To the Graduate Council:

I am submitting herewith a dissertation written by Cynthia Jo Wawrzyniak entitled "Regulation of the Expression of Murine Adult β-globin Genes." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Raymond A. Popp, Major Professor

We have read this dissertation
and recommend its acceptance:

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Lisa B. Russell

Julian Preston

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

REGULATION OF THE EXPRESSION OF MURINE ADULT β-GLOBIN GENES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Cynthia Jo Wawrzyniak

August 1986

DEDICATION

TO MOM AND DAD

for loving each other enough to have me and
see me through this accomplishment

and

TO MY BROTHERS

Mike, Tom and Billy

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ABSTRACT

Hemoglobin switching is ideal for studying the basic regulation of genes during development. Here, a mouse model was used to demonstrate independent expression of the hemoglobin genes $\beta 1^{major}$ and $\beta 2^{minor}$ during erythroid differentiation and in response to anemia. Mice homozygous for the $\underline{Hbb}^{s2}$ haplotype code for two adult β-globins, β-s2major and β-sminor, which can be distinguished from other embryonic and adult hemoglobins by cellulose acetate electrophoresis and ion exchange chromatography. At 11.5 days of gestation, β-s2major hemoglobin comprises under 20 percent and β-sminor hemoglobin over 80 percent of the adult β-globin present in circulating erythrocytes. The relative level of β-sminor hemoglobin decreases through fetal development; at birth β-sminor represents 34 percent of the β-globin in circulation. The adult value of 70 percent β-s2major and 30 percent β-sminor hemoglobin in circulation is already expressed in mice at 6 days after birth.

To investigate whether the rapid shift in the ratio of β-sminor to β-s2major hemoglobin observed during gestation was due to a change in erythrocyte populations, the larger primitive and the smaller definitive red cells were separated according to size via centrifugal elutriation. Analysis of fetal samples at daily intervals from 12.5 through 17.5 days of gestation revealed that the larger red cells, which are yolk sac-derived, have relatively greater amounts of

β-sminor hemoglobin than the smaller red cells, which are derived from the fetal liver.

Dot blot hybridization was used to determine the relative amounts of the β-s2major and β-sminor globin RNAs present in circulating reticulocytes of homozygous $\underline{Hbb}^{s2}$ mice at 14.5 and 17.5 days of gestation and at birth relative to the amount of these RNAs in induced reticulocytes of adults. The level of β-sminor RNA in reticulocytes at 14.5 and 17.5 days of gestation is nearly the same as that in induced reticulocytes of adult mice. In contrast, the level of β-s2major RNA in reticulocytes at 14.5 days of gestation is 23 percent, and at 17.5 days of gestation is 66 percent, of the amount found in induced reticulocytes of adult mice. These data indicate that the differential expression of β-sminor and β-s2major hemoglobins during development is regulated at the level of transcription.

Perturbation of the relative quantities of the two adult hemoglobins and the two adult globin RNAs by hematopoietic stress was evaluated in adults. β-sminor hemoglobin constitutes 29 percent of the total β-globin of normal adults. In anemic mice, this proportion is raised to the following levels: 34 percent in β-thalassemic heterozygotes; 38 percent in α-thalassemic heterozygotes; and 41 percent in doubly heterozygous α-thalassemic, β-thalassemic mice. The altered levels of globin RNAs in thalassemic mice demonstrate that the increase in the β-sminor hemoglobin is accompanied by an increase in

β-sminor RNA. Thus, a level of murine adult β-globin gene expression can be regulated transcriptionaly, which differs in the subpopulations of erythrocytes.

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CHAPTER I

INTRODUCTION

Hematopoiesis

Most vertebrates have multiple sites of erythropoiesis. The mouse, not being an exception, has four different tissues which at some time during development have the ability to produce cells that synthesize hemoglobin. In addition to the various locations of a functional erythron, the kinds of α- and β-globin polypeptides produced also change during development. In the mouse, the first site of erythropoiesis is the yolk-sac blood islands. Between the 8th and 14th days of gestation the yolk-sac blood islands give rise to the primitive nucleated erythrocytes that synthesize mostly embryonic hemoglobins (Fantoni et al., 1967). However, a small quantity of adult hemoglobin is produced in the primitive erythrocytes between 12 and 14 days of gestation (Chui et al., 1982). The second site of erythropoiesis is the fetal liver; fetal hepatic erythrocytes are non-nucleated and begin to synthesize adult hemoglobin at 12 days of gestation. The liver continues to function as an erythropoietic tissue until near birth (Rifkind et al., 1969). The bone marrow and spleen are the major sites of erythropoiesis in juvenile and adult mice; in these tissues, erythrocyte production and hemoglobin synthesis begin at 17 days of gestation. The various locations that

produce hemoglobin during development are probably a reflection of the regulation occurring at the level of the DNA.

Hemoglobin, whether of embryonic or adult type, is a tetrameric molecule. The three embryonic hemoglobins of mice are designated EI, EII and EIII (Fantoni et al., 1967). EI (x_2y_2) is comprised of the pre-alpha, x-globin polypeptide and the pre-beta, y-globin polypeptide. Hemoglobins EII (α_2y_2) and EIII (α_2z_2) are comprised of adult alpha-globin and the pre-beta, y- and z-globin polypeptides, respectively. Adult mouse hemoglobins ($\alpha_2\beta_2$) are encoded by one or both of the two α- and two β-globin genes (Russell, 1979) that are described further under Nomenclature.

Nomenclature

Mouse adult hemoglobin types are commonly described as "single" or "diffuse" according to their electrophoretic pattern (Ranney et al., 1960; Russell, 1979). These patterns are governed by alleles at the hemoglobin β-globin locus, Hbb, that is linked with albinism, c, on chromosome 7 (Popp and Amand, 1960). These patterns are also correlated with amino acid sequences in β-globin polypeptides (Popp, 1973; Popp and Bailiff, 1973; Gilman, 1976) and nucleotide sequences in the adult β-globin genes (Konkel et al., 1978; Konkel et al., 1979). BALB/c mice of the Hbb^d haplotype synthesize the "diffuse" type of adult hemoglobin, and C57BL/6 mice of the Hbb^s haplotype synthesize the "single" type adult hemoglobin. Since the electrophoretic pattern of mouse hemoglobin is determined by the kind of β-globin

polypeptide present in the tetrameric hemoglobin molecule ($\alpha_2\beta_2$), each hemoglobin is often designated simply by its β-globin type. Thus, $\alpha_2\beta_2^{dmajor}$ and $\alpha_2\beta_2^{dminor}$ hemoglobins are commonly called the β-dmajor and β-dminor hemoglobins, respectively; and $\alpha_2\beta_2^{single}$, often abbreviated $\alpha_2\beta_2^{s}$, is commonly called the β-single hemoglobin.

Mice of the Hbb^s haplotype produce a single kind of adult β-globin (Popp, 1973) even though they possess two adult β-globin genes, which Weaver et al. (1981) originally called β^s and β^t. These genes have now been assigned the symbols $\beta1^s$ and $\beta2^s$, respectively. Lewis et al. (1985) recently characterized a point mutation induced by ethylnitrosourea (ENU) in a C57BL/6 mouse of the Hbb^s haplotype. It appears that the mutation occurred in the $\beta1^s$ gene. Therefore, the $\beta1^s$ gene will be called $\beta1^{smajor}$ and the $\beta2^s$ gene will be called $\beta2^{sminor}$. The ENU-induced mutant $\beta1^s$ gene will be called $\beta1^{s2major}$ where the 2 indicates that it is the second form of that gene to be described. The hemoglobin molecule with the mutant form of the β-globin subunit, from the $\beta1^{s2major}$ gene, will be called the β-s2major hemoglobin. Mice of the Hbb^p haplotype have the $\beta1^{pmajor}$ and $\beta2^{pminor}$ genes which code for β-pmajor and β-pminor hemoglobins, respectively.

Thalassemia is a hereditary anemia caused by deficient synthesis of either the α- or β-globin subunit of hemoglobin. Two different mouse mutants, one α- and the other β-thalassemic, and combinations of them were used here. The β-thalassemic mutant arose spontaneously due to a deletion of a 3.7 kilobase segment of DNA that

includes the entire $\beta 1^{dmajor}$ globin gene (Skow et al., 1983). This mutation will be identified by $\underline{Hbb}^{d3(th)}$. Animals carrying the β-thalassemic mutation are viable both in the heterozygous and homozygous conditions. Refer to Figure 1.1 for a summary of β-globin nomenclature.

The α-thalassemic mutant involves the α-globin complex, designated Hba, which is located on chromosome 11 of the mouse (Russell and McFarland, 1974). This locus consists of three genes, one embryonic x-globin and two adult α-globin genes (Whitney and Russell, 1980), which are arranged 5'-x-$\alpha 1^b$-$\alpha 2^b$-3' in mice of the $\underline{Hba}^b$ haplotype. The mutant first was found in X-radiation-induced α-thalassemic 352HB mice (Russell et al., 1976); the entire α-globin complex is deleted (Whitney et al., 1981). The symbol $\underline{Hba}^{b2(th)}$ will be used to identify this deletion of the normal $\underline{Hba}^b$ allele. The α-thalassemic mutants survive only when the mutation is present in the heterozygous condition; homozygous α-thalassemic mice die in utero (Popp et al., 1980; Popp et al., 1981).

In Vitro Systems

Mouse erythroleukemia (MEL) cells, which were originally virus induced (Friend et al., 1974), have been extensively used to study the mechanism by which globin genes are activated during erythropoiesis. MEL cells are arrested in a proerythrocyte stage of erythropoiesis, but they can be induced to differentiate in culture by various chemicals (Marks et al., 1978). Differentiation is

HAPLOTYPE DESIGNATION	5' DNA 3'	ADULT β-GLOBINS PRODUCED
Hbb^{d}	-----β1^{dmajor}-------β2^{dminor}-----	β-dmajor and β-dminor
Hbb^{s}	-----β1^{smajor}-------β2^{sminor}-----	β-smajor and β-sminor
Hbb^{s2}	-----β1^{s2major}------β2^{sminor}-----	β-s2major and β-sminor
Hbb$^{d3(th)}$	----- X ------β2^{dminor}-----	β-dminor only

Figure 1.1. Summary of β-globin nomenclature.

The four β-globin haplotypes used for this study are listed. The order of the adult globin genes in the DNA is diagrammed, and the adult type polypeptides produced are shown. The X represents the 3.7 kb deletion.

defined here as the ability to undergo terminal cell division and synthesize hemoglobin. Prior to induction, MEL cells express low levels of β-minor hemoglobin (Nudel et al., 1977). Different inducing agents have different effects on the relative amounts of β-minor and β-major hemoglobin produced by the differentiated MEL cell. Polar compounds, such as hexamethylene bisacetamine (HMBA) and dimethyl-sulfoxide, induce a higher quantity of β-major than β-minor hemoglobin, fatty acids (i.e., butyric acid) induce approximately equal quantities of β-major and β-minor hemoglobin, and hemin induces synthesis of primarily β-minor hemoglobin (Marks et al., 1985). Within hours of exposure to chemical agents, there is an increased accumulation of globin mRNA (Ross et al., 1972). Many studies indicate that the globin synthesis in cell lineages is regulated primarily at the level of transcription (Groudine et al., 1981; Landes et al., 1982; Villeponteau et al., 1982). The structure of the chromatin in uninduced versus HMBA-induced MEL cells has been investigated (Mark et al., 1985). The two cell types showed no obvious differences in DNA methylation. However, a new upstream DNaseI and SI nuclease hypersensitivity site was found in the DNA of induced cells, and there was some increase in the level of nucleosome disruption as measured by a chromatin fractionation procedure.

Organization of the DNA Sequence

The *Hbb*^{d} haplotype, characteristic of BALB/c mice, and *Hbb*^{s} haplotype, characteristic of C57BL mice, are common in wild mouse

populations as balanced polymorphisms (Berry, 1978). The β-globin gene complex (haplotype) spans approximately 65 kilobases of DNA and is composed of four functional genes which are active at some time during hematopoiesis, as well as of three inactive pseudogenes βh0, βh2 and βh3. The structural arrangement of these β-homologous sequences is 5'-y-βh0-βh1-βh2-βh3-$\beta 1^{dmajor}$-$\beta 2^{dminor}$-3' in BALB/c mice of the $\underline{Hbb}^{d}$ haplotype (Jahn et al., 1980; Leder et al., 1980; Farace et al., 1984) and 5'-y-βh0-βh1-βh2-βh3-$\beta 1^{smajor}$-$\beta 2^{sminor}$-3' in C57BL/6 mice of the $\underline{Hbb}^{s}$ haplotype (Weaver et al., 1981). The DNA sequences of the $\beta 1^{dmajor}$ and $\beta 2^{dminor}$ genes in BALB/c mice (Konkel et al., 1978; Konkel et al., 1979; Jahn et al., 1980) and of the $\beta 1^{smajor}$ and $\beta 2^{sminor}$ genes in C57BL mice (Erhart et al., 1985) are known. The adult globin genes, $\beta 1^{major}$ and $\beta 2^{minor}$, from all haplotypes have two introns and three exons. Comparisons of the $\underline{Hbb}^{d}$ and $\underline{Hbb}^{s}$ haplotypes suggest that they have distinct evolutionary histories (Erhart et al., 1985). The allelic genes $\beta 1^{dmajor}$ and $\beta 1^{smajor}$ are 95 percent homologous in their 5' and 3' flanking sequence. Comparison of the $\beta 2^{dminor}$ and $\beta 2^{sminor}$ genes reveals a 95 percent similarity in 5' flanking sequence and 99 percent similarity in 3' flanking sequence. Overall the $\beta 1^{dmajor}$ and $\beta 1^{smajor}$ genes are highly homologous in terms of genomic location and DNA sequence, as are the $\beta 2^{dminor}$ and $\beta 2^{sminor}$ genes; in addition, the data to be presented will demonstrate a functional homology (Whitney, 1977; Alter and Goff, 1980; Wawrzyniak et al., 1985; Wawrzyniak and Popp, 1985). The β-dmajor and β-dminor globins, which are synthesized in different

amounts (Whitney, 1977), differ at nine amino acid positions (Popp and Bailiff, 1973) which are accounted for in the DNA sequence. The $\beta 1^{smajor}$ and $\beta 2^{sminor}$ genes are 99.6 percent homologous from their transcriptional start to stop codons and code for identical functional polypeptide chains. On the other hand, of the 3' side of the termination codon, there is a large increase in variation between $\beta 1^{smajor}$ and $\beta 2^{sminor}$. This area is only 70 percent homologous and thus provides a region for which the messenger RNAs may be distinguished.

The new hemoglobin mutant ($\underline{Hbb}^{s2}$) coding for the β-s2major and β-sminor hemoglobins differ at amino acid position 60 (Lewis et al., 1985). This amino acid change of a valine at position 60 in the β-smajor globin to a glutamic acid in the β-s2major globin allows for separation of the protein products. This change was probably caused by a transversion of a thymine to an adenine nucleotide in the $\beta 1^{smajor}$ gene. With the reasonable assumption that ENU induced only one point mutation in this haplotype, a model is provided to study the multiple regulatory components of hemoglobin switching in C57BL mice.

CHAPTER II

EXPRESSION OF β-S2MAJOR AND β-SMINOR GLOBIN GENES AT THE LEVEL OF THE PROTEIN DURING DEVELOPMENT

Summary

Mice of the mutant haplotype ($\underline{Hbb}^{s2}$) produce a variant β-smajor hemoglobin (β-s2major) which can be distinguished from β-smajor and β-sminor hemoglobin by electrophoresis and ion exchange chromatography. Mice homozygous for this mutation were used to study the relative quantities of the mutant β-smajor and normal β-sminor hemoglobins specified by the two adult β-globin genes of the $\underline{Hbb}^{s2}$ haplotype during development. At 11.5 days of gestation, β-s2major hemoglobin comprises under 20 percent and β-sminor over 80 percent of the adult β-globin in circulation. The relative level of β-sminor hemoglobin decreased through fetal development; at birth β-sminor represented 33.7 percent of the β-globin. The adult values of 70 percent β-s2major and 30 percent β-sminor hemoglobin are expressed in mice at six days after birth. The relative levels of expression of the $\beta 1^{s2major}$ and $\beta 2^{sminor}$ globin genes of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice correlate well with the expression of the $\beta 1^{dmajor}$ and $\beta 2^{dminor}$ globin genes of $\underline{Hbb}^{d}/\underline{Hbb}^{d}$ mice, both in adults and during development. Expression of the $\beta 1^{sminor}$ globin gene should not be affected by the ENU-induced base substitution in the $\beta 1^{smajor}$ gene. Therefore, it is proposed that the $\beta 1^{sminor}$ gene is also expressed in mice of the $\underline{Hbb}^{s}$

haplotype. The results also indicate that the two adult β-globin genes of the $\underline{Hbb}^{s2}$ and, presumably, $\underline{Hbb}^{s}$ haplotypes are regulated independently as are the $\beta 1^{major}$ and $\beta 2^{minor}$ genes of the $\underline{Hbb}^{d}$ and $\underline{Hbb}^{p}$ haplotypes.

Introduction

In vertebrates, the sites of erythropoiesis (yolk sac, fetal liver, spleen and bone marrow), and the kinds of hemoglobins produced (3 embryonic and 2 adult types) change during development. A hemoglobin mutation has provided a model system to study the expression of the two adult mouse β-globin genes throughout fetal and postnatal development at the protein level.

Both of the adult β-globin genes are expressed in mice of the $\underline{Hbb}^{d}$ and $\underline{Hbb}^{p}$ haplotypes (Russell and McFarland, 1974). In adults, β-dmajor accounts for 80 percent and β-dminor 20 percent of the hemoglobin present; however, relatively more β-dminor than β-dmajor hemoglobin is present between 17 days of gestation and postnatal life (Whitney, 1977; Alter and Goff, 1980). A systematic study of the relative quantities of the β-dmajor and β-dminor hemoglobins in mice of the $\underline{Hbb}^{d}$ haplotype during earlier stages of development was not done because the embryonic EII and the β-dminor hemoglobins can not be resolved by electrophoresis.

Mice of the $\underline{Hbb}^{s}$ haplotype also have two adult β-globin genes (Weaver et al., 1981). However, these mice produce a single kind of β-globin polypeptide (Popp, 1962). Until recently it was not possible

to distinguish between two possibilities: either both genes produce the same kind of globin or only one of the two β-globin genes in mice of the $\underline{Hbb}^{s}$ haplotype is functional. The recent recovery and characterization of an ethylnitrosourea induced mutation at the $\underline{Hbb}^{s}$ locus have now shown the former possibility to be the correct one (Lewis et al., 1985). In mice that carry this mutation, designated $\underline{Hbb}^{s2}$, both of the adult globin genes are functional. The present study shows that both of the adult hemoglobins produced by $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice are electrophoretically separable from the embryonic hemoglobins so that the relative quantities of the products encoded by these adult β-globin genes could be measured beginning with the onset of their expression.

