γ chains

are synthesized instead of ε chains begins resulting in fetal hemoglobin (Hb F). After birth, the production of γ chains is gradual decrease and begin synthesis of β and small amount of δ chains from chromosome 11 results in the production of adult hemoglobin ($\alpha 2\beta 2$) (Keren, 2003).



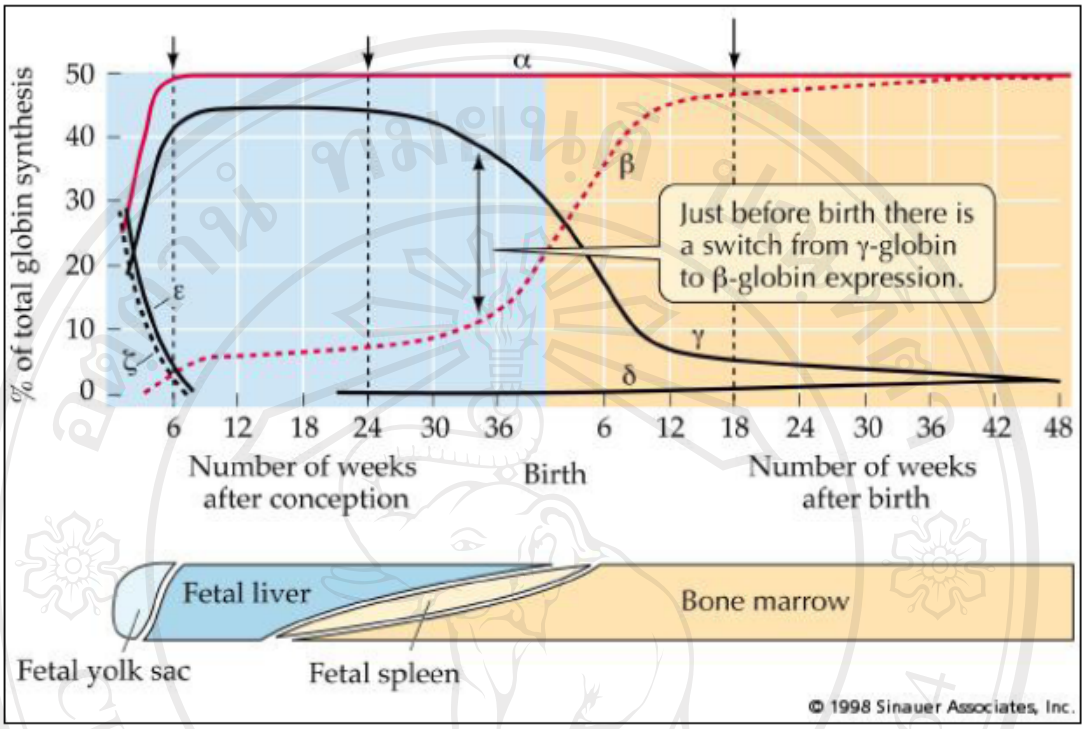


Figure 1.6 Developemental change in globin chains and production organs from fetus to adult.

(http://www.mie .utoronto.ca/labs/lcdlab/biopic/fig/14.10.jpg accessed 20 August 2006)

1.2.4 Thalassemia

1.2.4.1 Introduction of thalassemia syndromes

Thalassemia is a genetic decrease in globin chain synthesis. Theoretically, there are as many types of thalassemias as there are types of globin chains (Laosombat *et al.*, 2001). They are the most common genetic defect among man. The resulting of hematological disorders ranged from asymptomatic to severe anemia, which can cause the significant morbidity and mortality of humankind. All type of thalassemias are considered quantitative hemoglobin diseases (Hartwell et al., 2005). Although each can be further classified into different subgroups, all these disorders have one thing in common, which is always imbalanced of globin chain synthesis. Thalassemia can be categorized into three classes according to severity of the symptoms: major, intermediate and minor thalassemia. The two main syndromes (thalassemia major) are α- and β-thalassemia which involve homozygous genetic defects in the α-globin and β-globin chain productions, respectively. β-thalassemia and sickle cell anemia have wide distribution in tropical areas due to natural selection by malaria (Weatherall and Clegg, 2001). α-thalassemia is the most common found in Southeast Asia and Africa. Related thalassemia minors or carriers are α-thalassemia 1 (2 out of 4 globin gene deletion) and α-thalassemia 2 (1 out of 4 globin gene deletion) (Asawamahasakda, 1994). This is the hallmark of thalassemia, and it is the deleterious consequence of the globin that is produced in excess that are responsible for the ineffective erythropoiesis and shorten red cell survival that characterizes all the severe forms of the disease.

The incidence of thalassemia syndrome is usually found accountably in malarial epidemic areas (Figure 1.7). To date, the increase in population migration all

over the world results in expanding of the thalassemia heterozygotes out side of the epidemic area. Under improper prevention, in the future the thalassemia patients will be increased from these thalassemia heterozygotes (Hofstaetter *et al.*, 1993). Thus, the thalassemia syndrome is increasingly becoming the health problem of the world and great concerns are required.

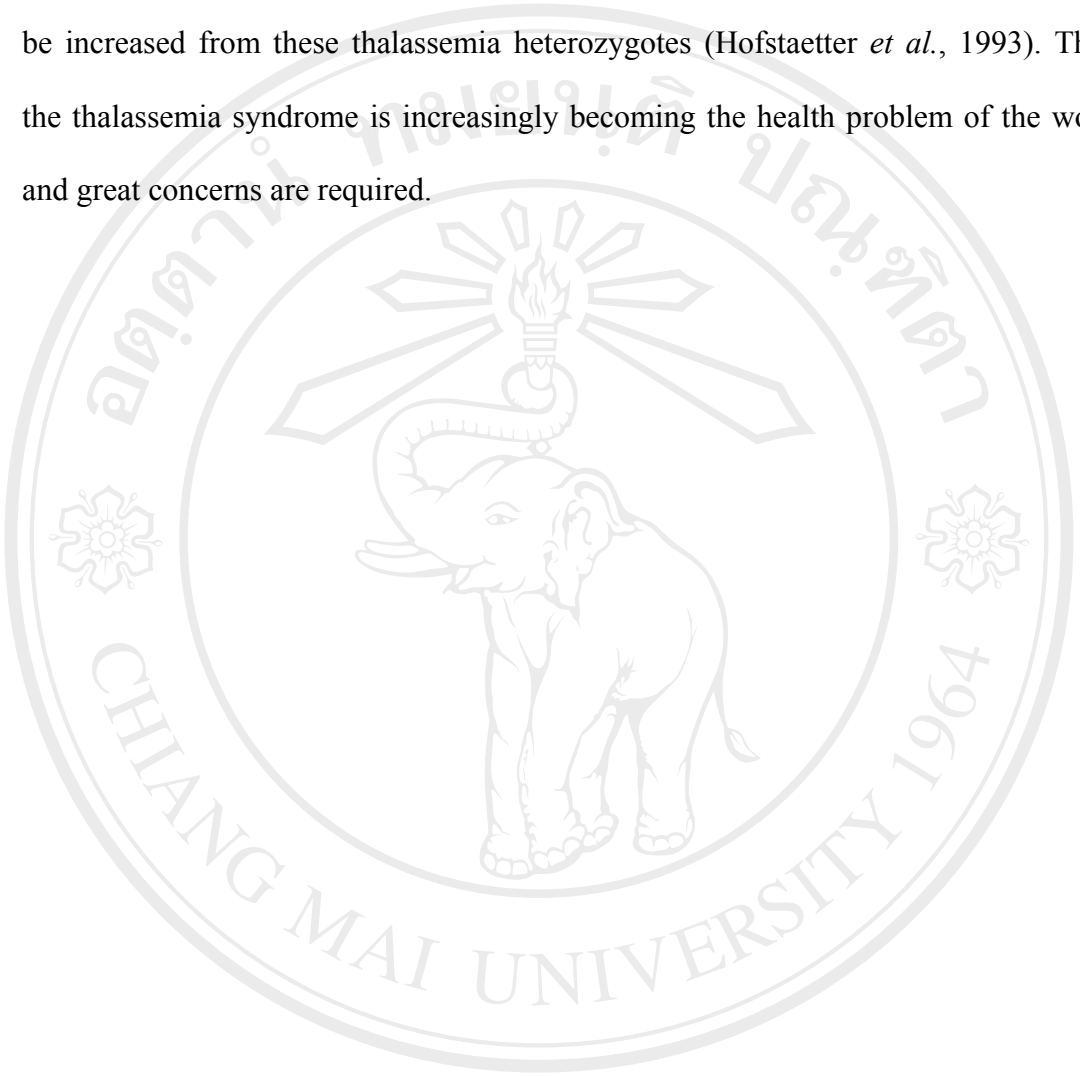


Figure 1.7 Incidence of thalassemia syndrome worldwide. The global distribution of thalassemia syndrome is represented in the orange-color areas.

(http://www.abanet.it/fondazioneberloni/images/mondo.gif)

1.2.4.2 β-thalassemia

The cause of the β-thalassemia is defective β-globin chain synthesis, which leads to imbalanced globin chain production and excess of α-globin chains (Nopparatana, 1998). Nearly 200 different mutations have been described in patients with β-thalassemia and related disorders (Higgs *et al*, 2001). The principal characteristic of homozygous β-thalassemia is a quantitative deficiency of β-globin chains in the erythroid cells of affected individuals. The heterozygous state of β-thalassemia is essentially asymptomatic, although such individuals can be identified by typical abnormalities of red cell morphology and indices. However, in homozygous β- thalassemia, also known as Cooley's anemia, there is greater imbalance of α to β-globin chain synthesis than in the heterozygote. The excess α-globin chains tend to precipitate, damaging the cell membrane of the β-thalassemic red cell. As a result, erythropoiesis is ineffective, with cell membrane damage, phagocytosis and destruction of nucleated erythrocyte precursors in the bone marrow, and with premature removal of surviving red cells from the circulation by the spleen. Thus, individuals with homozygous β-thalassemia manifest a chronic severe microcytic hemolytic anemia and hepatosplenomegaly. Untreated patients may develop a characteristic face due to bony distortion from expansion of the marrow in facial bones. β-thalassemia is divided into three types that also range from mild to severe in their effect on the body.

Thalassemia minor or thalassemia trait. In this condition, the lack of β-globin is not great enough to cause problems in the normal functioning of the hemoglobin. A person with this condition simply carries the genetic trait for thalassemia and will

usually experience no health problems other than a possible mild anemia. As in mild alpha thalassemia, physicians often mistake the small red blood cells of the person with beta thalassemia minor as a sign of iron-deficiency anemia and incorrectly prescribe iron supplements. The patient will have an increased fraction of Hemoglobin A2 (>2.5%) and a decreased fraction of Hemoglobin A (<97.5%).

Thalassemia intermedia, in this condition the lack of β-globin in the hemoglobin is great enough to cause a moderately severe anemia and significant health problems, including bone deformities and enlargement of the spleen. However, there is a wide range in the clinical severity of this condition, and the borderline between thalassemia intermedia and the most severe form, thalassemia major, can be confusing. The deciding factor seems to be the amount of blood transfusions required by the patient. The more dependent the patient is on blood transfusions.

Thalassemia major or Cooley's anemia, this is the most severe form of β-thalassemia in which the complete lack of β-globin in the hemoglobin causes a life-threatening anemia that requires regular blood transfusions and extensive ongoing medical care. These extensive, lifelong blood transfusions lead to iron-overload which must be treated with chelation therapy to prevent early death from organ failure.

The classical β-thalassemias are of two general types: β^0- and β^+-thalassemia. In homozygous β^0-thalassemia, β-globin chains are completely absent, although small quantities of β-globin mRNA may be either present or absent (Old, 2003). In homozygous β^+-thalassemia, however, β-globin chains are present at 5%-30% of normal levels, and there is a proportional deficiency of β-globin mRNA in the erythroid cells of affected individuals.