Procedures

Mice

Four heterozygous carriers of an ethylnitrosourea induced mutation at the $\underline{Hbb}^{s}$ locus (Lewis et al., 1985) were obtained from Dr. Susan E. Lewis, Research Triangle Institute, RTP, NC. They were brother-sister mated to produce the stock of homozygous $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice used in this study.

The age of the fetus was timed by designating the presence of a copulation plug in the morning as day .5 of gestation. The date of birth was used to determine the age of newborn, juvenile and adult mice. Blood from an average of 18 fetuses was sampled for each day of development and blood from an average of 13 mice was sampled for

each day between birth and 42 days of age; the minimum sample size was 7. The values obtained from each individual were averaged for each day. The same mouse could be used to provide hemoglobin for the analysis at more than one time point after birth, so long as it had not been bled within a period of five days.

Collection of Blood

Erythrocytes were obtained from individual fetuses. On successive days of gestation starting with day 11.5 through birth, blood was obtained at daily intervals from at least 13 fetuses taken from two or more pregnant females. Pregnant females were sacrificed, the uterus was removed, and each fetus was dissected from the uterus with care to keep the placenta and yolk sac intact. For the earlier times in gestation, it was necessary to remove the decidua capsularis to expose the fetus enveloped in the amnion and yolk sac. Once the decidua capsularis was removed, a forceps was placed at the junction of the yolk sac and the placenta—the placenta was on one side and the fetus and yolk sac on the other side of the tips of the forceps. The forceps was closed and a second sharp-edged forceps was used to sever the placenta from the fetus. While maintaining a secure hold on the forceps, the yolk sac and fetus were serially rinsed in 0.85 percent saline to remove any maternal blood that might contaminate the sample. The fetus was then placed in 10 ml of 0.85 percent saline and the yolk sac vessels and major sinuses of the fetus were ruptured to allow the blood to flow into the saline. The suspended erythrocytes were collected by centrifugation at 600 xg for 10 minutes at 5°C and

the hemoglobin in the pelleted erythrocytes was separated by electrophoresis.

Blood for electrophoresis was removed from the tail vein of neonates and older mice. Repeated retro-orbital sinus bleeding was done to elevate the peripheral blood reticulocyte level for studies on α- and β-globin synthesis.

Electrophoresis

Erythrocytes in approximately 5 µl of whole blood from juvenile and adult mice or in 5 µl of pelleted erythrocytes from each fetus were lysed in 10 µl of water, and the hemoglobin was modified by adding 5 µl of 66.7 mM cystamine (Whitney, 1978). Eight samples of approximately 2 µl each were loaded with an applicator on buffer-moistened Helena Lab Titan III cellulose acetate plates and electrophoresed at 300V for 27 minutes. An electrophoresis buffer of 0.15M Tris : 0.0833M boric acid : 0.0017M ethylenediaminetetracetic acid (EDTA), pH 8.6 was used to separate hemoglobins in prenatal blood samples; and 0.15M Tris : 0.0833M boric acid : 0.0033M EDTA, pH 8.6 buffer was used to analyze hemoglobin from postnatal blood samples.

Scanning Densitometry

Immediately following electrophoresis, the cellulose acetate plate was placed in 5 percent trichloroacetic acid for 5 minutes, rinsed in 5 percent glacial acetic acid for 5 minutes, and air dried overnight. This procedure fixed the hemoglobin so that it did not

diffuse, and quantitation of the separated hemoglobins could be done the following day. The hemoglobin bands were quantitated using a Flur-Vis transmission densitometer (Helena Lab Autoscan) equipped with an electronic integrator.

In Vitro Globin Synthesis

Reticulocytosis was induced by removing 0.4 ml of blood from each adult mouse every other day for 5 days. Reticulocytes from several mice were pooled, washed twice in 0.85 percent saline and incubated in leucine-deficient medium containing 1.8×10^7 cpm of ^{3}H-leucine for 90 minutes at 37°C in a 5 percent CO_2 incubator as described by Martinell et al. (1981). After incubation, the cells were again washed twice in 0.85 percent saline to remove nonincorporated ^{3}H-leucine, and globin was prepared by precipitation in cold, acidified (0.012N HCl) acetone (Rossi-Fanelli et al., 1958). The globin chains were separated by carboxymethylcellulose column chromatography using a 1.9 x 25 cm column, running 3 liters of a 10 to 35 mM gradient of Na_2HPO_4 - NaH_2PO_4 buffer, pH 6.9, containing 50 mM β-mercaptoethanol and 8M deionized urea (Clegg et al., 1966). The ^{3}H-leucine incorporated into the newly synthesized polypeptides was determined for each 15 ml fraction by liquid scintillation spectrometry (Packard Tri-Carb 3255).

Results

The cellulose acetate electrophoreogram (Figure 2.1) illustrates the specific types of adult hemoglobins produced in mice

Figure 2.1. Cellulose acetate electrophoreogram of adult hemoglobins in mice of four different hemoglobin genotypes.

The globin chains present in each lane are designated at the left. Lane 1 contains the $\alpha_2\beta_2^{smajor}$ and $\alpha_2\beta_2^{sminor}$ tetramers. In lane 2 the cathodal band contains the $\alpha_2\beta_2^{smajor}$ and $\alpha_2\beta_2^{sminor}$ tetramers and the anodal band the $\alpha_2\beta_2^{s2major}$ tetramers. In lane 3 the cathodal band contains the $\alpha_2\beta_2^{sminor}$ tetramers and the anodal band the $\alpha_2\beta_2^{s2major}$ tetramers. In lane 4 the cathodal band contains the $\alpha_2\beta_2^{dminor}$ tetramers and the anodal band the $\alpha_2\beta_2^{dmajor}$ tetramers.

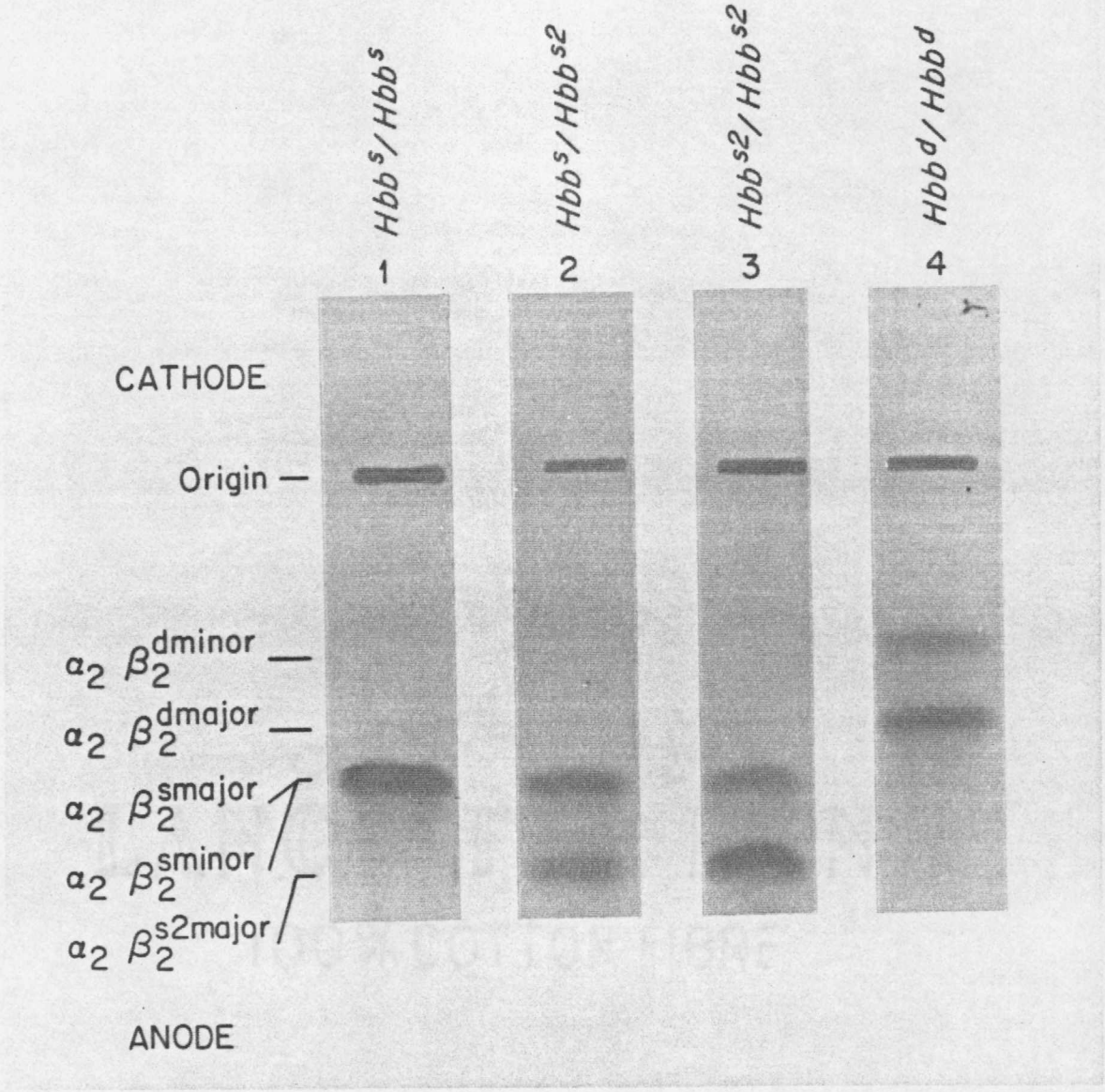

1 Hbb^s/Hbb^s
2 Hbb^s/Hbb^s2
3 Hbb^s2/Hbb^s2
4 Hbb^d/Hbb^d
CATHODE
Origin
α2 β2dminor
α2 β2dmajor
α2 β2smajor
α2 β2sminor
α2 β2s2major
ANODE

Figure 2.1

of various genotypes. Hemoglobin from an adult $\underline{Hbb}^{s}/\underline{Hbb}^{s}$ mouse is shown in lane 1; it electrophoreses as a single band, yet its β-globin is produced from two different β-globin genes, $\beta 1^{single}$ and $\beta 2^{single}$. Hemoglobin in lane 3 is from a homozygous mutant $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$; due to the substitution of glutamic acid for valine at position 60 in the β-smajor globin chain, the more anodal band ($\alpha_2\beta_2{}^{s2major}$) is electrophoretically separable from the more cathodal band ($\alpha_2\beta_2{}^{sminor}$). The hemoglobin pattern from a heterozygous $\underline{Hbb}^{s}/\underline{Hbb}^{s2}$ mouse (lane 2) has a cathodal band consisting of identical $\alpha_2\beta_2{}^{smajor}$ and $\alpha_2\beta_2{}^{sminor}$ tetramers, and an anodal band of $\alpha_2\beta_2{}^{s2major}$ tetramers. Lane 4 shows the two hemoglobins produced by a homozygous $\underline{Hbb}^{d}/\underline{Hbb}^{d}$ mouse; the cathodal one is $\alpha_2\beta_2{}^{dminor}$ and the anodal one is $\alpha_2\beta_2{}^{dmajor}$.

Analysis by transmission densitometry of hemoglobin from a 14.5 day $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ fetus produced the autoscan shown in Figure 2.2A. This illustrates the separation and quantitation of the five hemoglobins present at this age: the two anodal bands are the adult hemoglobins ($\alpha_2\beta_2{}^{s2major}$ and $\alpha_2\beta_2{}^{sminor}$) and the three cathodal bands are the embryonic hemoglobins (EI, EII and EIII). Densitometric analysis of the hemoglobin from an adult $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mouse (e.g., lane 3 of Figure 2.1) produced the autoscan shown in Figure 2.2B, where 70 percent of the hemoglobin is $\alpha_2\beta_2{}^{s2major}$ and 30 percent is $\alpha_2\beta_2{}^{sminor}$.

Figure 2.3 plots the percent of embryonic and adult hemoglobins in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ fetuses from 11.5 days of gestation through birth. Some adult hemoglobin is already present in these fetuses at 11.5 days of gestation. At this time the two adult hemoglobins jointly

Figure 2.2. Scanning densitometry.

Scanning densitometric results of hemoglobin of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice after cellulose acetate electrophoresis and fixation in 5 percent TCA, 5 percent glacial acetic acid, and allowed to air dry. (A) The relative quantities of β-s2major, β-sminor, EI, EII, and EIII hemoglobins at 14.5 days of gestation are shown. (B) The relative quantities of β-s2major and β-sminor hemoglobins in an adult mouse are shown.

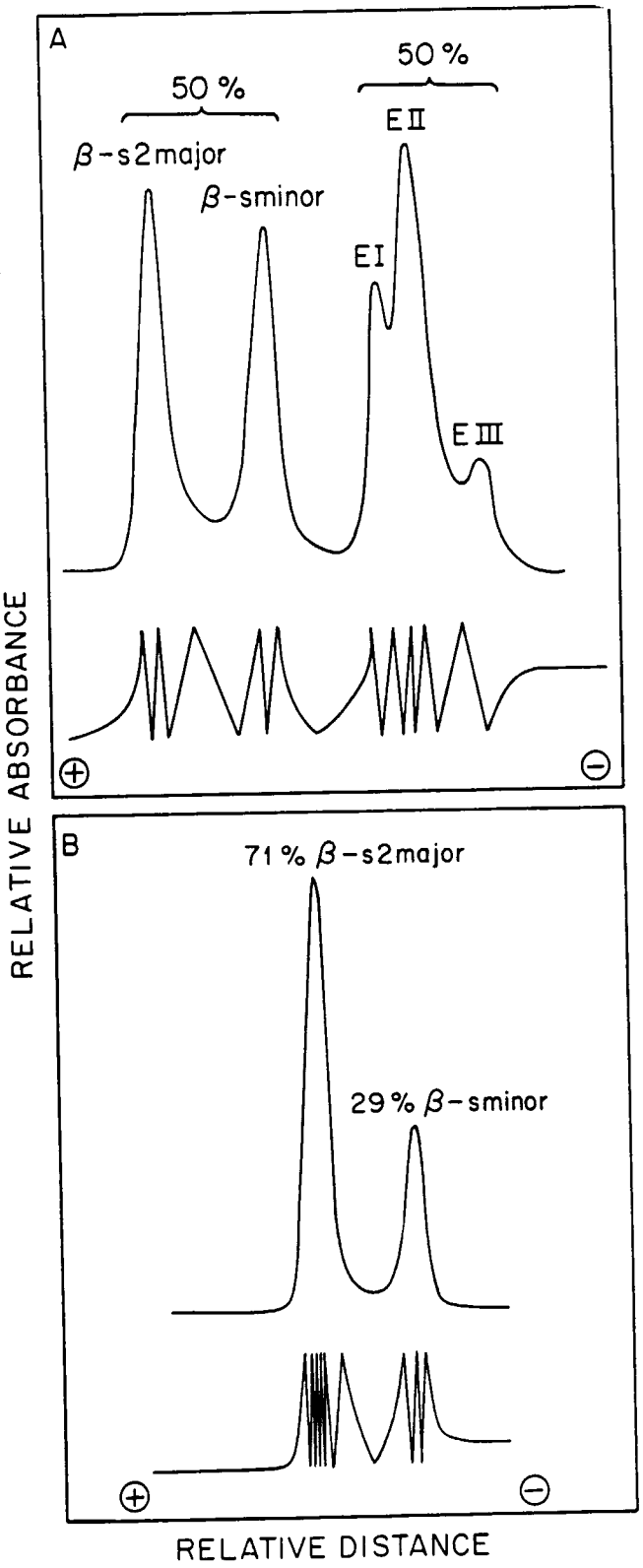

A
50 %
50 %
β-s2major
β-sminor
EII
EI
EIII
⊕
⊖
B
71 % β-s2major
29 % β-sminor
⊕
⊖
RELATIVE ABSORBANCE
RELATIVE DISTANCE

Figure 2.2

Figure 2.3. Quantitation of embryonic and adult hemoglobins during development of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice.

The percentage of the sum of the three embryonic hemoglobins (EI, EII and EIII) (- - - -) versus the sum of the two adult hemoglobins ($\alpha_2\beta_2^{s2major}$ and $\alpha_2\beta_2^{sminor}$) (———) are shown. Quantitation was done from cellulose acetate electrophoreograms of lysed erythrocytes sampled on successive days between day 11.5 of gestation and birth. Error bars indicate standard error of the mean.