β-thalassemia usually result from mutations that affect transcription, translation, or RNA stability (Olivieri,1999). Mutations in or close to the conserved promoter sequences and in the 5' untranslated region downregulate transcription, usually resulting in mild β^+-thalassemia. Transcription is also affected by deletions in the 5' region, which completely inactivate transcription and result in β^0-thalassemia. Both splicing of the messenger RNA (mRNA) precursor and ineffective cleavage of the mRNA transcript result in β-thalassemia. In some mutations, no normal message is produced, whereas other mutations only slightly reduce the amount of normally spliced mRNA. Mutations within invariant dinucleotides at intron–exon junctions, critical to the removal of intervening sequences and the splicing of exons to produce functional mRNA, result in β^0-thalassemia. Mutations in highly conserved nucleotides flanking these sequences, or in "cryptic" splice sites, which resemble a donor or acceptor splice site, result in severe as well as mild β^+-thalassemia. Substitutions or small deletions affecting the conserved AATAAA sequence in the 3' untranslated region result in ineffective cleavage of the mRNA transcript and cause mild β^+-thalassemia.

Mutations that interfere with translation involve the initiation, elongation, or termination of globin-chain production and result in β^0-thalassemia. Approximately half of all β-thalassemia mutations interfere with translation; these include frame-shift or nonsense mutations, which introduce premature termination codons and result in β^0-thalassemia. A more recently identified family of mutations, usually involving exon 3, results in the production of unstable globin chains of varying lengths that, together with a relative excess of α-globin chains, precipitate in red-cell precursors

and lead to ineffective erythropoiesis, even in the heterozygous state. This is the molecular basis for dominantly inherited (β^+) thalassemia. In addition, missense mutations, resulting in the synthesis of unstable β-globin chains, cause β-thalassemia (Higgs *et al*, 2001).

1.2.4.3 Laboratory diagnosis of thalassemia

Carrier or heterozygote screening and mutation identification are the best way of prevention program for the hemoglobin disorders including thalassemia. The strategy for carrier screening and mutation analysis is based on that fact that although heterozygotes are symptom free, they present specific hematologic characteristics that are useful for their identification (Figure 1.8). The accurate determination of the carrier phenotype is essential for the selection of the appropriate molecular tests to determine the carrier genotype and exclude from the other acquire anemia disorders such as iron deficiency anemia (Old, 2003). The basic hematological tests required are the measurement of the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH) value and the quantification of HbF by alkaline denaturation test (Betke, 1953) or High Performance Liquid Chromatography (HPLC) and HbA_2 by ion-exchange chromatography (Moors et al., 1979). In addition, the hemoglobin pattern needs to be examined. The traditionally electrophoresis methods and isoelectric focusing electrophoresis (IFE) have been used for this purpose. HPLC is also used to detect most of the common, clinically relevant hemoglobin variants, such as HbS, HbC, HbD-Punjab, HbO-Arab, HbE and abnormal hemoglobin such as Hb Bart's, HbH at the same time (Old, 2003; Sanguansermsri et al., 2001). Detecting the mutant gene using polymerase chain reaction (PCR) can be identified the majority of the common thalassemia mutations and abnormal hemoglobins.

The variable severity of the different β-thalassemia alleles is reflected in their phenotypic effect in heterozygotes, in the degree of hypochromia and microcytosis as indicated by the mean cell hemoglobin (MCH) and mean cell volume (MCV) values, respectively. The β^0-thalassemia alleles demonstrated lower MCVs that were also within a tighter range (mean 63.1 fL, SD = 3.4) while the β^+ alleles were associated with higher MCVs within a wider range (mean 69.3 fL, SD = 5.6). Similarly, there was a wider range of MCH values for β^+-mutations (mean MCH 21.8 pg, SD = 2.03) compared to that for the β^0-mutations (mean MCH 19.7 pg, SD = 1.26) The broader range of MCV and MCH in β^+-thalassemia when compared to β^0-thalassemia, is not surprising given the broad range in the deficit of production, from barely detectable levels at the severe end, to just a little short of normal in the 'silent' β-thalassemia alleles (Thein, 2005).

Erythrocytosis and a mild hypochromic microcytic anemia characterize this heterozygous condition. HPLC analysis shows an increase in HbA_2 and, in some cases, HbF. Homozygous β-thalassemia may lead to a marked reduction or absence of normal β chain production. Affected individuals typically present midway through the first year of life with a severe hypochromic microcytic anemia. HPLC and electrophoretic findings include absence or marked reduction in HbA with a variable Hb F concentration. Interestingly and unlike the pattern in the heterozygotes, HbA_2 is not increased in homozygous β-thalassemia.