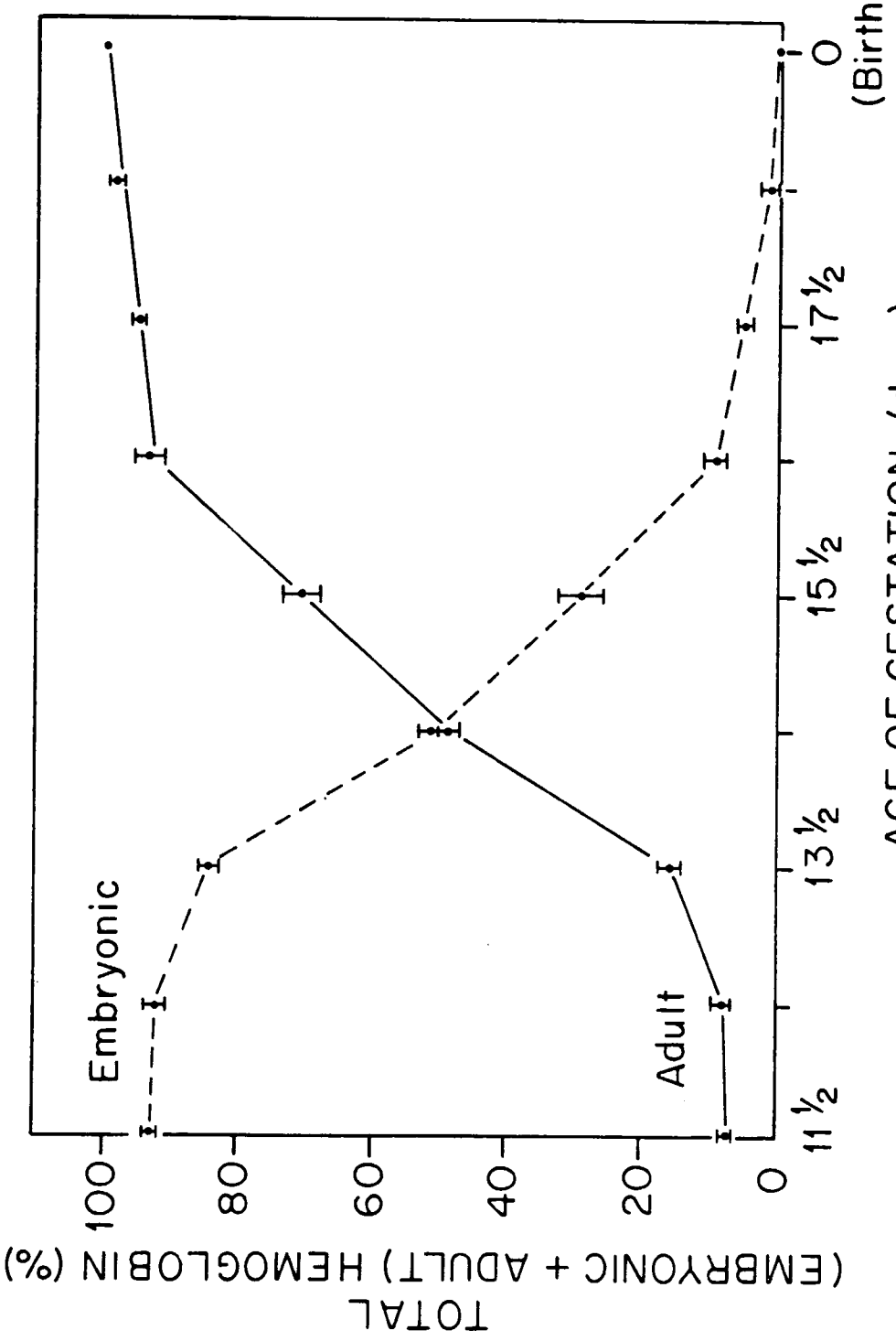

TOTAL
(EMBRYONIC + ADULT) HEMOGLOBIN (%)
100
80
60
40
20
0
Embryonic
Adult
11½
13½
15½
17½
0
(Birth)
AGE OF GESTATION (days)

Figure 2.3

account for 7.2 ± 0.8 percent of total (adult and embryonic) hemoglobin. Between 11.5 and 13.5 days of gestation the relative quantity of adult hemoglobin increases slowly, but by 14.5 days of gestation there are about equal amounts of adult and embryonic hemoglobins. The largest shift from embryonic to adult hemoglobin expression occurs between 13.5 through 16.5 days of gestation. At birth, the level of embryonic hemoglobins is insufficient to be detected by the methods employed.

The relative quantities of the two kinds of adult hemoglobin in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice from 11.5 days of gestation to 42 days after birth are plotted in Figure 2.4, where 100 percent = β-s2major + β-sminor. The quantities of $\alpha_2\beta_2^{s2major}$ and $\alpha_2\beta_2^{sminor}$ are proportional to the amounts of the β-s2major and β-sminor globins synthesized in these mice. At 11.5 days of gestation, the β-sminor hemoglobin accounts for 80 percent of the adult hemoglobin present, although adult hemoglobin represents only 10 percent of the total hemoglobin at this time (Wawrzyniak et al., 1985). As the fetus develops, there is a shift in the ratio of β-s2major : β-sminor; at 14.5 days of gestation these products are present in equal amounts, and at 6 days after birth the adult level is achieved, which is 70 percent β-s2major and 30 percent β-sminor hemoglobin.

The β-s2major and β-sminor hemoglobins can also be separated and quantitated by CMC column chromatography (Figure 2.5). Data from 4 separate determinations from CMC columns gave mean values of 71.0 ± 1.6 percent β-s2major and 29.0 ± 1.6 percent β-sminor, which

Figure 2.4. Quantitation of the two kinds of adult hemoglobins during development.

These values were obtained by scanning densitometry and are presented as though 100 percent = β-s2major + β-sminor. The percentage of the adult β-s2major (●---●) and β-sminor (o---o) globins in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice from 11.5 days of gestation past weaning are shown. Error bars are the standard error of the mean.

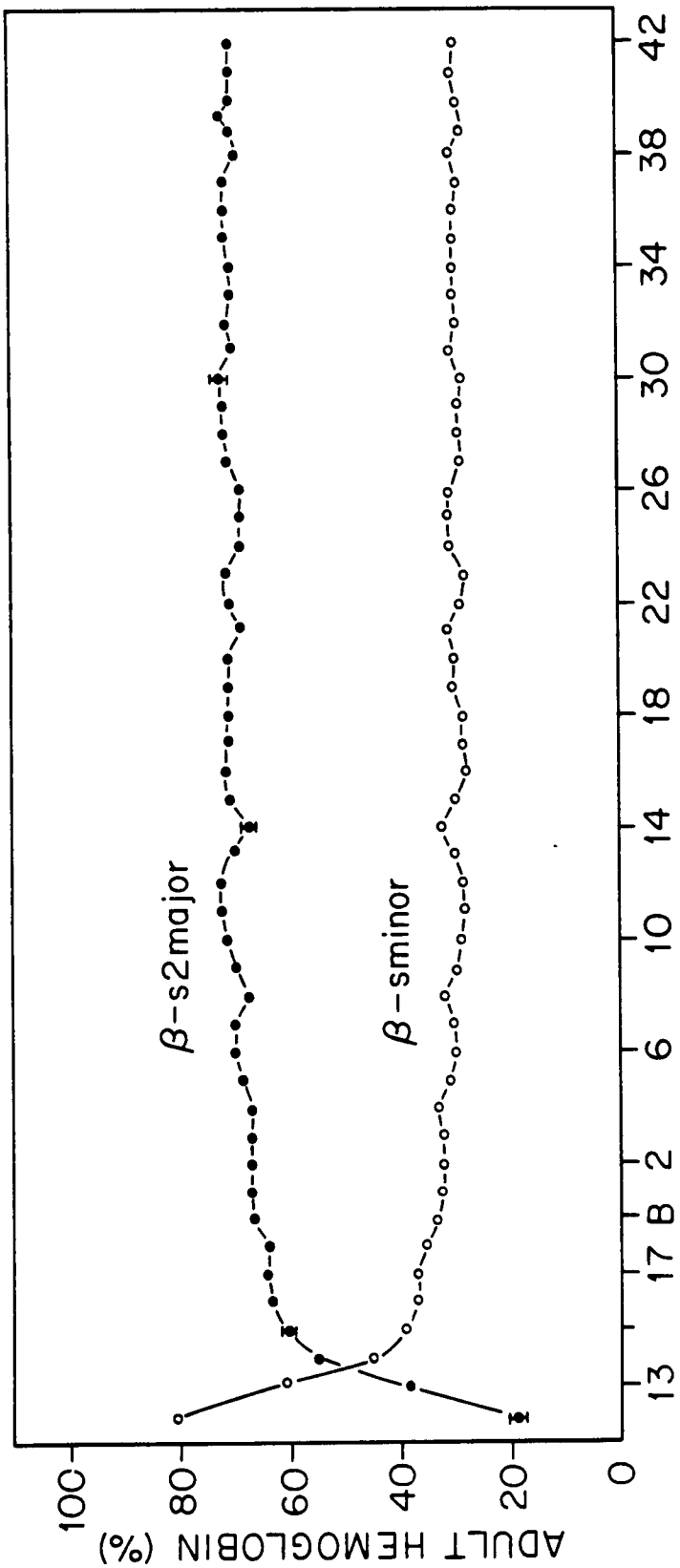

ADULT HEMOGLOBIN (%)
100
80
60
40
20
0
β-s2major
β-sminor
13
17
B
2
6
10
14
18
22
26
30
34
38
42
GESTATION
AGE (days)

Figure 2.4

Figure 2.5. Separation and quantitation of globins by carboxymethylcellulose column chromatography.

Reticulocytes from an adult $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mouse were incubated in medium containing [^{3}H]-leucine. The globin chains were separated by carboxymethylcellulose column chromatography using 8M urea and a 10-35mM Na_2HOP_4-NaH_2PO_4 gradient, pH 6.0. The [^{3}H]-leucine incorporated was determined by scintillation spectrometry, and absorbance at 280nm was measured by a spectrophotometer.

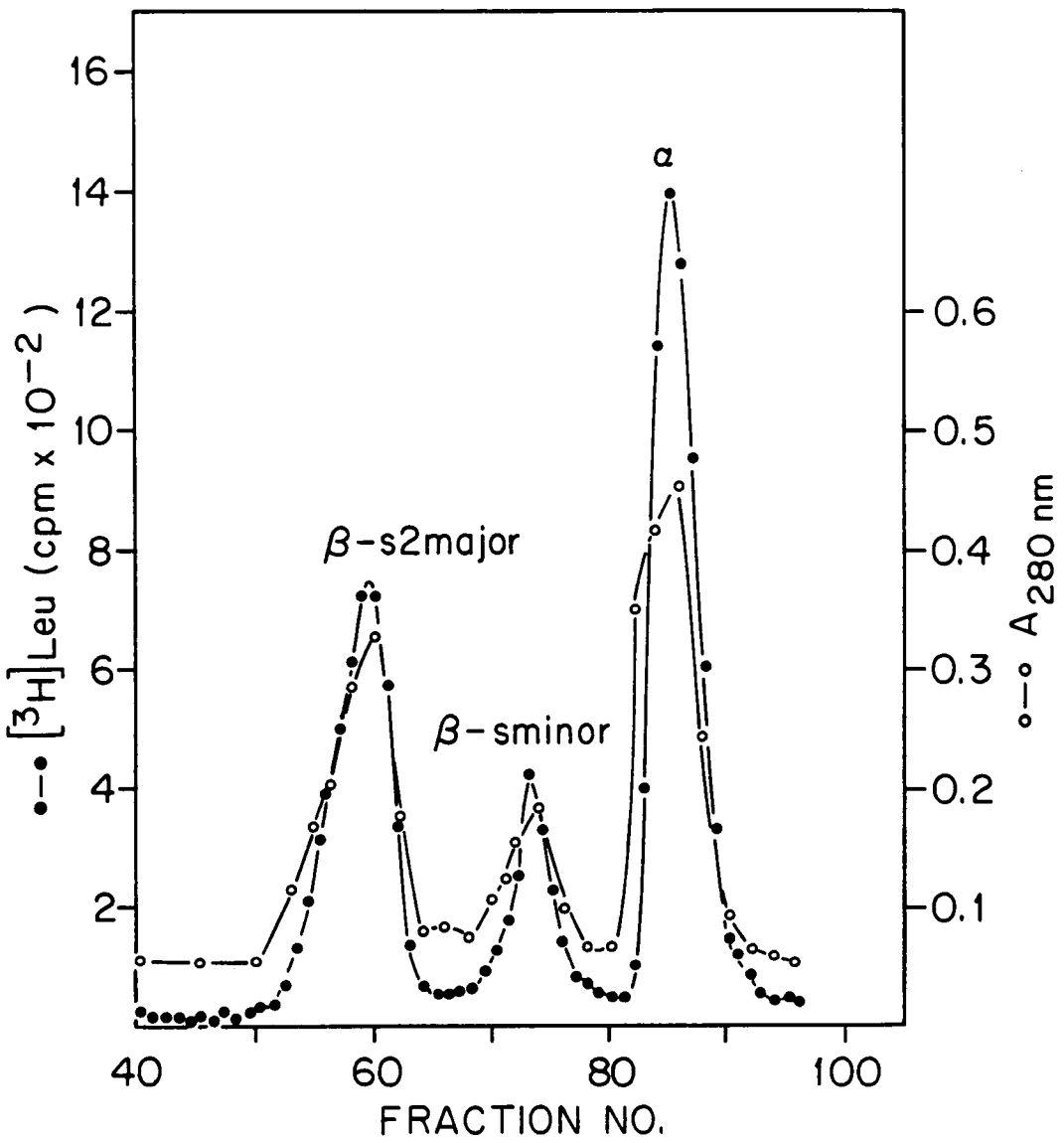

16
14
12
10
8
6
4
2
0.6
0.5
0.4
0.3
0.2
0.1
α
β-s2major
β-sminor
•—• [3H]Leu (cpm x 10−2)
°—° A280 nm
40
60
80
100
FRACTION NO.

Figure 2.5

are quantitatively the same as those obtained by the cellulose acetate electrophoresis and scanning densitometry method (Figures 2.1 and 2.2).

Discussion and Conclusions

The results presented here establish that the relative expression of the two adult β-globin genes of the $\underline{Hbb}^{s2}$ haplotype varies with the developmental stage of the mouse. The gene coding for β-sminor globin is expressed at a relatively low level (< 30 percent) in the adult and a relatively high level (> 80 percent) in the fetal circulation at 11.5 days of gestation (Figure 2.4). Correspondingly, the gene coding for β-s2major globin is expressed at a relatively high level (> 70 percent) in the adult and at a relatively low level (< 20 percent) at 11.5 days of gestation. At 11.5 days of gestation, mainly primitive nucleated erythrocytes derived from the yolk sac and very few definitive non-nucleated erythrocytes derived from the fetal liver are present in circulating blood. The yolk sac-derived red cells synthesize mostly embryonic hemoglobin and a small amount of adult type hemoglobin (Chui et al., 1979), but the red cells of fetal liver origin produce only adult type hemoglobin (Wong et al., 1983). The change from embryonic to adult hemoglobin expression in C57BL/6 mice correlates well with the number of nucleated and non-nucleated erythrocytes in the fetus between 12 and 16 days of gestation (Craig and Russell, 1964). The rapid shift in the ratio of β-s2major and β-sminor hemoglobin observed between 12.5 and 16.5 days

of gestation (Figure 2.4) might be reflecting the change in the red blood cell population occurring during this period (refer to Chapter V).

This study also indicates that the adult β-globin genes of the $\underline{Hbb}^{d}$ haplotype (which codes for the adult hemoglobins β-dmajor and β-dminor), $\underline{Hbb}^{s2}$ haplotype (which codes for the adult hemoglobins β-s2major and β-sminor), and presumably the $\underline{Hbb}^{s}$ haplotype (which codes for the adult hemoglobins β-smajor and β-sminor) have homologous functions. From analysis of heteroduplexes (Weaver et al., 1981), the $\beta 1^{single}$ and $\beta 1^{dmajor}$ genes are allelic as too are the $\beta 2^{single}$ and $\beta 2^{dminor}$, genes. The present study has extended to periods of gestation earlier than those investigated by others, but studies of comparable periods on mice of the $\underline{Hbb}^{d}$ haplotype gave parallel results (Whitney, 1977; Alter and Goff, 1982). Thus, the independent regulation and expression of the $\beta 1^{major}$ and $\beta 2^{minor}$ genes through the course of development appear to be similar for the $\underline{Hbb}^{d}$ and $\underline{Hbb}^{s}$ haplotypes. The circulating levels of β-s2major and β-dmajor hemoglobin are both lower during the prenatal period and increase until shortly after birth, whereas the β-sminor and β-dminor hemoglobin levels are higher before birth and decrease until shortly after birth.

It is interesting to note that the developmental profile of the β-sminor hemoglobin resembles that of a fetal hemoglobin in other mammals. The mouse has no fetal hemoglobin. In $\underline{Hbb}^{s}/\underline{Hbb}^{s}$ mice, both the $\beta 1^{single}$ and $\beta 2^{single}$ genes appear to be functional (Lewis et al., 1985; Wawrzyniak et al., 1985), but each produces the same polypeptide

(Popp, 1973). The adult β-globin genes were duplicated prior to speciation of rodents and the β-dmajor and β-dminor polypeptides differ by 9 amino acids (Popp, 1973; Popp and Bailiff, 1973; Gilman, 1976). Gene convergence could explain why the $\beta 1^{single}$ and $\beta 2^{single}$ genes are not presently divergent (Erhart et al., 1985). The signals that regulate the expression of the two adult β-globin genes of the $\underline{Hbb}^{s}$ haplotype, due to nucleotide sequence alone, apparently lie outside the segment involved in gene conversion. For this reason the $\beta 1^{sminor}$ gene continues to be expressed at higher levels in fetal than in adult mice; this mimics the expression of the fetal gamma globin genes in humans (Stamatoyannopoulos et al., 1981). An understanding of the mechanism(s) involved in changing the expression of the globin genes might provide insights into the regulation of other genes during development as well as how their natural expression might be altered in adults.

CHAPTER III

PERTURBATION OF ADULT HEMOGLOBIN RATIOS IN ASSOCIATION WITH HEMATOPOIETIC STRESS

Summary

The differential expression of the two adult hemoglobins coded for in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice has been demonstrated in Chapter II. A perturbation in the adult values of 70 percent β-s2major and 30 percent β-sminor hemoglobin is found in association with hematological stress in adult mice. In mildly anemic α-thalassemic heterozygotes ($\underline{Hba}^{b2(th)}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$), the level of β-sminor hemoglobin increases to 37.9 ± 0.5 percent, but in asymptomatic β-thalassemic heterozygotes ($\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{d2(th)}/\underline{Hbb}^{s2}$) β-sminor is elevated to a lesser extent (34.1 ± 0.4 percent). The relative quantity of β-sminor hemoglobin is increased the most (41.4 ± 0.5 percent) in doubly heterozygous α-thalassemic, β-thalassemic mice ($\underline{Hba}^{b2(th)}/\underline{Hba}^{b}$; $\underline{Hbb}^{d3(th)}/\underline{Hbb}^{s2}$). In mice homozygous for a macrocytic anemia, which does not alter the β-globin gene complex ($\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2};\underline{W}/\underline{W}^{v}$), the level of β-sminor hemoglobin is increased to 36.0 ± 0.8 percent. There is also an elevation in the β-sminor hemoglobin level after prolonged phlebotomy.

Introduction

There are many mechanisms by which the normal physiology of erythropoesis may be disturbed. The hematological stress associated with thalassemia, congenital macrocytic anemia, and phlebotomy has been studied to assess the effects on the expression of adult β-globins.

Thalassemia is a hereditary anemia caused by a decreased synthesis of either the α- or β-globin subunit of hemoglobin. In thalassemic mice the hematocrit and hemoglobin concentration are decreased.

Mice with dominant white spotting ($\underline{W}/\underline{W}^{v}$) have a congenital macrocytic anemia, and the number of erythrocytes is significantly reduced (Silvers, 1979). The basis of this macrocytic anemia is a defect in the number and the differentiation capacity of the erythropoietic stem cells.

Phlebotomy is the act of removal of blood from a vessel. Bleeding creates an anemia and puts a demand on the erythropoietic system to replace the lost blood.

This study establishes that the relative levels of the adult β-globins in red cells can be perturbed to various degrees depending on the extent of hematopoetic stress encountered.

Procedures

Mice

The stock of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice described in Chapter II was also used here. These mice were mated with one of the X-radiation-induced α-thalassemia mutants, 352HB ($\underline{Hbb}^{b}/\underline{Hbb}^{b2(th)}$) (Russell et al., 1976; Popp and Enlow, 1977), or with the spontaneous β-thalassemia mutant (Skow et al., 1983) to obtain mice that carried multiple mutations at the hemoglobin loci. Identification of heterozygous α-thalassemia was done by microscopic inspection of blood smears for abnormal erythrocytes, i.e., anisocytosis and poikilocytosis (Popp and Enlow, 1977). The Hbb alleles were identified by electrophoresis of hemoglobin as described below.

To obtain $\underline{Hbb}^{s2}/\underline{Hbb}^{s2};\underline{W}/\underline{W}^{v}$ mice, the appropriate matings were made between stocks of C57BL/6-$\underline{W}^{v}$/+ and WB/Re-W/+ and $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$;+/+ mice. The dominant spotting genotypes were classified by the unique coat colors of the progeny (Silvers, 1979), and the Hbb genotypes were classified by electrophoresis as discussed below.

Collection of Blood

Blood for electrophoresis was removed from the tail vein. Phlebotomy was performed by drawing blood from the retro-orbital sinus. Blood for hematology was also drawn from the retro-orbital sinus.

Electrophoresis

Erythrocytes in approximately 5 µl of whole blood of adult mice were lysed in 10 µl of water, and the hemoglobin was modified by adding 5 µl of 66.7 mM cystamine (Whitney, 1978). Eight samples of approximately 2 µl each were loaded with an applicator on buffer-moistened Helena Lab Titan III cellulose acetate plates and electrophoresed at 300V for 27 minutes. An electrophoresis buffer of 0.15M Tris:0.083M boric acid:0.0017M EDTA, pH 8.6, was used to separate hemoglobin tetramers based on their β-globin subunit. This buffer system was also used to detect the absence of the β-dmajor hemoglobin in β-thalassemic mice.

Scanning Densitometry

Immediately following electrophoresis, the procedure described in Chapter II Procedures was followed to quantitate the hemoglobin bands on the cellulose acetate plates.

Scanning Electron Microscopy

For scanning electron microscopy, fresh blood was fixed in 1 percent glutaraldehyde in 0.85 percent NaCl, and post-fixed in 0.5 percent OsO_4 in 0.85 percent NaCl. Red cells were then dehydrated through a graded series of increasing ethyl alcohol concentrations at room temperature. The red cells were critical-point-dried, layered with gold/palladium, and examined under the scanning electron microscope.

Hematology

Hematological analysis was performed on five to eight mice of each genotype presented in Table 3.1. The red cell counts were made with a hemacytometer, hematocrits were obtained via the microhematocrit method, and peripheral blood hemoglobin was determined by the cyanmethemoglobin colorimeteric method (Wintrobe et al., 1981). The mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCHb) values were calculated from the above measurements: MCV = hematocrit/rbc count, and MCHb = (gHb/dl)/(rbc/dl).

Phlebotomy

To study the effects of phlebotomy, 8 adult male mice of the $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ genotype were subjected to repeated bleeding. Approximately 0.4 ml of whole blood was removed from the orbital sinus every other day for 20 days, then every third day for an additional 18 days. Quantitation of the two adult hemoglobins was performed at each bleeding.

Results

The cellulose acetate electrophoreogram in Figure 3.1 illustrates the specific types of adult hemoglobins produced in mice of various hemoglobin genotypes. Hemoglobin from a $\underline{Hbb}^{s}/\underline{Hbb}^{s}$ mouse is shown in lane 1; the two adult β-globin genes, $\beta 1^{single}$ and $\beta 2^{single}$, code for identical β-globin polypeptides. Hemoglobin in lane 2 is from a homozygous mutant $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$. The more anodal band

TABLE 3.1

HEMATOLOGIC VALUES FOR ADULT MICE OF VARIOUS HEMOGLOBIN GENOTYPES

	(Control)[a]	(α-Thal)[b]	(β-Thal)[c]	(α-Thal, β-Thal Double Heterozygote)[d]	(Macrocytic Anemia)[e]
Hematology					
RBC x 10^6/mm^3	12.7 ± 0.4[f]	12.9 ± 0.5	12.5 ± 0.4	13.8 ± 0.4	5.6 ± 0.4
Hematocrit (%)	51.3 ± 1.1	46.1 ± 1.6	51.2 ± 1.2	47.7 ± 1.0	33.2 ± 2.2
gHb/dl	17.2 ± 0.2	15.1 ± 1.5	16.3 ± 0.4	15.1 ± 0.4	9.3 ± 1.1
MCV (fl)	41.2 ± 1.0	35.8 ± 0.9	41.2 ± 0.8	34.6 ± 0.6	59.3 ± 2.1
MCHb (pg/rbc)	13.6 ± 0.3	11.8 ± 0.5	13.1 ± 0.3	11.0 ± 0.4	16.7 ± 1.6
Quantitative Analysis					
β-dminor globin (%)			30.8 ± 0.6	35.8 ± 0.3	
β-sminor globin (%)	29.0 ± 0.5	37.9 ± 0.5	23.6 ± 0.4 (34.1 ± 0.4)[g]	26.6 ± 0.5 (41.4 ± 0.5)[g]	36.0 ± 0.8
β-2smajor globin (%)	71.0 ± 0.5	62.1 ± 0.5	45.6 ± 0.7 (65.9 ± 0.4)[g]	37.6 ± 0.5 (58.6 ± 0.5)[g]	64.0 ± 0.8
n	> 100	23	32	13	23

TABLE 3.1 (continued)

[a] $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$.

[b] $\underline{Hba}^{b2(th)}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$.

[c] $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{d3(th)}/\underline{Hbb}^{s2}$.

[d] $\underline{Hba}^{b2(th)}/\underline{Hba}^{b};\underline{Hbb}^{d3(th)}/\underline{Hbb}^{s2}$.

[e] $\underline{Hba}/\underline{Hba};\underline{Hbb}^{s2}/\underline{Hbb}^{s2};\underline{W}/\underline{W}^{v}$.

[f] Standard error of the mean.

[g] Percentages for the hemoglobins produced by $\underline{Hbb}^{s2}$ only.

Figure 3.1. Cellulose acetate electrophoreogram of adult hemoglobins in mice of eight different hemoglobin genotypes.

The hemoglobin tetramers present are: a, $\alpha_2\beta_2^{dminor}$, b, $\alpha_2\beta_2^{dmajor}$; c, $\alpha_2\beta_2^{smajor}$ and $\alpha_2\beta_2^{sminor}$; and d, $\alpha_2\beta_2^{s2major}$. O is the point of sample application.

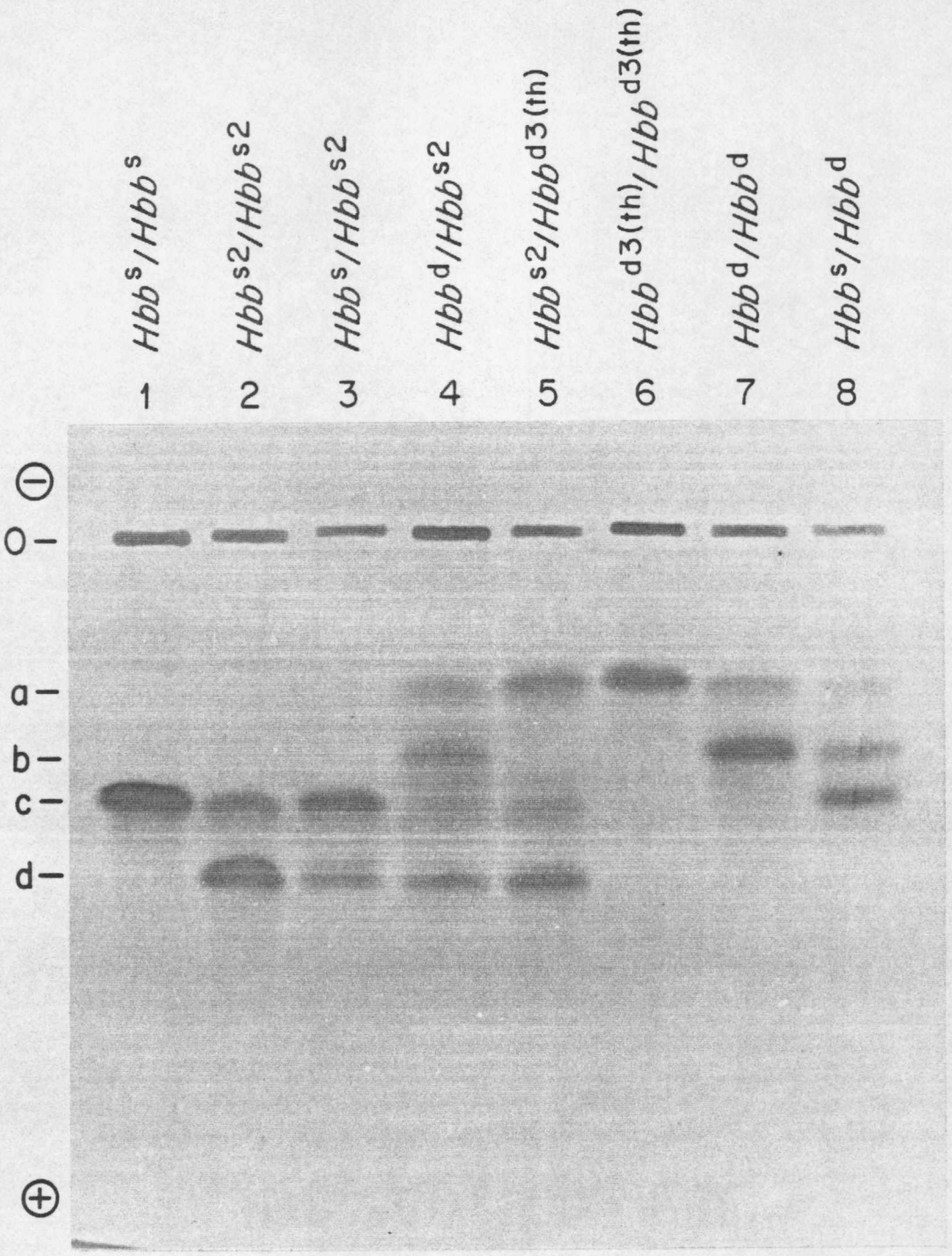

1 Hbb^s/Hbb^s
2 Hbb^s2/Hbb^s2
3 Hbb^s/Hbb^s2
4 Hbb^d/Hbb^s2
5 Hbb^s2/Hbb^d3(th)
6 Hbb^d3(th)/Hbb^d3(th)
7 Hbb^d/Hbb^d
8 Hbb^s/Hbb^d
⊖
O
a
b
c
d
⊕

Figure 3.1

($\alpha_2\beta_2^{s2major}$) is electrophoretically separable from the more cathodal band ($\alpha_2\beta_2^{sminor}$). The hemoglobin pattern from a heterozygous $\underline{Hbb}^s/\underline{Hbb}^{s2}$ mouse (lane 3) has a cathodal band consisting of identical $\alpha_2\beta_2^{smajor}$ and $\alpha_2\beta_2^{sminor}$ tetramers, and an anodal band of $\alpha_2\beta_2^{s2major}$ tetramers. Lane 7 shows the separation of the two hemoglobins produced by a homozygous $\underline{Hbb}^d/\underline{Hbb}^d$ mouse; the cathodal one is $\alpha_2\beta_2^{dminor}$ and the anodal one is $\alpha_2\beta_2^{dmajor}$. A heterozygous $\underline{Hbb}^d/\underline{Hbb}^{s2}$ mouse has four hemoglobins (lane 4); the $\underline{Hbb}^d$ haplotype contributes $\alpha_2\beta_2^{dminor}$ and $\alpha_2\beta_2^{dmajor}$, and the $\underline{Hbb}^{s2}$ haplotype contributes the $\alpha_2\beta_2^{sminor}$ and $\alpha_2\beta_2^{s2major}$. The hemoglobin produced by a homozygous β-thalassemic ($\underline{Hbb}^{d3(th)}/\underline{Hbb}^{d3(th)}$) mouse is shown in lane 6. The $\beta 1^{dmajor}$ globin gene is deleted so the $\alpha_2\beta_2^{dmajor}$ band is missing. Lane 5 shows the hemoglobin from a heterozygous $\underline{Hbb}^{s2}/\underline{Hbb}^{d3(th)}$ mouse, in which the $\alpha_2\beta_2^{dmajor}$ tetramer is also absent.

The deficiency of normal globin polypeptides in thalassemic mice affects several hematological parameters and the red blood cell morphology. The scanning electron micrograph in Figure 3.2 demonstrates the homogeneous population of biconcave red cells present in normal adult mice. In contrast, red cells from a heterozygous β-thalassemic mouse (Figure 3.3) and a homozygous β-thalassemic mouse (Figure 3.4) represent the anisocytosis and poikilocytosis associated with thalassemia.

The relative quantities of the two adult hemoglobins obtained by transmission densitometry in control $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice is 70 percent β-s2major and 30 percent β-sminor. The effects of α- and

Figure 3.2. Scanning electron micrograph of red cells from a normal adult mouse.

The erythrocytes in normal adult mice form a homogeneous population of biconcave cells. These red cells have smooth membrane surfaces.

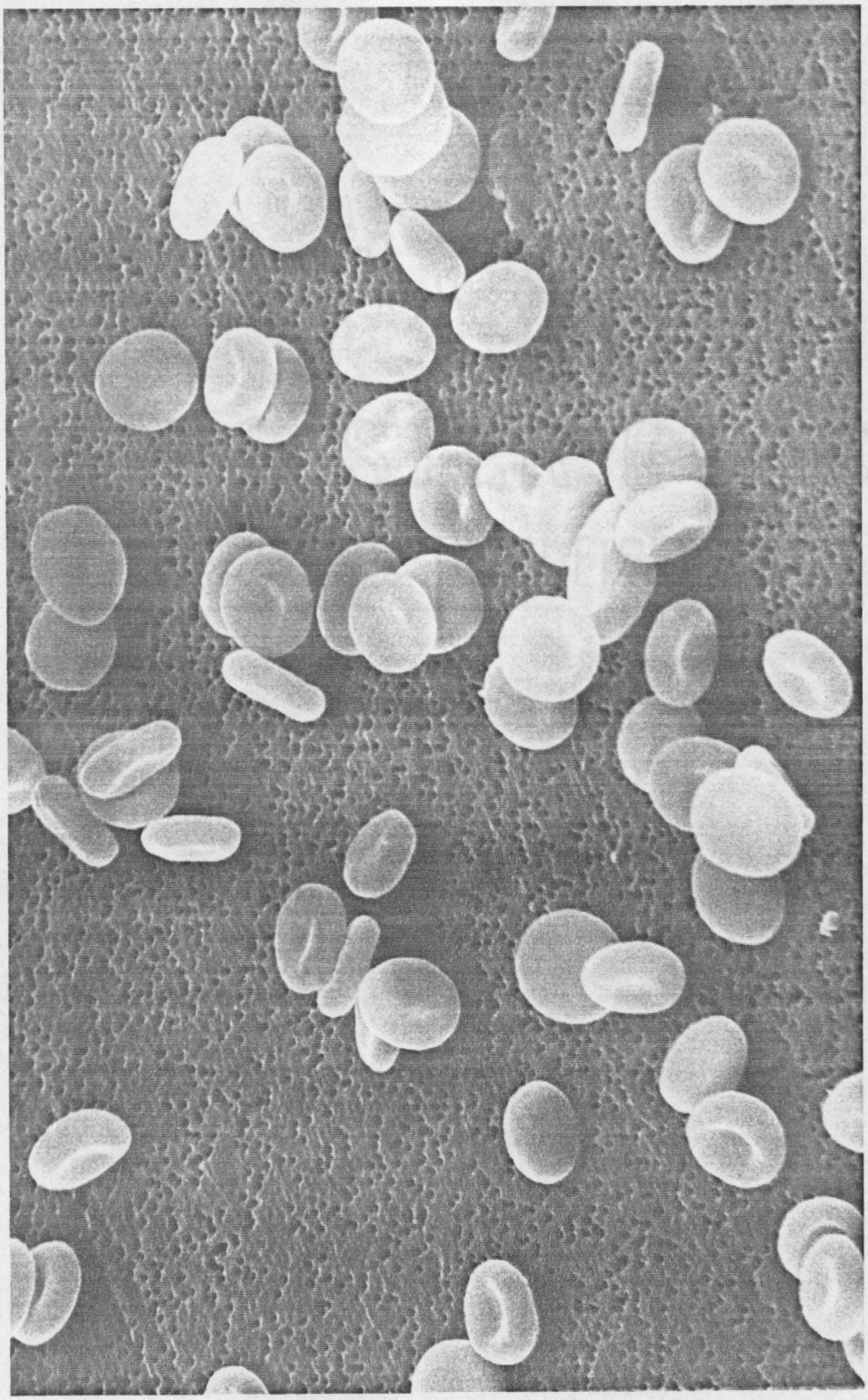


Figure 3.2

Figure 3.3. Scanning electron micrograph of red cells from an adult heterozygous β-thalassemic mouse.

The vast majority of red cells in heterozygous β-thalassemic mice have normal biconcave morphology. Occasionally an abnormally shaped red cell is observed as is depicted in the center of the micrograph.