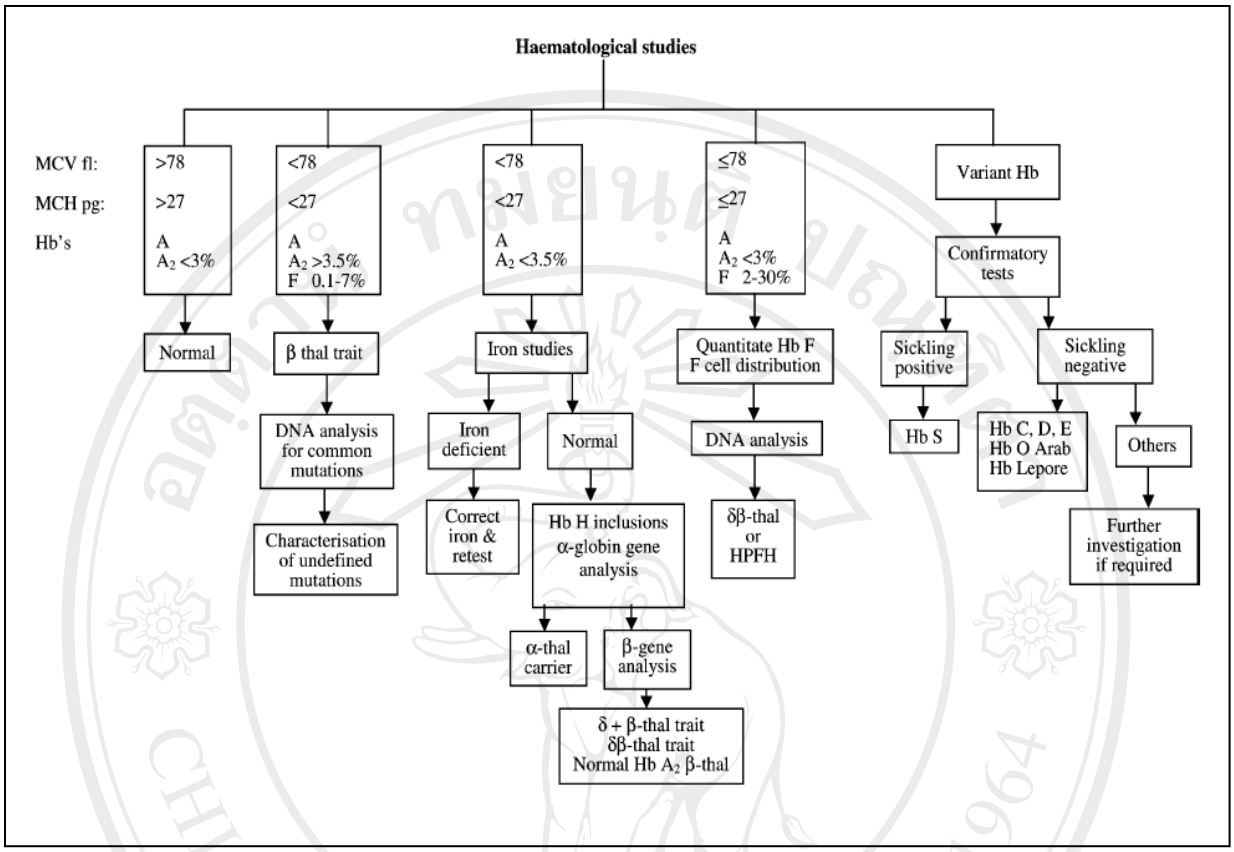


Figure 1.8 Simplified flow chart for screening of thalassemia carriers. (Old, 2003)

1.2.4.4 Screening techniques

Screening techniques are the group of techniques that can initially indicate a defect in hemoglobin synthesis. Positive results from these tests need confirmation by a more extensive analysis technique. Negative results normally help in cutting down the number of subjects that need to be further diagnosed by more advanced and complicated testing. Initial screening techniques are defined as techniques that are simple and relatively low cost, which can indicate the possibility of having thalassemia. These techniques should involve the least sample pretreatment and is rapid sample preparation, and may not need special instrumentation. This would lead to low cost and high sample throughput analysis. They provide a "yes/no" type answer. Positive samples need further confirmatory test while negative samples can be eliminated from further complicated and expensive testing. The red blood cell indicies generated by automated complete blood count (CBC) or the alternative one-tube osmotic fragility test (OF test) can be used to screen for α-thalassemia. The negative result eliminates the possibility of having thalassemia. These screening techniques, however, cannot provide the information on the exact type of thalassemia of the positive persons. (Hartwell *et al.*, 2005).

Complete blood count (CBC)

Red cell indices are critical to the diagnosis of thalassemias. The key components of the CBC include: Hb, red blood cell (RBC) number, mean corpuscular volume (MCV), and red cell distribution width (RDW). Among these parameters, MCV and MCH are the most important. Individual with hypochromic microcytic red blood cell indices that is with an MCH below 27 pg or an MCV below 80 fL should be investigated further (Old, 2003).

The RDW is a measure of the degree of variation in red cell size. Some causes of microcytic anemia, most notably iron deficiency, are characterized by an increase in RDW. The thalassemias**,** in contrast, tend to produce a uniform microcytic red cell population without a concomitant increase in RDW. This observation is variable among the thalassemia syndromes, however, with notable increases in RDW in δβ-thalassemia minor (Hedlund, 1980). Therefore, the RDW may provide information useful as an adjunct to diagnosis but is not useful as a lone indicator.

The RBC count is also useful as a diagnostic adjunct because the thalassemias produce a microcytic anemia with an associated decrease in the RBC number. Other causes of microcytic anemia, including iron deficiency and anemia of chronic disease, are more typically associated with a decrease in the RBC number that is proportional to the degree of decrease in Hb concentration.

The Hb concentration typically is decreased in thalassemia. The thalassemia minor conditions produce minimal decrements in the Hb concentration, whereas thalassemia intermedia and thalassemia major may be associated with moderate to severe decreases in Hb concentration.

One-tube osmotic fragility test (OF test)

This simple test utilizes osmosis, the movement of water from lower to higher salt concentration region, to test for the osmotic resistance of the red blood cells. Whole blood is thoroughly mixed with 0.36% buffered saline solution. In a hypotonic condition, the concentration of salt on the outside of a cell is lower than that on the inside, resulting in net water movement into cells. Normal red blood cells are lysed within 1-2 minutes and the mixture then turns clear and reddish. Abnormal red blood cells have deviated osmotic resistances as compared to normal red cells.

Red blood cells of thalassemia have higher osmotic resistance and thus have slower rupture rate, therefore the mixture remains turbid even after 1-2 hr. This technique can be carried out in one test tube and it is therefore called one tube method. Different laboratories may be using slightly different recipes for preparation of hypotonic salt solution, but all are normally based on the same concept of kinetic osmotic fragility (Hartwell *et al*., 2005). Although the OF test is a quick preliminary and very economic test before performing further studies of the red blood cells and carrying out thalassemia in large populations. However, the specificity of OF test is not so high (74.9%) and its false positive rate is rather high (25%). This means increase the workload of the later conformational PCR analysis (Sirichotiyakul *et al.*, 2004). Recently, the use of a 0.34% modified OF test was suggested for reducing the workload of PCR by about 70% (Panyasai *et al.*, 2002).

Quantification of HbF

More precise methods for quantification of hemoglobin F was also routinely performed by alkaline denatulation test. This is required because HbF is normally present in such small proportion, and mild elevations of HbA_2 above 3.5% confirm the diagnosis of beta-thalassemia trait. HbF is increased in some patients with beta-thalassemia, as well as other thalassemic syndromes, sickle cell anaemia, and some acquired conditions.

Electrophoresis

Traditionally, electrophoresis has been the method of choice for identification and quantification of variant Hbs. Electrophoretic methods have been developed that allow for separation at pH 8.4 (alkaline) and pH 6.2 (acid) on agarose gels. provide a clear background, allowing for quantification of the Hb present by densitometric

scanning. Visualization of the Hb bands is made possible by staining with Amino Black and Acid Violet (or similar stains). At alkaline pH, electrophoretic migration of HbE and HbA_2 is similar (Clarke and Higgins, 2000).