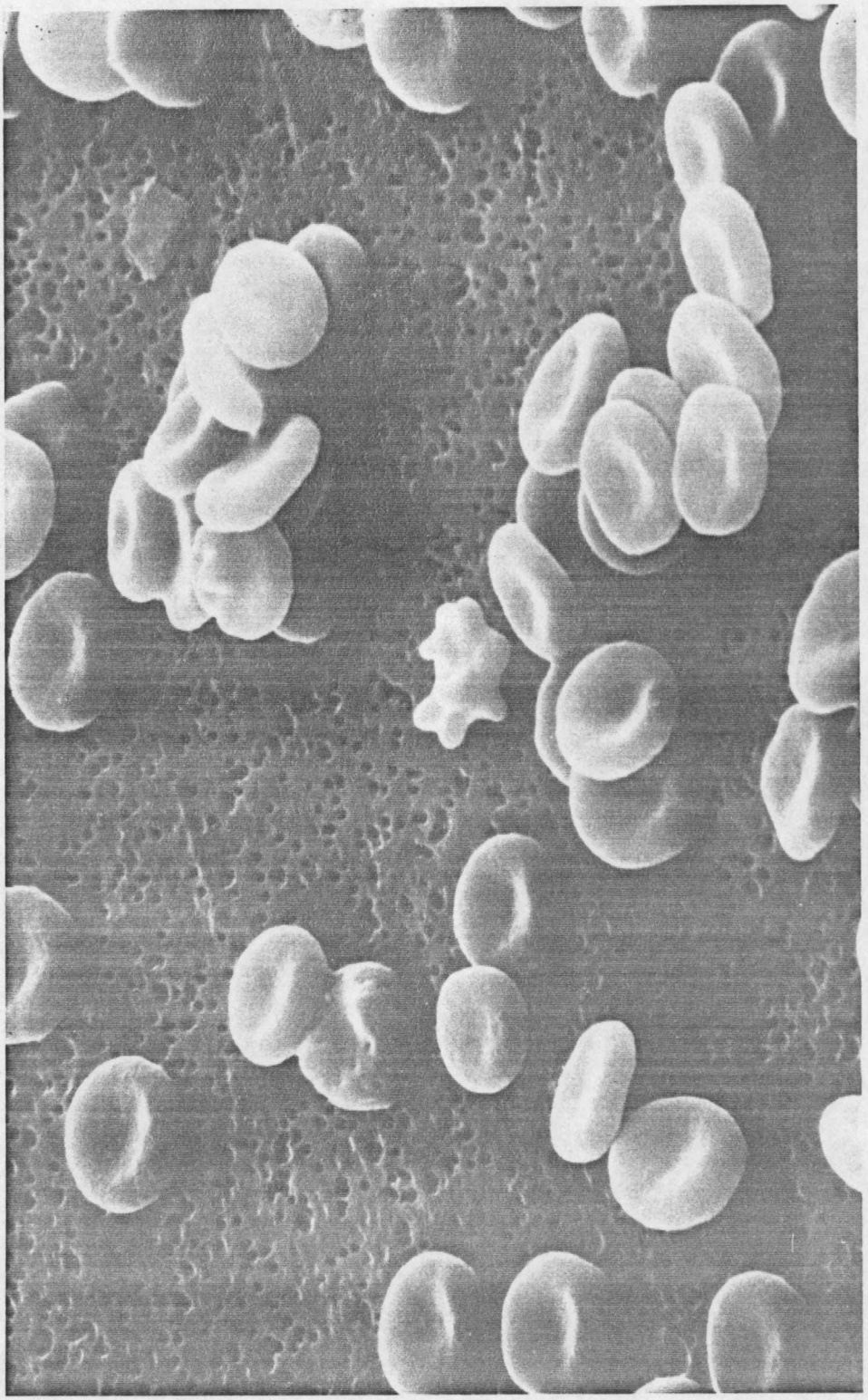


Figure 3.3

Figure 3.4. Scanning electron micrograph of red cells from an adult homozygous β-thalassemic mouse.

A heterogenous population of erythrocytes is present in homozygous β-thalassemic mice. The morphology of many of the red cells pictured here is abnormal. In addition, membrane pitting can be seen on the surface of many cells.

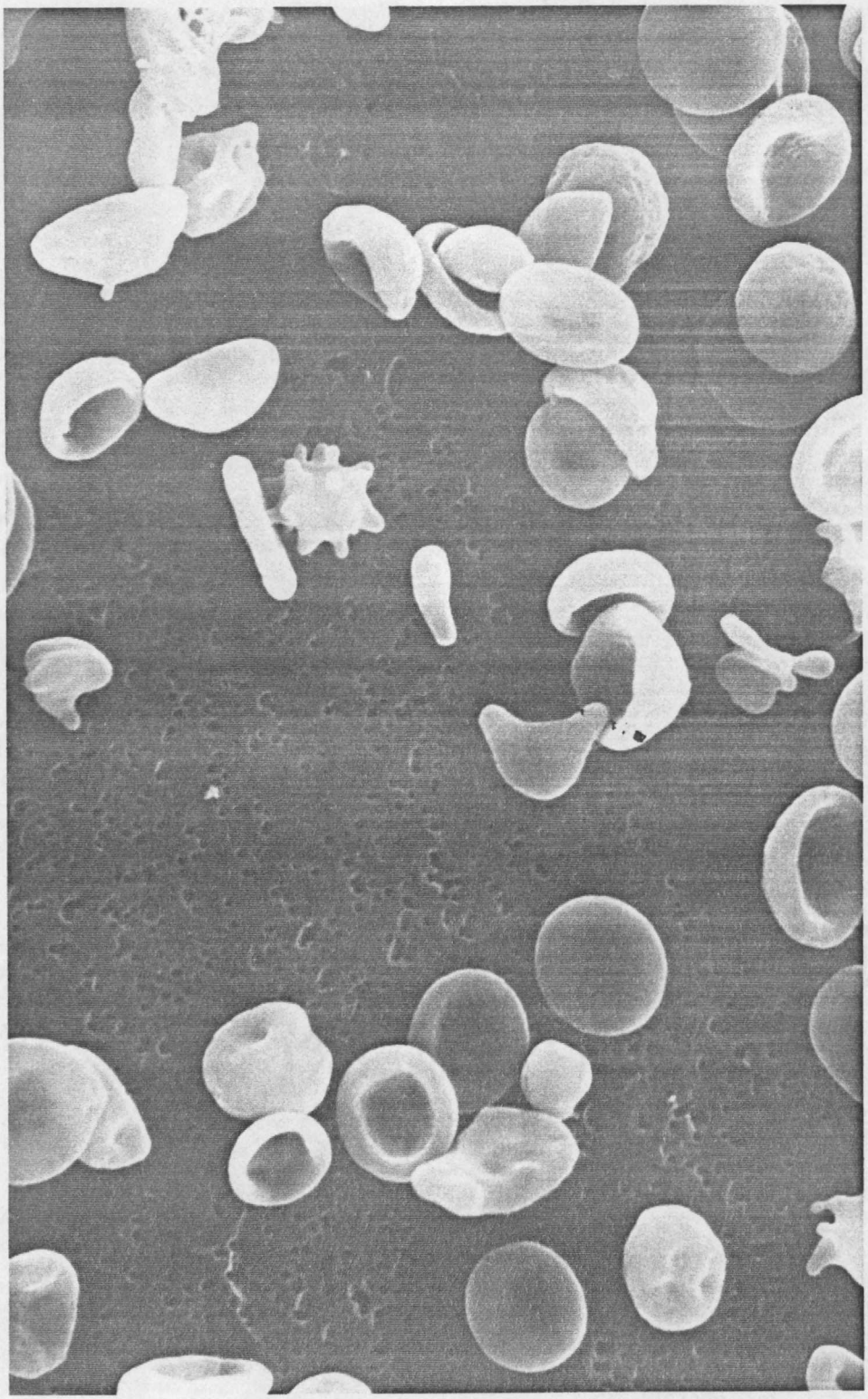


Figure 3.4

β-thalassemia on the hematological indices and on the ratio of β-2smajor to β-sminor hemoglobins are presented in Table 3.1. The α-thalassemic heterozygote displays an hypochromic, microcytic anemia (MCHb of 11.8 ± 0.5 pg/rbc, hematocrit of 46.1 ± 1.6 percent, and MCV of 35.8 ± 0.9 fl). In addition, anisocytosis and poikilocytosis are observed in blood films. The hematological values of a β-thalassemic heterozygote are within normal ranges, and very few abnormal red cells are observed in blood films. Doubly heterozygous α-thalassemic, β-thalassemic mice have elevated red cell counts (13.8 ± 0.4 x $10^6/mm^3$); however, these red cells are microcytic (34.6 ± 0.6 fl) and hypochromic (11.0 ± 0.4 pg Hb/rbc). In addition, the most profound anisocytosis and poikilocytosis are seen in blood smears from mice of the doubly heterozygous thalassemic genotype.

In heterozygous α-thalassemic mice, the level of β-sminor hemoglobin increased from 30 to 38 percent (Table 3.1). If one considers only the hemoglobins produced by the $\underline{Hbb}^{s2}$ allele, the expression of β-sminor hemoglobin is increased to 34 percent in heterozygous β-thalassemic mice. In an α-thalassemic, β-thalassemic double heterozygote, the level of β-sminor hemoglobin is increased from 30 to 41 percent and β-s2major is decreased accordingly from 70 to 59 percent.

The hematology parameters and results of quantitative analysis for mice with the macrocytic anemia ($\underline{W}/\underline{W}^v$) are listed in Table 3.1. This type of anemia is associated with low red cell counts (5.6 ± 0.4 x $10^6/mm^3$); however, these cells are extremely large (59.3 ± 2.1 fl)

and hyperchromic (16.7 ± 1.6 pg Hb/rbc). The macrocytic anemia perturbs the ratio of adult globins, increasing the β-sminor hemoglobin component to 36 percent, and correspondingly decreasing the β-s2major to 64 percent.

The effects of phlebotomy in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice are shown in Figure 3.5. There was a gradual increase in β-sminor relative to β-s2major hemoglobin during the period of the first 10 to 15 bleedings, with the maximum response of 36 percent β-sminor hemoglobin after 33 days.

Discussion and Conclusions

The relative expression of the adult β-globin genes of the $\underline{Hbb}^{s2}$ haplotype is perturbed in hematopoietically stressed adult mice. Five kinds of hematopoietic stress were analyzed in this study.

In regard to the thalassemias, the hematopoietic stress is most severe in doubly heterozygous α-thalassemic, β-thalassemic mice, these also showed the greatest increase in β-sminor hemoglobin. Heterozygous α-thalassemic mice are mildly anemic, whereas heterozygous β-thalassemic mice are not anemic. In the former mice, the quantity of β-sminor hemoglobin is higher than in mice with asymptomatic heterozygous β-thalassemia. An alteration in the expression of the multiple hemoglobins in α-thalassemic mice of the $\underline{Hba}^{b2(th)}/\underline{Hba}^{b}$; $\underline{Hbb}^{s}/\underline{Hbb}^{d}$ genotype had been observed earlier (Popp et al., 1976), but the basis for the change was not determined. It is now clear that

Figure 3.5. Effects of phlebotomy in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice.

Approximately 0.4 ml of whole blood was removed every other day for 20 days and every second or third day for an additional 18 days. Quantitation of the β-sminor globin is plotted for each bleeding. The error bars represent the standard error of the mean.

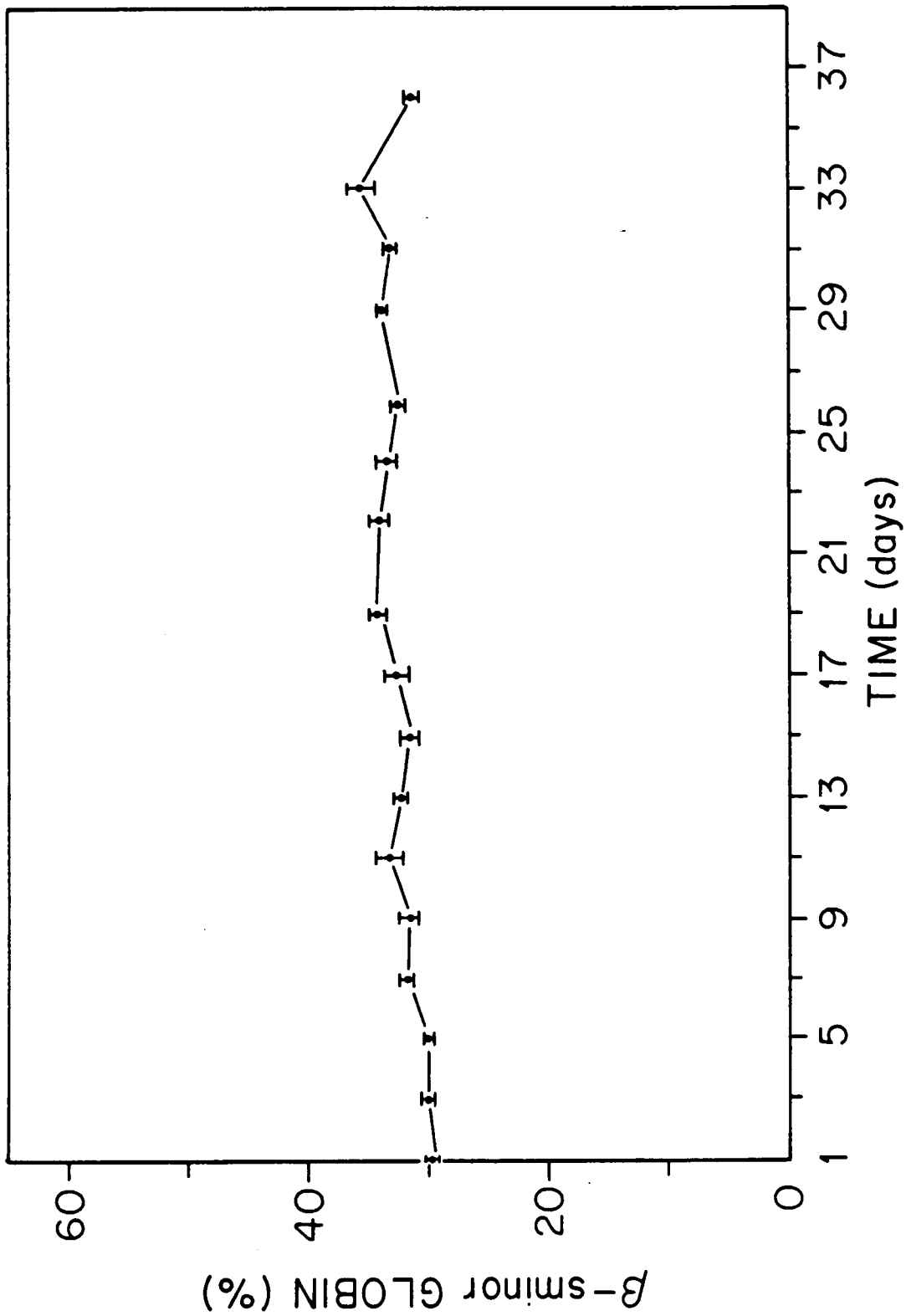


Figure 3.5

the relative increase in β-single hemoglobin in the $\underline{Hba}^{b2(th)}/\underline{Hba}^{a}$; $\underline{Hbb}^{s}/\underline{Hbb}^{d}$ mice is due to an increased expression of the $\beta 2^{sminor}$ gene of the $\underline{Hbb}^{s}$ haplotype.

The β-sminor hemoglobin, coded for by the $\beta 2^{sminor}$ gene, is increased relative to β-s2major hemoglobin in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice that had a macrocytic anemia ($\underline{W}/\underline{W}^{v}$). Expression of the homologous $\beta 2^{dminor}$ gene of the $\underline{Hbb}^{d}$ haplotype has also been shown to be elevated in mice with congenital macrocytic anemias (Whitney, 1981). Thus, in response to the stress of macrocytic anemia, mice of the $\underline{Hbb}^{d}$ and $\underline{Hbb}^{s2}$ haplotypes exhibit an increase in the expression of the β-minor globin gene, and it may be assumed that this is also true for $\underline{Hbb}^{s}$.

In response to phlebotomy, the level of β-sminor hemoglobin is increased gradually during the period of the first 10 to 15 bleedings, and the general level remains more or less steady after 19 days. A maximum elevation in β-sminor hemoglobin from 30 to 36 percent was obtained after 33 days of bleeding (Wawrzyniak et al., 1985). A similar but greater response to phlebotomy was demonstrated for the β-dminor hemoglobin product of the $\underline{Hbb}^{d}$ haplotype when 20 percent more blood was removed at each bleeding (Alter et al., 1982).

Collectively, these studies establish that expression of the two adult β-globin genes is independently regulated. The common physiological response to hematopoietic stress of thalassemia, macrocytic anemia and phlebotomy is an increase in the quantity of β-minor hemoglobin and a reduction in the quantity of β-major hemoglobin. The functional response of the $\beta 1^{dmajor}$ and $\beta 2^{dminor}$ globin genes of

the $\underline{Hbb}^{d}$ haplotype and the $\beta 1^{smajor}$ and $\beta 2^{sminor}$ globin genes of the $\underline{Hbb}^{s}$ haplotype are homologus, and parallel the structural data comparing the specified genes. The specific nucleotide sequences that are involved in regulating globin gene expression in response to hematopoietic stress will be of interest as a model system which is readily assessable at the protein level.

CHAPTER IV

EXPRESSION OF β-S2MAJOR AND β-SMINOR GLOBIN GENES AT THE LEVEL OF THE RNA

Summary

Dot blot hybridization was used to determine the relative amounts of the β-s2major and β-sminor globin RNAs present in reticulocytes of mice at 14.5- and 17.5-day fetuses, newborns, and adult mice of the $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ globin genotype. RNAs isolated from embryonic yolk sac, fetal liver, and adult reticulocytes were hybridized with the following labeled DNA probes: α-1, β-minor specific, or β-major specific. The level of β-sminor RNA in reticulocytes of 14.5- and 17.5-day fetuses is nearly the same as that in reticulocytes of adult mice. In contrast, the level of β-s2major RNA in reticulocytes of 14.5- and 17.5-day fetuses is, respectively, 23 percent and 66 percent the amount found in reticulocytes of induced adult mice. These results correlate well with the observations presented in Chapter II which indicate that the $\beta 2^{sminor}$ globin gene approaches its normal adult level of expression by 14.5 days of gestation, whereas the $\beta 1^{s2major}$ globin gene expression increases between 14.5 days of gestation and 6 days postnatally. The two types of data together indicate that the differential expression of β-sminor and β-s2major hemoglobins during development is regulated at the level of transcription. Expression of the $\beta 2^{sminor}$ globin gene in

reticulocytes of adult normal mice is not maximal, however, because the levels of the β-sminor RNA and hemoglobin are increased further in reticulocytes of thalassemic mice.

Introduction

The globin genes as a family are considered to have descended from duplication and variation of an ancestral gene (Collins and Weissmann, 1984). Although the active members may share related or identical functions, they can be expressed at different times or in different cell types. The adult $\beta 1^{major}$ and $\beta 2^{minor}$ globin genes, located on chromosome 7, demonstrate differential expression during development (Whitney, 1977; Alter and Goff, 1980; Wawrzyniak et al., 1985). Mice of the $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ genotype code for two adult β-globins, β-sminor and β-s2major (Lewis et al., 1985), which are functionally (refer to Chapter III) and structurally (Weaver et al., 1981) homologous to the β-dminor and β-dmajor adult hemoglobins coded for by the $\underline{Hbb}^{d}$ haplotype. In $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice, the β-sminor hemoglobin is the predominant form of adult-type β-globin in erythrocytes at the early stages of fetal life. At 11.5 days of gestation, β-sminor hemoglobin comprises over 80 percent of the adult β-globin present, and β-s2major hemoglobin under 20 percent. A rapid switch in the amounts of adult β-globins expressed, β-sminor versus β-s2major, occurs between 14.5 and 17.5 days of gestation, and by 6 days after birth the ratio of the two adult hemoglobins is 30 percent β-sminor and 70 percent β-s2major. This rapid switch might be regulated at the

transcriptional or translational level. Results of this study demonstrate that the differential expression of the $\beta 1^{s2major}$ and $\beta 2^{sminor}$ globin genes is correlated with the relative levels of their specific RNAs in reticulocytes during development, and that transcription of the $\beta 2^{sminor}$ gene is elevated in reticulocytes of thalassemic mice.