High Performance Liquid Chromatography (HPLC)

In high performance liquid chromatography, particles size of the stationary phase packed in the column is quiet small. High pressure is required to force the mobile phase to continuously flow through the column. As the samples solution flows with the liquid mobile phase through the stationary phase, the components of the sample will migrate according to the non-covalent interactions of the compounds with the stationary phase. The degree of interactions determines the degree of migration and separation of the components (i.e., the component with a stronger interaction with the mobile phase than with the stationary phase will have a shorter retention time and thus will be eluted from the column first and vice versa) (Hartwell *et al.*, 2005). HPLC has become a very important tool for thalassemia and Hb variants diagnosis because of its ability to accurately and rapidly qualitative and quantitative different types of Hbs. However, in most laboratories, HPLC has been used for diagnosis of Hb variants rather than for quantification of normal Hb or thalassemia, except for the case of prenatal analysis. HPLC is very suitable for prenatal screening diagnosis and has been used to diagnose thalassemia and hemoglobinopathies (Fucharoen *et al.*, 1998).

DNA analysis

After presumptive identification of thalassemia syndromes, and particularly for purposes of genetic counseling, defining the mutation or deletion present may be required. Several molecular techniques are available. DNA from white blood cells,

amniocytes, or chorionic tissue may be utilized for diagnosis of various β-globin chain abnormalities. Typically, deletional mutations causing β-thalassemia is diagnosed using Southern blot hybridization of particular restriction enzyme digests to labeled complementary gene probes.

PCR techniques using allele-specific probes after globin gene amplification, allele-specific primers, or deletion-dependent amplification with flanking primers are used in definition of known globin chain mutations/deletions of several β-thalassemia (Vaysal and Huisman, 1994; Lundeberg *et al*, 1999)

1.2.5 Alpha Hemoglobin Stabilizing Protein

Many genes and metabolic pathways that participate in hemoglobin synthesis are controlled by GATA-1, a DNA-binding protein that is essential for the survival and maturation of lineage-committed erythroid precursors (Fujiwara, 1996). Known GATA-1 target genes include those encoding α- and β-globins and heme biosynthetic enzymes (Weiss, 1995). Kihm *et al.* (2002) therefore reasoned that other facets of hemoglobin production and regulation must also be controlled by GATA-1. They performed subtractive hybridization to identify transcripts that were upregulated after genetic rescue of the GATA-1 null erythroid cell line G1EER2. The screen verified known GATA-1 targets and identified numerous other genes of unknown function. Among the latter group, erythroid differentiation-related factor (EDRF) (GenBank accession number AF060220) messenger RNA and protein were strongly and rapidly induced by GATA-1. The EDRF was originally identified as a protein that showed a decrease in expression in the hematopoietic tissues of rodents and cattle infected with transmissible spongiform encephalopathies (Miele *et al,* 2001) and proposed as a specific marker for TSE (Aguzzi, 2001). Currently, EDRF was renamed as AHSP after it was found that it binds to two specific helices on α Hb at overlapping sites in the same region that interacts with β Hb. The α Hb-AHSP complex converts α Hb to a unique, stable structure, dramatically altered in its 3D state, with its Fe ion configuration changing from Fe^{2+} to a more stable Fe^{3+} form (Gell *et al,* 2002; Feng *et al,* 2004; Creuza *et al,* 2004; Hamdane *et al,* 2007). In the presence of sufficient concentrations of β Hb, the greater affinity of α Hb for β Hb compared with its affinity for AHSP facilitates the optimal "hand-off " of α Hb from AHSP to β Hb and the subsequent formation of normal Hbαβ dimers and HbA tetramers. As shown in

Figure 1.9, AHSP does not react with apo-β-globin, β Hb, or HbA tetramers (Kihm *et al,* 2002). The AHSP encodes a 102-amino-acid protein that is highly conserved in human, pig, cow and rat and contains no recognizable signature motifs.

The structure of alpha-Hemoglobin Stabilizing Protein (AHSP), a molecular chaperone for free alpha-hemoglobin, has been determined using NMR spectroscopy (Figure 1.10). The protein native state shows conformational heterogeneity attributable to the isomerization of the peptide bond preceding a conserved proline residue. The two equally populated cis and trans forms both adopt an elongated antiparallel three alpha-helix bundle fold but display major differences in the loop between the first two helices and at the C-terminus of helix 3. Proline to alanine single point mutation of the residue Pro 30 prevents the cis/trans isomerization. The structure of the P30A mutant is similar to the structure of the trans form of AHSP in the loop 1 region. Both the wild-type AHSP and the P30A mutant bind to alpha-hemoglobin and the wild-type conformational heterogeneity is quenched upon complex formation suggesting that just one conformation is the active form. Changes in chemical shift observed upon complex formation identifies a binding interface comprising the C-terminus of helix 1, the loop 1 and the N-terminus of helix 2, with the exposed residues Phe 47 and Tyr 51 being attractive targets for molecular recognition. The characteristics of this interface suggest that AHSP binds at the intradimer alpha(1)beta(1) interface in tetrameric HbA (Santiveri *et al*, 2004).