Procedures

Mice and Hemoglobin Genotypes

Mice with seven different α- and β-globin genotypes were generated by appropriate matings. The β-globin alleles (Hbb) were identified by electrophoresis on Helena Lab Titan III cellulose acetate plates in 0.15M Tris : 0.0833M boric acid : 0.0033M ethylenediaminetetracetic acid (EDTA), pH 8.6 buffer. This system separates hemoglobin tetramers on the basis of differences among the β-globin subunits, so that tetramers containing β-dminor, β-dmajor, β-sminor/β-smajor, or β-s2major hemoglobin are clearly distinguished (Wawrzyniak et al., 1985). The Hbb locus is compound. Considering only the adult-type hemoglobins, the Hbb^{d} allele codes for β-dmajor and β-dminor hemoglobins, the Hbb^{s} allele codes for the β-smajor and β-sminor hemoglobins (which are structurally identical to each other), and the Hbb^{s2} allele codes for the β-s2major and β-sminor hemoglobins. The $Hbb^{d3(th)}$ allele codes only for β-dminor hemoglobin because the $\beta 1^{dmajor}$ gene was lost by a spontaneous deletion of a 3.7 kilobase

segment of DNA that includes the entire $\beta 1^{dmajor}$ globin gene (Skow et al., 1983).

Since a portion of these studies was designed to detect alterations in the amount of β-globin under different conditions, the genetic heterogeneity introduced by the α-globin locus was minimized by using only two different alpha haplotypes. Thus all mice were either homozygous or hemizygous for the $\underline{Hba}^{b}$ allele. Identification of heterozygous α-thalassemia ($\underline{Hba}^{b2(th)}/\underline{Hba}^{b}$) was done by microscopic inspection of blood smears for large numbers of abnormal erythrocytes, i.e., anisocytosis and poikilocytosis, which are not found in normal ($\underline{Hba}^{b}/\underline{Hba}^{b}$) mice (Popp and Enlow, 1977). The Hba locus is also compound. The $\underline{Hba}^{b}$ allele codes for two adult α-globins, chains 2 and 3 (Whitney, 1979). Hemoglobin tetramers with either of these chains have identical electrophoretic mobility on cellulose acetate. The $\underline{Hba}^{b2(th)}$ allele does not code for α-globin due to a deletion mutation induced by X-radiation carried in 352HB mice (Russell et al., 1976). The deletion includes both adult α-globin and the embryonic α-like x-globin genes (Whitney et al., 1981).

The gestational age of the fetus was timed by inspecting females in the morning for the presence of a copulation plug. The day of the plus was designated as day .5 of gestation.

RNA Preparation and Dotting

The RNAs were prepared by a modification of the procedure reported by Martinell et al. (1981). Reticulocytosis was induced in

adult mice by removing 0.4 ml of blood from the retro-orbital sinus every other day. Reticulocyte values of 40 to 50 percent were obtained after 5 bleedings. Blood from the retro-orbital sinus of adult mice was suspended in 2 percent Na-citrate and 0.5 percent NaCl. After centrifugation at 600 xg for 10 minutes, all white blood cells were removed by aspiration. The reticulocytes were then washed 3 times in Ca^{++}- and Mg^{++}-free phosphate-buffered saline. The packed cells were lysed in an equal volume of water, and the membranes were pelleted by centrifugation at 20,000 xg for 20 minutes at 4°C. The RNA was extracted from the resulting reticulocyte lysate with Tris-HCl pH 9.0 buffered phenol-chloroform mixture containing sodium dodecyl sulfate (SDS). To 1 ml of lysate was added 100 µl of pH 9.0 Tris-HCl, 100 µl of 5 percent SDS, and 0.6 ml of a 1:1 mixture of phenol: chloroform. The preparation was vortexed for 30 seconds, mixed by manual shaking for an additional 4 minutes, and centrifuged at 20,000 xg for 10 minutes at 4°C. The precipitate was suspended in 1 ml of water and re-extracted as above. The supernatants of the first and second extractions were pooled and the aqueous layer was re-extracted three times with a 1:1 mixture of phenol:chloroform. The RNA was precipitated by making the sample 0.1M in NaCl and 66 percent in ethanol and storing at -20°C overnight. The resulting RNA was pelleted by centrifugation at 20,000 xg for 30 minutes at -20°C, and washed twice in 66 percent ethanol without NaCl. At this point the RNA pellet was lyophilized and stored at -70°C until it was applied on nitrocellulose.

RNA was also obtained from reticulocytes of fetuses and newborn mice. Each fetus was dissected from the uterus with care taken to keep the yolk sac intact and to remove any maternal blood that might contaminate the fetus. The fetus was then placed in 10 ml of 0.85 percent saline, and the yolk sac vessels and major sinuses of the fetus were ruptured to allow the blood to flow into the saline. Blood from several fetuses at either 14.5 or 17.5 days of gestation was pooled and collected by centrifugation at 600 xg for 10 minutes at 5°C and the RNA in the pelleted reticulocytes was isolated as described above.

When the RNA was removed from storage, it was dissolved in water. Absorbancies at 260 nm and 280 nm were recorded, and the quantity of RNA was calculated from the abosrbance of each sample. The concentration of RNA in an Eppendorf tube was adjusted to 0.5 µg/µl of water. The solution containing 3 parts 20x SSC (SSC=0.15M NaCl, 0.015M Na-citrate) and 2 parts of 37 percent formaldehyde was added. The contents were vortexed for 20 seconds and placed in a 60°C water bath for 15 minutes, followed by rapid cooling on ice for 2 minutes. The RNA in solution was applied to a nitrocellulose filter with the aid of a Bethesda Research Laboratory (BRL) Hybri-Dot Manifold (Cat. No. 1050MM). Dotted serial dilutions, ranging in concentrations from 10 µg to 0.078 µg of total reticulocyte RNA, were applied in 400 µl of 20x SSC. Each RNA-dotted nitrocellulose filter was dried under a heat lamp, then baked at 80°C in a vacuum for 2-3 hours, and stored at 4°C until it was prepared for hybridization.

RNA Hybridization

The baked nitrocellulose filter that contained the reticulocyte RNA was wetted in 6x SSC, followed by prehybridization in 10 ml of 10x SSC, 100mM NaH_2PO_4-Na_2HPO_4 pH 7, 500 µg/ml sheared salmon sperm DNA, 0.04 percent bovine serum albumin, 0.04 percent Ficoll 400, and 0.04 percent polyvinylpyrrolidone for 4-12 hours at 42°C (Thomas et al., 1983). Following nick translation (Maniatis et al., 1982), the ^{32}P-labeled DNA probes were denatured at 100°C for 7 minutes in 0.5 ml of 80 percent deionized formamide, and quick chilled on ice for 2 minutes. To this solution of the denatured probe, 0.5 ml of deionized formamide and 2.0 ml hybridization buffer (same as prehybridization buffer, but with the addition of 20 percent sodium dextran sulfate) was added. The prehybridization mixture was removed from the sealable plastic bag, and 3 ml of hybridization buffer containing a labeled probe was added and allowed to hybridize for 24 hours at 42°C. After hybridization, the unbound probe was removed by rinsing the nitrocellulose filter 6 times in 2x SSC and 0.1 percent SDS, followed by soaking in 0.5x SSC and 0.1 percent SDS at 42-55°C until background radioactivity in the washing solution was consistently less than 100 cpm/ml. The washed and dried nitrocellulose filters were exposed to Kodak X-Omat AR X-ray film between Kodak Lanex and X-Omatic intensifying screens at 15°C for 1-7 days. The hybridot films were analyzed by scanning with a laser densitometer. The area under each peak was integrated with an Apple II computer and the least squares linear regression was calculated; comparisons were made in the linear response area.

In order to hybridize the RNA on the nitrocellulose filter with a different probe, the first hybridization probe was removed by pouring a boiling solution, which contained 0.1 percent SDS and 0.1x SSPE (SSPE=0.15M NaCl, 0.01M $NaH_2PO_4 \cdot H_2O$, 0.001M EDTA), onto the nitrocellulose filter and allowing it to cool for 30 minutes (Gatti et al., 1984). The above procedure was repeated. A rinse was then done in 2x SSC, 0.1 percent SDS, and the nitrocellulose filter was stored at 4°C until rehybridization.

DNA Probes

The 0.45 kb β-minor and 1.0 kb β-major specific probes used were prepared from DNA clones kindly provided by Sheldon Z. Goldberg, National Heart, Lung, and Blood Institute, Bethesda, Maryland. The terminal 3' untranslated nucleotide regions of the $\beta 1^{major}$ (Kunkel et al., 1978) and $\beta 2^{minor}$ (Jahn et al., 1980) genes are markedly divergent. The $\underline{Hbb}^{d}$, $\underline{Hbb}^{s}$, and $\underline{Hbb}^{s2}$ haplotypes, however, are highly homologous to one another in the 3' untranslated sequences of the $\beta 2^{minor}$ genes as well as in the 3' untranslated regions of the $\beta 1^{major}$ genes (Erhart et al., 1985). Therefore, the 3' terminal region of the $\beta 2^{dminor}$ gene beginning at the Rsa I site (which is 5 bp away from the 5' end of the termination codon) and extending 0.45 kb in the 3' direction to the Pst I site was used as the β-minor specific probe. An analogous 3' terminal region of the $\beta 1^{dmajor}$ gene extending between two Rsa I sites was used as the 1.0 kb β-major specific probe. The β-major specific probe does not hybridize with reticulocyte RNA isolated from a $\underline{Hbb}^{d3(th)}/\underline{Hbb}^{d3(th)}$ mouse (data not shown). A pure

source of β-major RNA is not available to establish an absolute specificity for the β-minor probe, but the level of hybridization with RNA isolated from control mice indicates that the β-minor probe is also highly specific.

The 9.7 kb Eco RI fragment containing the α-1 globin gene of the $\underline{Hba}^{b}$ haplotype (Nishioka and Leder, 1979) cloned into pBR322 was used as the probe for reticulocyte α-globin RNA, designated as the α-1 probe.

The DNA probes were nick translated to specific activities of 0.5-4 x 10^{6} cpm/μg DNA as described by Maniatis et al. (1982), using α-^{32}P-dCTP obtained from New England Nuclear.

Results

An autoradiograph demonstrating the intensity of hybridization of the β-minor specific probe to RNAs bound to a nitrocellulose filter is shown in Figure 4.1A. These RNAs were isolated from reticulocytes of $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice at different ages in development and were immobilized onto a nitrocellulose filter. The first dot in each row contained 4 μg of RNA which was serially diluted as shown. The intensity of hybridization with the β-minor specific probe is nearly the same at 14.5 and 17.5 days of gestation as that of induced reticulocytes of adult mice, but the amount of β-minor RNA present in peripheral blood of newborn mice is much less than at the other times shown.

Figure 4.1. Autoradiogram of RNA isolated from mouse reticulocytes at different stages of development.

A. RNA hybridized with the 0.45 kb β-minor specific probe. The ages of the $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice from which the reticulocyte RNAs were isolated are indicated from top to bottom: induced adult, birth, 17.5 day fetus, and 14.5 day fetus. The first dot contained 4 μg of RNA and its serial dilutions (1/2) were applied to successive dots. B. RNA hybridized with the 1.0 kb β-major specific probe. Rows 1-4 contain the same RNA samples as rows 1-4 in A.

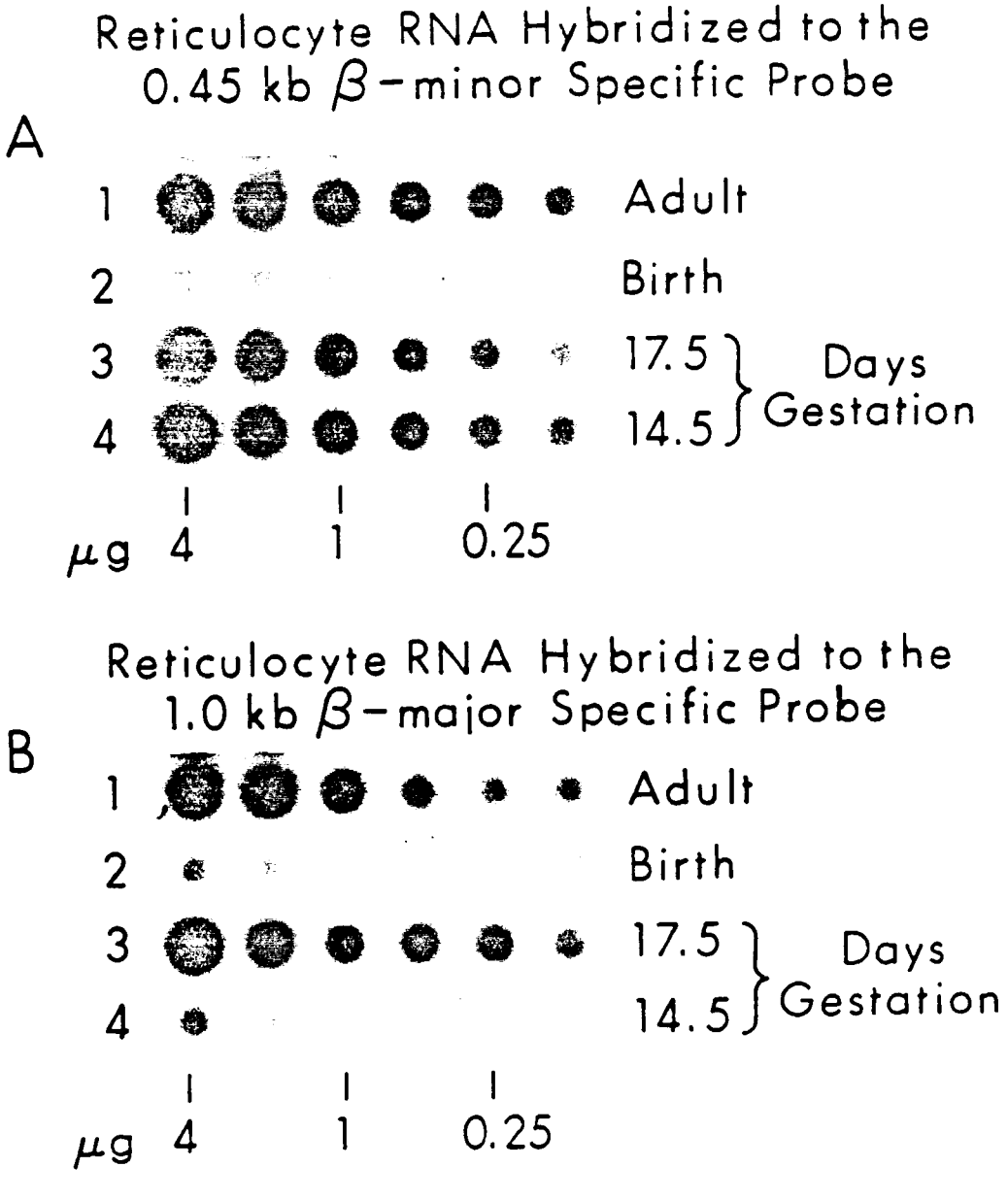

Reticulocyte RNA Hybridized to the
0.45 kb β-minor Specific Probe
A
1
2
3
4
Adult
Birth
17.5
14.5
Days
Gestation
μg 4
1
0.25
Reticulocyte RNA Hybridized to the
1.0 kb β-major Specific Probe
B
1
2
3
4
Adult
Birth
17.5
14.5
Days
Gestation
μg 4
1
0.25

Figure 4.1

A second nitrocellulose fiber (Figure 4.1B) dotted with the same RNAs as those in Figure 4.1A was hybridized with labeled β-major specific probe. It can be seen that the amount of β-s2major RNA in reticulocytes of fetuses increases between 14.5 and 17.5 days of gestation compared to the RNA in the induced reticulocytes of adult mice. The amount of β-s2major RNA present in peripheral blood of newborn mice is low, as was the β-sminor RNA at this age.

The relative levels of β-s2major and β-sminor RNA present at various times during development were measured by scanning the autoradiogram with a laser densitometer and analyzing the data with an Apple II computer. To decrease error that might be introduced by replicate dotting of RNA, the nitrocellulose filter in Figure 4.1A was dehybridized of β-minor specific probe and rehybridized with β-major specific probe so that the hybridization intensities with the two probes could be compared. These results are plotted in Figure 4.2, where 100 represents the intensity of hybridization detected for RNA obtained from induced reticulocytes of normal adult mice. The amounts of RNA that hybridized with the β-minor specific probe at 14.5 and 17.5 days of gestation are 93 and 100 percent of the adult value, respectively. In contrast, the amounts of RNA that hybridized with the β-major specific probe at 14.5 and 17.5 days of gestation are only 23 and 66 percent that of the adult value, respectively. The level of globin RNA of both β-major and β-minor are low at birth, although there is more major than minor relative to the level in an induced adult. Each observation described above was repeated.

Figure 4.2. Bar graph of reticulocyte RNA hybridization versus age.

The same RNA dot blot was hybridized first with the β-minor specific probe, dehybridized, and then rehybridized with the β-major specific probe. The level of hybridization was determined by laser densitometric analysis followed by comparison of least squares regression slopes. The results were shown relative to the 100 percent level of hybridization for these specific RNAs in reticulocytes of induced adult mice. (A) The relative amount of β-sminor RNA present at 14.5 and 17.5 days of gestation, and at birth. (B) The relative amount of β-s2major RNA present at 14.5 and 17.5 days of gestation, and at birth.