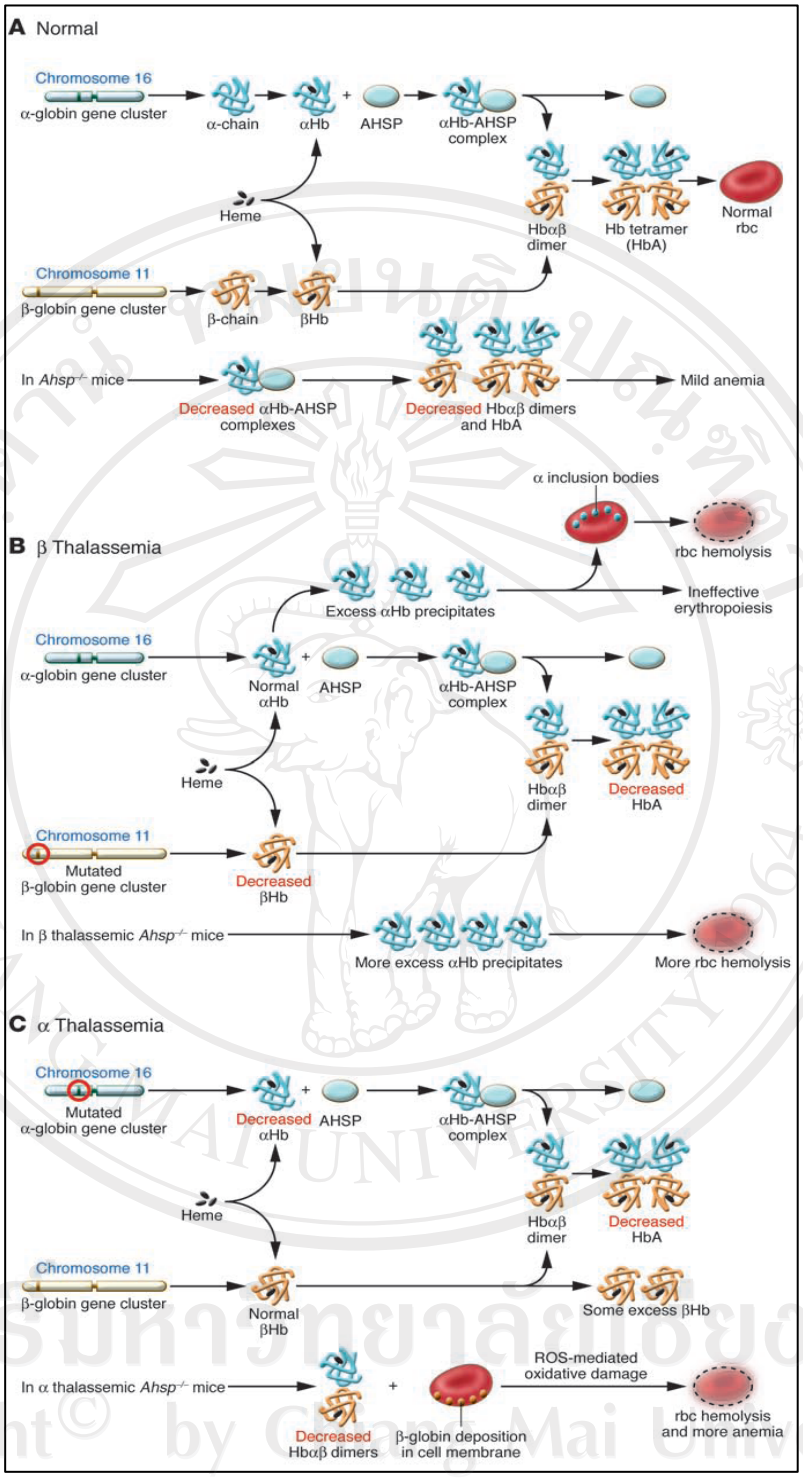


Figure 1.9 Hemoglobin formations with and without AHSP. (A) In normal cells, (B) In β-thalassemia, (C) In α-Thalassemia. (Bank, 2007)

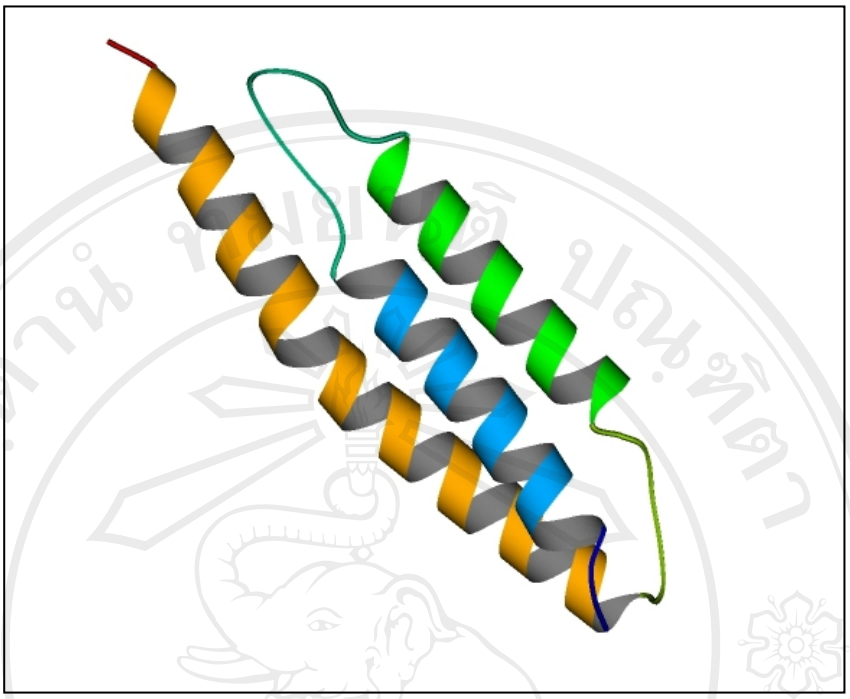


Figure 1.10 The structure of alpha-Hemoglobin Stabilizing Protein (AHSP) has been determined using NMR spectroscopy. (Clara, 2004)

AHSP mRNA is expressed exclusively in hematopoietic tissues including bone marrow, spleen and fetal liver. RNA *in sit*u hybridization analysis of early and mid-gestation murine embryos demonstrated strong expression in embryonic yolk-sac blood islands and fetal liver, respectively (Gell *et al*, 2002; Kihm *et al*, 2002; Feng *et al*, 2004). No mutations or specific associations were found between extended AHSP haplotypes and disease severity in these patients (Viprakasit *et al*, 2004). Although AHSP is highly expressed in erythroid precursors, studies have shown that its expression is most abundant during late-stage erythroid maturation (Kong *et al*, 2004; dos Santos *et al*, 2004). Santos *et al*. (2005) reported a non-synonymous single nucleotide polymorphism (SNP) in exon 3 that may impact on the severity of β-thalassemia. In addition, 120 Thai patients with severities variable of Hb E/β thalassemia were genotyped for the common sequence variants in AHSP. Galanello *et al,* (2003) reported that reduced expression of AHSP was associated with a more severe phenotype among individuals with identical β-thalassemia and α-globin genotypes although no mutations or polymorphisms in the gene could be implicated. Furthermore, AHSP knock-out mice showed ineffective erythropoiesis and pathological features similar to those of β-thalassemia and loss of AHSP exacerbates the hematological abnormalities in β-thalassemic mice (Kong *et al*, 2004). The evidence suggests that AHSP acts as a molecular chaperone for free α-Hb, and that altered levels or function of AHSP might account for some of the clinical variability observed in patients with β-thalassemia (Luzzatto & Notaro, 2002). Several studies have attempted to assess the relationship between AHSP level and the severity of β-thalassemia.

1.2.5 Principle of the production of BCCP fusion protein

The use of fusion tags in recombinant protein purification has a long tradition. In addition to facilitate purification strategies, fusion tags have been observed to improve protein yield, to prevent proteolysis and to increase solubility in *vivo* (Soren and Mortensen, 2005). Currently, bacterial expression systems are commonly used to produce recombinant proteins. The production of recombinant proteins involves in the cloning of appropriate genes into a prokaryotic expression vector (Koths, 1995; Hannig and Makrides, 1998; Duilio *et al*., 2004). A well-designed prokaryotic expression vector contains a set of optimally configured genetic elements that affect both transcriptional and translational aspects of protein production. The promoter is typically positioned approximately 10 to 100 bp upstream of the ribosome-binding site (RBS) and is under the control of a regulatory (repressor) gene, which may be presented on the vector itself or integrated in the host chromosome. The RBS consists of the Shine-Dalgarno (SD) sequence followed by an AT rich translational space that has an optimal length of approximately 8 bases. The SD sequence interacts with the 3' end of the 16S rRNA during translation initiation. The transcription terminator serves to stabilize the mRNA and the vector. In addition, the inclusion of an antibiotic-resistance gene facilitates phenotypic selection of the vector and the origin of replication (Ori) determines the vector copy number. Multi copy plasmids have been extensively used as vectors for recombinant protein expression. Expression vectors commonly used for overexpression of foreign genes in *E. coli* can be categorized according to the type of promoter used. The systems commonly used are driven by IPTG-inducible, bacteriophage T7, or bacteriophage lambda pL promoters.

Gene fusion techniques permit the assembly of recombinant protein with a protein fusion partner that has been designed for purification or detection purpose (LaVallie and McCoy, 1995). Fusion proteins was widely used include polyhistidine (His6), glutathione-S-transferase (GST), maltose-binding protein (MBP) and biotin carboxyl carrier protein (BCCP).

Biotin carboxyl carrier protein (BCCP) is one of the three functional subunits in the acetyl-CoA carboxylase (ACC) of *E.coli*. This enzyme catalyses the biotin dependent carboxylation of acetyl-CoA to malonyl-CoA, the first committed step in the biosynthesis of fatty acids. Post-translational addition of biotin to a single lysine residue of apoBCCP is catalyzed by the biotin holoenzyme synthetase (BHS). The biotin ligation reaction is highly specific and in *E.coli* only the BCCP subunit of acetyl-CoA carboxylase is biotinylated (Fall, 1972). In its biotinylated form, holoBCCP carries the activated CO_2 from the biotin carboxylase region to the active site of the transcarboxylase in the reaction in which the CO_2 moiety is transferred to acetyl-CoA to produce malonyl-CoA. Studies of the efficiency with which truncated forms of biotin carrier proteins expressed as protein fusions are biotinylated have shown that a minimum of 75–80 residues, ~ 35–40 residues on either side of the biotin attachment site, is necessary to specify biotinylation. The biotinylation process which involves in the formation of an amide bond between the carboxyl group of biotin and the ε-amino group of the lysine residue is catalyzed by the enzyme biotin ligase (BirA) in an ATP-dependent reaction. The lysine residue, which attaches to the biotin, is located at the C-terminal region of the BCCP protein in an independent domain of about 80 amino acids. This domain can be fused to recombinant proteins and it can be biotinylated *in vivo* by the endogenous biotin ligase of *E. coli*. The

BCCP domain fusion approach has previously been used for site-specific biotin labeling of various recombinant proteins (Sibler *et al*., 1999; Santala and Lamminmaki, 2004).

1.2.6 Monoclonal antibody production

Monoclonal antibodies (mAb) are antibodies produced by a single clone of hybridoma cells. These cells are derived from the fusion of B-cell and myeloma cell. (Kohler and Milstein, 1975). B cells, which will continually produce antibodies specific for a defined antigen, an immune response of mouse was raised by immunizing the protein of interest. Myeloma cell line is a tumor of the bone marrow that can be adapted to grow permanently in cell culture. All mouse myeloma cells commonly used for hybridoma production are of BALB/c origin, and it is generally easiest to use BALB/c mice as the spleen donor (Figure 1.11). Therefore, these cells can not use the salvage pathway for nucleotide synthesis. Normal animal cells synthesize DNA precursors, purine nucleotides and thymidylate, by *de novo* synthesis pathway which requires tetrahydrofolate. Hence, using of antifolate drugs, such as aminopterin, can block activation of tetrahydrofolate, thereby inhibits the synthesis of purine and rules out DNA synthesis by *de novo* pathway (Yelton and Scharff, 1980). Myeloma cell lines that used as fusion partner are defected in hypoxanthine guanine phosphoribosyltransferase, HGPRT, an enzyme of a salvage pathway. When the main biosynthetic pathway for guanosine, *de novo* pathway, is blocked by the folic acid antagonist aminopterin, there is an alternative salvage pathway in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate via the enzyme hypoxanthine guanine phosphoribosyl transferase Cells lacking HGPRT die in medium containing hypoxanthine, aminopterin and

thymidine (HAT medium), because both the main and the salvage pathways are blocked. However, an $HGPRT^-$ cell can be made to grow in HAT medium if it is provided the missing enzyme by fusion with an $HGPRT^+$ cells. Splenocytes of the immunized mouse are fused with myeloma cells. After cell fusion, un-fused B cells, un-fused myeloma cells and hybrid cells are randomly generated. Un-fused myeloma cells and myeloma-myeloma hydrids can be selected in a medium containing HAT medium. As myeloma fusion partners and myeloma-myeloma hydrids are deficient in an enzyme required for the salvage pathway of nucleotide synthesis, these cells are died in HAT medium because aminopterin blocks normal nucleotide synthesis and the enzyme deficiency blocks utilization of hypoxanthine or thymidine in the salvage pathway. By the HAT medium, thus non-fused myeloma cells and myeloma-myeloma hydrids are died and only those cells fused to normal cells survive (Figure 1.12). In the case of fusion of myeloma and normal cells, the outcome hybridomas can survive indefinitely in culture medium because the normal cells supply the missing enzyme for selection in HAT medium and the myeloma cells immortalize the hybrid cells. Un-fused normal lymphocytes can survive in culture medium for approximately 1 week then they die. Therefore, after long-term culture, only hydridomas of normal and myeloma cells grow in the selective medium. Fortunately, hybrid cells generated from B lymphocyte and myeloma cell fusion can grow continuously *in vitro* and secrete a single monoclonal antibody. By limiting techniques, the interest clones could be selected and they can be grown in tissue culture dish or *in vitro* or producing ascitic fluid in mouse or *in vivo*. These mAbs have been steady expansion into numerous ELISA developments. Its specificity and

reproducibility can solve the problems from traditional antisera (Yelton and Scharff, 1980; Galfre and Milstein, 1981).