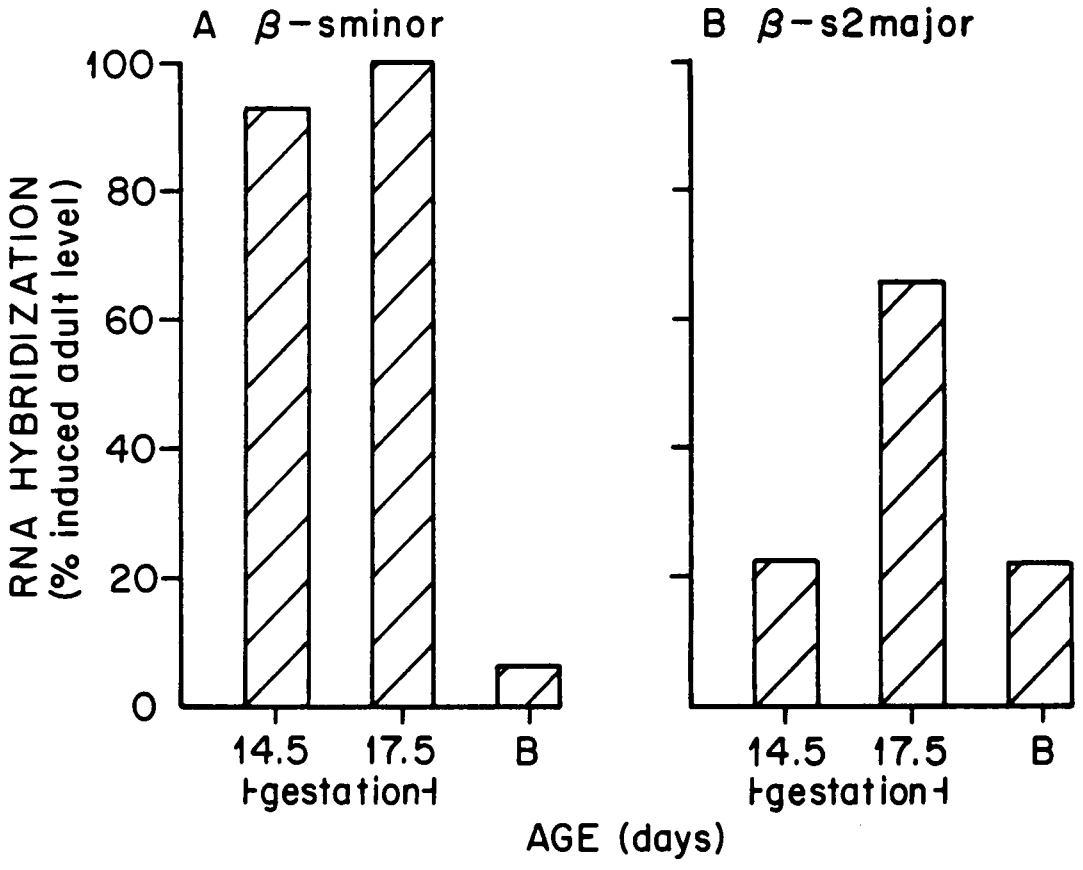
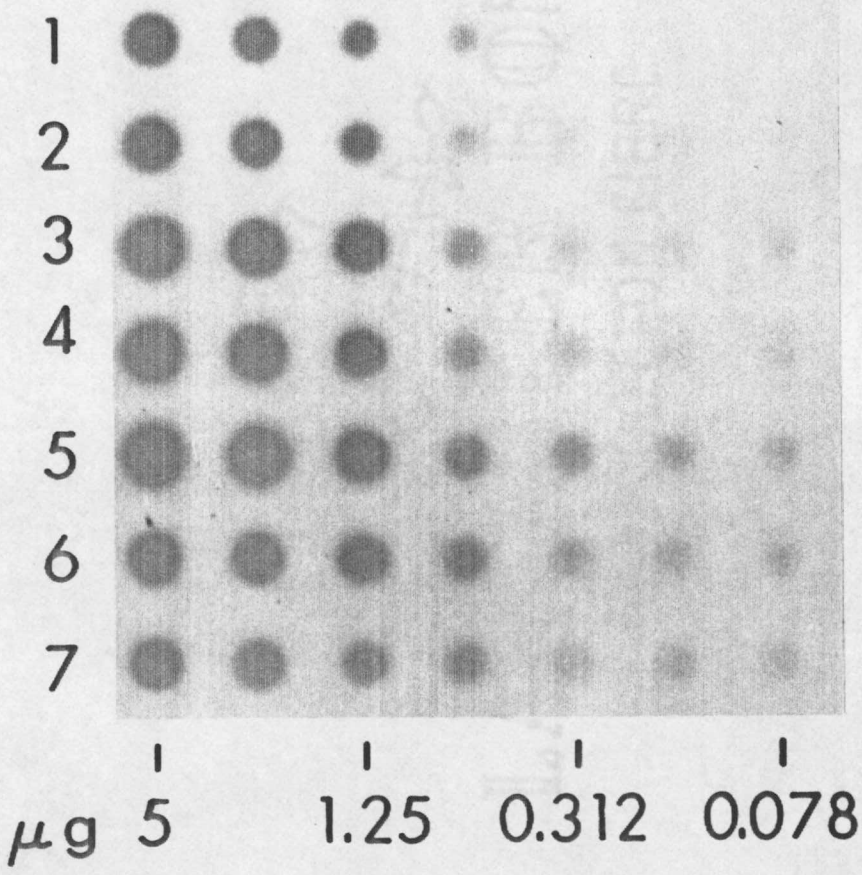

A β-sminor
B β-s2major
RNA HYBRIDIZATION
(% induced adult level)
100
80
60
40
20
0
14.5
17.5
B
14.5
17.5
B
Fgestation
Fgestation
AGE (days)

Figure 4.2

Another aspect of these experiments was to investigate the basis for the previously reported relative increase in the level of β-sminor hemoglobin in α-thalassemic ($\underline{Hba}^{b2(th)}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$) mice (Wawrzyniak and Popp, 1985). Figure 4.3 is an autoradiogram of reticulocyte RNA isolated from adult mice of 7 different hemoglobin genotypes. Rows 1, 3, and 5 contain RNA from normal mice of three different Hbb genotypes, and rows 2, 4 and 6 contain RNA from corresponding α-thalassemic mice of these Hbb genotypes. Row 7 has RNA isolated from homozygous β-thalassemic mice. Figure 4.3 demonstrates that the level of α-globin RNA is decreased in mice containing the $\underline{Hba}^{b2(th)}$ globin haplotype. The quantity of α-globin RNA, as measured by laser densitometry (White and Bancroft, 1982), in these α-thalassemic mice is 80 percent of that detected in induced reticulocytes of normal adult mice. When these same RNA samples are hybridized with a β-minor specific probe, as shown in Figure 4.4, the hybridization is more intense with RNA from α-thalassemic mice (rows 2, 4 and 6) than with RNA from normal mice (rows 1, 3 and 7). The level of β-dminor RNA, as measured by laser densitometry (White and Bancroft, 1982), is 1.8x greater in homozygous β-thalassemic mice (row 5) than in normal mice (row 1). Laser densitometer analysis of β-sminor in α-thalassemic mice showed the increase to be 1.3x that found in normal mice. There is also a slight increase in β-s2major RNA in α-thalassemic mice. In two experiments an average increase of 8 percent in β-minor RNA in α-thalassemic mice was found.

Figure 4.3. Autoradiogram of reticulocyte RNA hybridized to the α-1 probe.

RNA obtained from reticulocytes of mice of 7 different hemoglobin genotypes (as indicated to the right of each row) was hybridized with the α-1 probe. The first dot contained 5 μg of RNA and its serial dilutions (1/2) were applied to successive dots.



1 $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{d}/\underline{Hbb}^{d}$

2 $\underline{Hba}^{b2(th)}/\underline{Hba}^{b};\underline{Hbb}^{d}/\underline{Hbb}^{d}$

3 $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$

4 $\underline{Hba}^{b2(th)}/\underline{Hba}^{b};\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$

5 $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{s}/\underline{Hbb}^{s}$

6 $\underline{Hba}^{b2(th)}/\underline{Hba}^{b};\underline{Hbb}^{s}/\underline{Hbb}^{s}$

7 $\underline{Hba}^{b}/\underline{Hba}^{b};\underline{Hbb}^{d3(th)}/\underline{Hbb}^{d3(th)}$

Figure 4.3

Figure 4.4. Autoradiogram of reticulocyte RNA hybridized to the β-minor specific probe.

RNA obtained from reticulocytes of mice of 7 different hemoglobin genotypes (as indicated to the right of each row) was hybridized with the β-minor specific probe. The first dot contained 5 μg of RNA and its serial dilutions (1/2) were applied to successive dots.

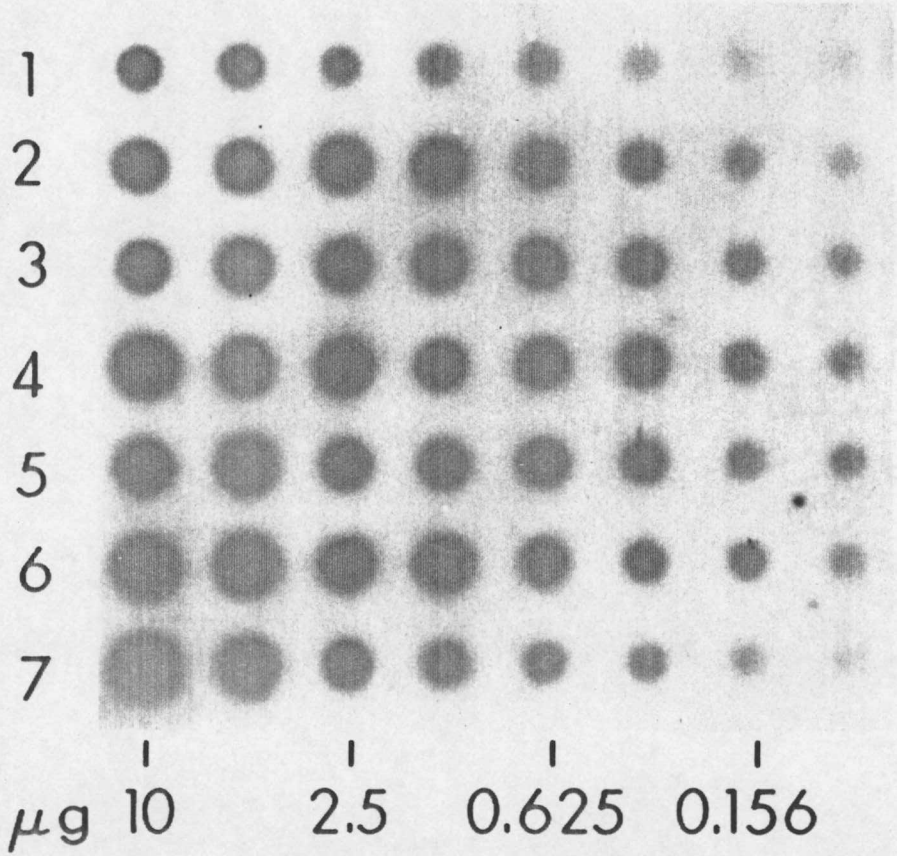

1
2
3
4
5
6
7
μg 10
2.5
0.625
0.156
Hba^b/Hba^b;Hbb^d/Hbb^d
Hba^b2(th)/Hba^b;Hbb^d/Hbb^d
Hba^b/Hba^b;Hbb^s/Hbb^s
Hba^b2(th)/Hba^b;Hbb^s/Hbb^s
Hba^b/Hba^b;Hbb^d3(th)/Hbb^d3(th)
Hba^b2(th)/Hba^b;Hbb^s2/Hbb^s2
Hba^b/Hba^b;Hbb^s2/Hbb^s2

Figure 4.4

Discussion and Conclusions

Mice of the $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ genotype code for two electrophoretically distinguishable adult hemoglobins called β-sminor and β-s2major. It was demonstrated in Chapter II that the relative level of β-sminor hemoglobin in circulation decreases as the level of β-s2major hemoglobin increases between 12.5 days of gestation and 6 days after birth, when the adult level of 30 percent β-sminor and 70 percent β-s2major hemoglobin expression is reached. The intensities of hybridization of the β-minor specific probe with RNAs isolated from natural reticulocytes of fetuses at 14.5 and 17.5 days of gestation and from induced reticulocytes of adult mice were similar (Figure 4.1A on page 61). This demonstrates that the $\beta 2^{sminor}$ gene already has reached a high level of transcription by 14.5 days of gestation. Analyses at earlier stages in development were not attempted because of the small quantities of blood available and the preponderance of nucleated yolk-sac-derived erythrocytes.

In contrast, the level of β-s2major RNA in natural circulating reticulocytes of the fetus during gestation is less than that in induced reticulocytes of adult mice, but the level increases during the later period of gestation (Figure 4.1B). At 14.5 and 17.5 days of gestation, the levels of β-s2major RNA in fetal circulating reticulocytes are 23 and 66 percent, respectively, of that found in the induced reticulocytes of adult mice (Figure 4.2). Thus, the quantity of β-s2major RNA in reticulocytes of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice

increases during gestation and neonatal life, as does the quantity of the β-s2major hemoglobin in erythrocytes.

It has been previously shown that the ratios of β-sminor and β-s2major hemoglobins in circulation are 50/50 in the 14.5 day fetus, 37/63 in the 17.5 day fetus, and 29/71 in the adult (Wawrzyniak and Popp, 1985). Since the relative quantities of α- and β-globins synthesized in reticulocytes agree well with the quantities of their respective RNAs (Martinell et al., 1981), the RNA ratio for the adult of 29 β-sminor/71 β-s2major was assigned in order to calculate the relative quantities of these RNAs in circulating reticulocytes of the fetus. Ratios for β-sminor/β-s2major RNAs were calculated to be 62/38 and 38/62 in circulating reticulocytes of 14.5 and 17.5 day fetuses, compared with the 50/50 and 37/63 ratios of β-sminor/ β-s2major hemoglobins cited above. Thus, changes in the relative quantities of the β-sminor and β-s2major specific RNAs and hemoglobin parallel one another during gestation.

The differential expression of the two adult β-globin genes during gestation might be explained if the $\beta 2^{sminor}$ gene is expressed either at a higher level or at an earlier time in development than the $\beta 1^{s2major}$ gene, or both. The results indicate that the $\beta 2^{sminor}$ gene is transcribed at a higher level than the $\beta 1^{s2major}$ gene at 14.5 and 17.5 days of gestation (Figure 4.2 on page 64). Progressively earlier stages of gestation will need to be studied to determine whether the $\beta 2^{sminor}$ gene is transcribed earlier than the $\beta 1^{s2major}$ gene. It seems, however, that the level of transcription of the

$\beta 1^{s2major}$ gene continues to increase after the $\beta 2^{sminor}$ gene has reached a constant level of expression. The results suggest that an increase in $\beta 1^{s2major}$ gene transcription, not a decrease in $\beta 2^{sminor}$ gene transcription, coincides with the increase in β-s2major hemoglobin relative to β-sminor hemoglobin during development. A similar shift in β-dminor and β-dmajor RNA levels probably accompanies the switch from substantial β-dminor to primarily β-dmajor hemoglobin synthesis during the later stages of development (Whitney, 1977; Alter and Goff, 1980).

The higher level of β-sminor versus β-s2major RNA in mouse reticulocytes during gestation, relative to the adult, may correspond to the cell types present at those times. At 14.5 days of gestation, the red cell population contains some primitive nucleated erythrocytes derived from the yolk sac, as well as erythrocytes derived from the fetal liver. The yolk sac-derived red cells produce primarily embryonic hemoglobin and a small amount ($\sim$ 10 percent) of adult hemoglobin (Chui et al., 1979), but the red cells from fetal liver produce only adult hemoglobin (Wong et al., 1983). The β-minor is considered to be an adult hemoglobin in the mouse, although its developmental profile resembles that of the fetal hemoglobin in other mammals. Thus, differences in the levels of the specific kinds of β-globin RNAs observed in the experiments reported here could be a reflection of a change in one or both erythroid populations occurring at this time (refer to Chapter V). At 14.5 days of gestation nearly equal amounts of embryonic and adult hemoglobins are present. At this time,

embryonic hemoglobin synthesis in the nucleated cells has nearly ceased; however, adult hemoglobin synthesis in the fetal liver-derived reticulocytes is very active (Fantoni et al., 1968). Thus, the majority of the RNA isolated at 14.5 days of gestation comes primarily from hepatic reticulocytes. This would be true also at 17.5 days of gestation, when very few nucleated erythrocytes remain and erythropoiesis in the spleen and bone marrow is just beginning (Russell, 1979). The data suggest that more $\beta 2^{sminor}$ than $\beta 1^{s2major}$ globin gene transcription occurs in earlier erythroid progenitors. An analogous situation occurs in humans where HbF synthesis decreases during the third trimester and gradually diminishes after birth (Bard, 1975).

A low level of β-globin RNA was found in reticulocytes at birth (Figures 4.1 and 4.2, pages 61 and 64). Fantoni et al. (1968) found that between 11 and 15 days of gestation RNA synthesis decreased 5-fold in erythroid cells derived from the yolk sac and fetal liver. Hemoglobin synthesis continues in these erythroid cells in the absence of new RNA synthesis due to the relatively long half-life of the RNA. In general, the fetal period leading up to term in mammals is very active in erythrocyte production (Russell et al., 1968); production is approximately 5-fold that found in the adult. Immediately after birth, however, erythropoiesis is markedly decreased in humans (Weatherall and Clegg, 1981) and mice (Russell and McFarland, 1966). Both the decreased globin RNA synthesis during the later periof of gestation and the overall decrease in erythropoiesis at birth might

account for the low level of globin RNA naturally present in circulating erythrocytes at birth. Globin RNA levels in non-induced erythroid cells of adult mice are also very low, so the adult RNA samples used in this study were isolated from reticulocytes induced by repeated bleeding. After 5 bleedings the reticulocyte count rises to 40-50 percent, but the ratio of β-sminor : β-s2major polypeptides remains at 30 : 70 (Wawrzyniak et al., 1985). The data reported represent the amount of globin RNAs naturally present during gestation and at birth relative to the amount of globin RNAs present in induced reticulocytes of adults.