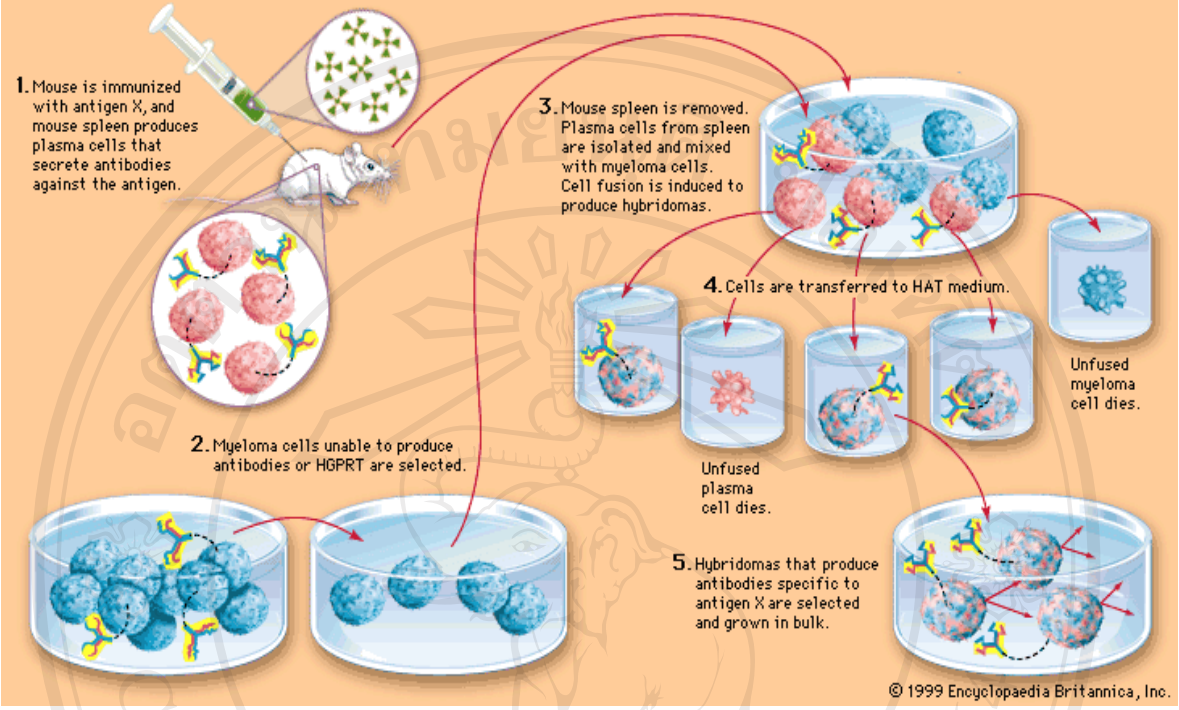


Figure 1.11 Monoclonal antibodies production. Spleen cells from immuned mice are fused with HGPRT$^-$ myeloma (plamacytoma) cells using polyethylene glycol. The binucleate fusion products are known as heterokaryons. At the next division, the nuclei fuse, generating hybrid cells, which grow in HAT medium. Unfused myeloma cells die in HAT medium, and unfused spleen cells can only survived a few day in culture. Hybrids are test for production of antibody of the desired specificity, and cloned by limiting dilution.

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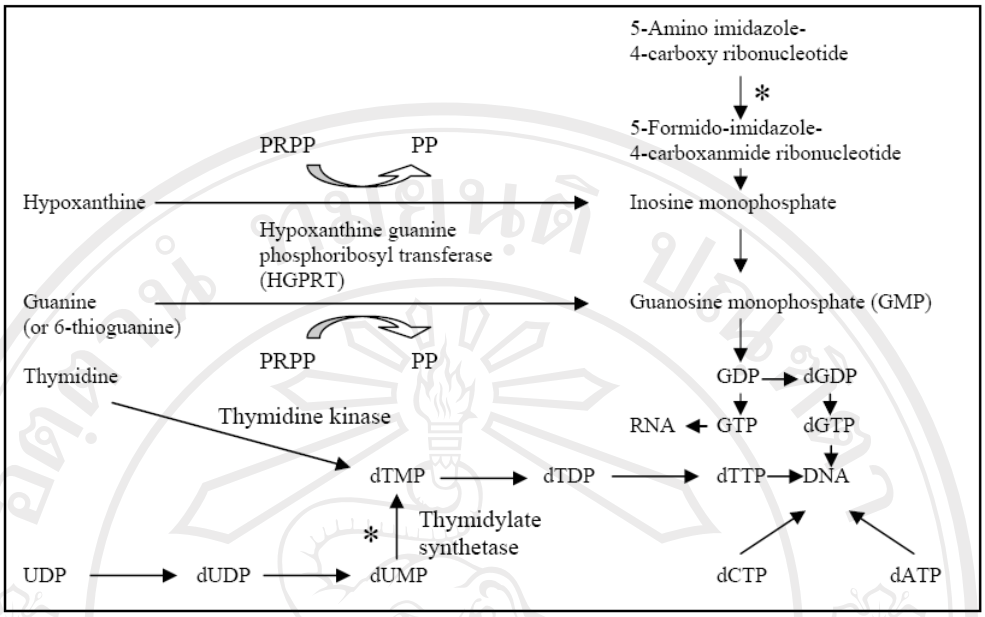

Figure 1.12 Metabolic pathways of DNA synthesis. When the *de novo* pathway are blocked with folic acid analogue (*), such as aminopterin, cell must depend on the salvage pathway (Goding, 1986).

1.3 Objectives

1. To produce monoclonal antibodies to Alpha Hemoglobin Stabilizing Protein (AHSP)
2. To characterize the produced monoclonal antibodies to Alpha Hemoglobin Stabilizing Protein (AHSP)