It has been demonstrated previously that the β-minor hemoglobin is elevated when the hematopoietic system is stressed by phlebotomy (Alter et al., 1982; Wawrzyniak et al., 1985), α-thalassemia (Popp et al., 1976; Wawrzyniak and Popp, 1985), or congenital macrocytic anemia (Whitney, 1981; Chapter III). Results in the present study demonstrate that the increase in β-minor globin found in association with α-thalassemia is accompanied by a relative increase in the ratio of β-sminor:β-s2major RNA. In heterozygous α-thalassemic mice, the quantity of α-globin RNA (Figure 4.3) is less due to the deletion of one of two α-globin complexes on chromosome 11 (Whitney et al., 1981). On a weight basis, total RNA isolated from reticulocytes of mice heterozygous for α-thalassemia had 1.3 times more β-sminor RNA and 1.2 times more β-s2major RNA than did RNA from non-thalassemic control mice (Figure 4.4). An increase in the relative amount of β-sminor RNA of approximately 8 percent, in reticulocytes of α-thalasemic mice may

explain the 9 percent elevation of β-sminor hemoglobin observed in these mice. These data also indicate that the quantity of β-globin versus total RNA in reticulocytes of normal and α-thalassemic mice is not a constant value. Actually, the elevated expression of β-minor hemoglobin in heterozygous α-thalassemic mice is associated with an increased expression of both β-globin genes (where expression of the $\beta 2^{sminor}$ gene is enhanced to a greater extent than that of the $\beta 1^{s2major}$ gene), rather than with a simple enhancement of only the $\beta 2^{sminor}$ gene. The natural level of expression of the $\beta 2^{dminor}$ gene in $\underline{Hbb}^{d}/\underline{Hbb}^{d}$ mice is also not maximal, because its expression is increased in homozygous β-thalassemic mice (Popp et al., 1985; Anderson et al., 1985; and Figure 4.4 rows 1 and 5).

The globin RNA levels in reticulocytes demonstrate that the $\beta 1^{major}$ and $\beta 2^{minor}$ globin genes are under independent transcriptional control. Hemoglobin synthesis in mouse erythroleukemia cell lineages has also been associated with transcriptional regulation (Groudine et al., 1981; Landes et al., 1982; Villepontaue et al., 1982). The nucleotide sequence(s) involved in controlling the expression of the $\beta 1^{s2major}$ and $\beta 2^{sminor}$ genes almost certainly lie outside their coding sequences, since the DNA sequences of their exons are highly homologous (Erhart et al., 1985). Further studies on the mechanisms that control the differential expression of these homologous β-globin genes will be pertinent to the understanding of natural hemoglobin switching and will aid attempts to induce specific hemoglobin switching in order to ameliorate hemoglobinopathies.

CHAPTER V

ADULT β-GLOBIN EXPRESSION IN SEPARATED ERYTHROCYTE POPULATIONS DURING GESTATION

Summary

The two adult β-globin genes ($\beta 1^{s2major}$ and $\beta 2^{sminor}$) of the $\underline{Hbb}^{s2}$ haplotype are differentially expressed during development. The ratio of expression of the β-sminor to β-s2major hemoglobin in the circulating erythrocytes is over 2-fold higher at 11.5 days of gestation than at 6 days after birth (Chapter II). Analysis of the hemoglobin in the two populations of erythrocytes present from 12.5 through 17.5 days of gestation showed that the larger cells yolk sac-derived) have relatively greater amounts of β-sminor hemoglobin than do the smaller cells (fetal liver-, spleen- and bone marrow-derived). The change in differential expression of the β-sminor and β-s2major hemoglobins in the two subpopulations of erythrocytes is most pronounced from 12.5 through 14.5 days of gestation. After 13.5 days of gestation, the level of the β-sminor component in non-nucleated cells plateaus at 39 percent, which is the level expressed in erythrocytes of adult mice.

Introduction

Erythrocytes that are formed during the fetal stages of mouse development differ in four major characteristics. (1) Tissue of

origin. Erythropoiesis occurs in the yolk sac, fetal liver, spleen, and bone marrow. (2) Cell size. The cells produced during earlier periods of gestation are larger than those produced during later periods. (3) Nucleation. Mature erythrocytes derived from the yolk sac are usually nucleated, whereas the others are non-nucleated. (4) Type of hemoglobin synthesized. Red cells arising from the yolk sac contain primarily embryonic hemoglobins (Fantoni et al., 1967), although a small quantity of adult hemoglobin is produced (Chui et al., 1979). Red cells that develop in the fetal liver, spleen, or bone marrow synthesize only adult hemoglobins (Wong et al., 1983). The murine embryonic hemoglobins, designated EI, EII and EIII (Fantoni et al., 1967), do not contain adult-type β-globins and are electrophoretically separable from adult hemoglobins, which do. In the homozygous $\underline{Hbb}^{s2}$ mice analyzed in this study, adult hemoglobins contain β-s2major or β-sminor globin.

The nucleated yolk sac-derived erythrocytes are 12.6 ± 1.2 μm in diameter (Steiner and Vogel, 1973) and are found in the circulation from 9.5 through 17.5 days of gestation. The non-nucleated fetal liver-derived red cells are smaller, 8.7 ± 0.7 μm in diameter (Steiner and Vogel, 1973), and appear in circulation beginning at 12 days of gestation. The fetal liver is a major site of red cell production until near birth (Rifkind et al., 1969). The non-nucleated spleen- and bone marrow-derived red cells are even smaller, 6.5 ± 0.3 μm in diameter (Steiner and Vogel, 1973) and are found in circulation from 17.5 days of gestation throughout the life span of the mouse.

It has been demonstrated that the nucleated yolk sac-derived erythrocytes synthesize small quantities of adult hemoglobin (Chui et al., 1979), but the type of adult hemoglobin was not determined. This study analyzes the levels of expression of the two adult β-globin genes in the nucleated and non-nucleated red cell populations at successive days during development.

Procedures

Mice

The stock of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice described in Chapter II was used. Mice homozygous for the mutant $\underline{Hbb}^{s2}$ haplotype have two adult β-globin polypeptides, β-s2major and β-sminor. All five, three embryonic and two adult, hemoglobins can be separated by cellulose acetate electrophoresis (Wawrzyniak and Popp, 1985). Homozygous fetuses were obtained by matings of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ x $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$.

The age of the fetus was timed by designating the presence of a copulation plug in the morning as day .5 of gestation. The females were on natural cycles, and removed from mating after a copulation plug was detected. Erythrocytes from several fetuses were pooled for quantitative analysis on each specified day of gestation. For 13.5 through 16.5 days of gestation red cells from a minimum of 3 litters were individually pooled and analyzed, and at 12.5 and 17.5 days of gestation red cells from 2 litters were individually pooled and analyzed. The average litter size was 7.

Collection of Blood

Pregnant females were sacrificed, and the procedure used to obtain fetal red cells was the same as described in Chapter II Procedures on page 11, with the exception that cold Ca^{++} and Mg^{++} free phosphate buffered saline (PBS) was substituted for 0.85 percent saline.

Blood from adult mice was obtained from the retro-orbital sinus, suspended in PBS and centrifuged at 600 xg for 10 minutes at 5°C to concentrate the red cells.

Elutriation

The process of elutriation can be used to separate heterogeneous populations of cells by using liquid flow versus centrifugal force (Lindahl, 1948; Thieulant and Duval, 1985). A Beckman model J2-21 centrifuge was used with a JE-6B elutriation rotor. While the rotor is spinning, red cells suspended in PBS are pumped into the bottom of the elutriation chamber at a constant flow rate of PBS through the chamber. When centrifugal and counter flow forces are balanced, cells larger than a specific size are retained in the chamber by centrifugal force while smaller cells are washed out by counter current flow of the PBS. As the rotor speed is decreased, red cells begin to exit the chamber according to size, with the largest cells exiting the chamber last.

The elutriator rotor was initially spun at 2,000 rpm (560 xg) at 10°C, with PBS flowing through the system at a constant 3 ml/minute.

The erythrocytes, suspended in 2.0 ml of PBS, were introduced into the inlet line via a three-way valve and pumped into the elutriator. A series of thirteen fractions were collected from the outlet line at the pump flow rates and elutriator rotor speeds given below. The flow rate was maintained at 3 ml/minute for fractions 1 through 10; the flow rate was increased to 13 ml/minute for fractions 11 through 13. The rotor speed for fractions 1 and 2 was 2,000 rpm; fraction 3, 1,750 rpm; fraction 4, 1,500 rpm; fraction 5, 1,250 rpm; fraction 6, 1,000 rpm; fractions 7 and 8, 750 rpm; and fraction 9 through 13, 500 rpm. The volume of each fraction was 30 ml. After the initial elutriation separation, fractions 9 through 13 were pooled. The erythrocytes were concentrated by centrifugation at 600 xg and run through a second elutriation cycle to enrich the nucleated red cell fraction. After the second cycle, the individual fractions were centrifuged at 600 xg for 10 minutes at 10°C to pellet the red blood cells. The fractions of interest were those which contained erythrocytes with the greatest quantity of embryonic hemoglobin (usually fraction 10 or 11 from the second elutriation cycle), versus fractions which had the least quantity of embryonic hemoglobin (usually fraction 5 or 6 from the first elutriation cycle).

Electrophoresis

For each fraction, a 2 µl sample of the erythrocyte pellet was lysed in 5 µl of water, and the hemoglobin was modified by adding 2 µl of 66.7 mM cystamine (Whitney, 1978). Eight samples were loaded

with an applicator on buffer-moistened Helena Lab Titan III cellulose acetate plates and electrophoresed at 300V for 35 minutes. An electrophoresis buffer of 0.15M Tris : 0.0833M boric acid : 0.0017M ethylenediaminetetracetic acid (EDTA), pH 8.6 was used to separate the various hemoglobin tetramers present.

Scanning Densitometry

Immediately following electrophoresis, the cellulose acetate plate was placed in Ponceau S stain for 6 minutes, rinsed repeatedly in 5 percent glacial acetic acid to clear the background of stain and they were air dried overnight. This procedure fixed and stained the hemoglobin so that it did not diffuse, and allowed quantitation of the separated hemoglobins to be done on the following day. The hemoglobin in each band was quantitated using a Flur-Vis transmission densitometer (Helena Lab Autoscan) equipped with an electronic integrator. There are five hemoglobin bands present in samples of fetal mouse blood, three are embryonic hemoglobins and two are adult hemoglobins. The quantities of the embryonic hemoglobins (EI + EII + EIII)/adult hemoglobins (β-s2major + β-sminor) were calculated to obtain the embryonic : adult ratios. Quantities of the two adult hemoglobins were compared to obtain the β-s2major : β-sminor ratios.

Blood Smears

Blood smears were made from individual elutriation fractions and from selected samples prior to elutriation, and were allowed to air dry. They were stained with Wright's stain and viewed under a

microscope, where a minimum of 500 red blood cells were scored as nucleated versus non-nucleated for individual elutriation fractions. The number of nucleated red blood cells/total red blood cells counted was calculated to obtain the percent nucleated red blood cells. For some fractions, blood smears were not made due to the paucity of cells. The priority for analysis of a sample was electrophoresis.

Results

The degree of separation of the two populations of red cells is presented in Table 5.1. At 12.5 days of gestation all fractions contained primarily nucleated cells. Prior to elutriation, 93.1 percent of the erythrocytes were nucleated. The elutriation concentrated the nucleated cells in the later fractions to a mean of 99.6 percent. The purity is based on microscopic enumeration of nucleated cells. At 13.5 days of gestation, when approximately 91.7 percent of the natural population of cells are nucleated, the elutriation yielded a fraction with 98.8 percent nucleated cells. At 14.5 days of gestation, when only 25.1 percent of the circulating cells are nucleated, an elutriation fraction was collected with 94.1 percent nucleated cells. At 15.5 and 16.5 days of gestation, two cycles of elutriation gave fractions with 66.2 and 10.6 percent nucleated cells, respectively. Some of the non-nucleated cells in these fractions were very large relative to other cells present, suggesting that some yolk-sac derived erythrocytes have become non-nucleated at this time.

TABLE 5.1

LARGE NUCLEATED CELLS IN FETAL BLOOD SAMPLES
BEFORE AND AFTER ELUTRIATION

Day of Gestation	Percent Large Nucleated Cells	
	In Fetal Circulation (Before Elutriation)	In Elutriation Fraction with the Largest Amount of Embryonic Hemoglobin[a]
12.5	93.1[b]	99.6[b] ± 0.3[c]
13.5	91.7	98.8 ± 0.7
14.5	25.1	94.1 ± 1.4
15.5	14.2	62.2 ± 2.9
16.5	9.7	10.6 ± 4.9
17.5	3.7	ND[d]

[a]The largest amount of embryonic hemoglobin was usually found in fractions 10 or 11.

[b]Calculated from blood smears stained with Wright's stain.

[c]Standard error of the mean.

[d]Not determined.

The relative quantities of embryonic hemoglobin in the large (nucleated) and small (non-nucleated) erythrocytes, after separation by elutriation, from fetuses at 12.5 through 17.5 days of gestation are plotted in Figure 5.1. The relative quantity of β-sminor in adult hemoglobin of erythrocytes in the fractions with the least and greatest quantity of embryonic hemoglobin (Table 5.2) are presented in Figure 5.2. From the data in Table 5.1 and Figure 5.1, it is clear that the large nucleated erythrocytes from 12.5 and 13.5 day fetuses, separated by elutriation to greater than 98 percent purity, contain more than 15 percent adult hemoglobin. At 14.5 days of gestation, fractions obtained after elutriation had an average of 94.1 percent nucleated erythrocytes and contained 25.7 percent adult hemoglobin. After 14.5 days of gestation two cycles of elutriation under the conditions used did not purify the nucleated erythrocytes sufficiently for measurement of the quantity of adult hemoglobin in the yolk sac-derived erythrocytes.

In the large nucleated cells (which contain primarily embryonic hemoglobin) from 12.5 through 14.5 days of gestation, the relative quantities of the two adult hemoglobins changed from approximately 12 percent β-s2major and 88 percent β-sminor to 39 percent β-s2major and 61 percent β-sminor. In contrast, in the small non-nucleated erythrocytes (which contain no embryonic hemoglobin), the relative quantities of the adult hemoglobins remain constant at about 60 percent β-s2major and 40 percent β-sminor between 14.5 and 17.5 days of gestation. This is also the level in adult mice.

Figure 5.1. Percent of embryonic hemoglobin in erythrocytes of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice for selected elutriation fractions.

Percentages were calculated on the bases that 100 percent hemoglobin = embryonic (EI, EII and EIII) + adult (β-s2major and β-sminor). For each day of gestation, and for the adult, data are plotted for the elutriation fraction with erythrocytes containing the largest quantity of embryonic hemoglobin (●———●), versus the elutriation fraction with erythrocytes containing the smallest quantity of embryonic hemoglobin (●- - -●). Error bars are the standard error of the mean.

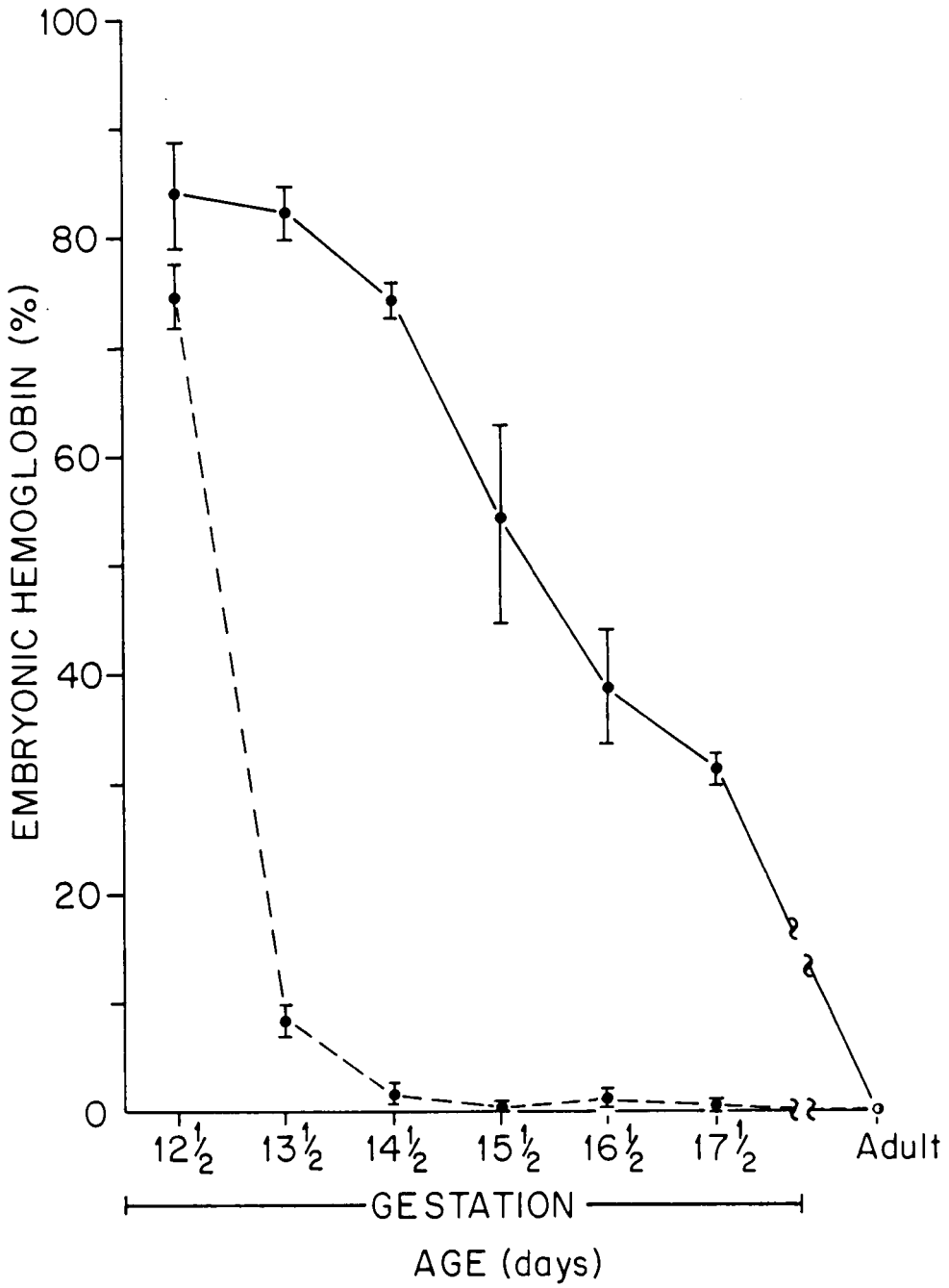

100
80
60
40
20
0
EMBRYONIC HEMOGLOBIN (%)
12½
13½
14½
15½
16½
17½
Adult
GESTATION
AGE (days)

Figure 5.1

TABLE 5.2

CHARACTERISTICS OF ERYTHROCYTES AND HEMOGLOBINS IN SELECTED ELUTRIATION FRACTIONS[a]

Day of Gestation	Cell Size in Fraction	Total Hemoglobin			Adult Hemoglobin		
		Percent Embryonic	Percent Adult	± SEM	Percent β-s2major	Percent β-sminor	± SEM
12.5	L[b]	84.1	15.9	5.5	11.9	88.1	6.1
	L>S	74.9	25.1	2.9	26.2	73.8	1.1
13.5	L	82.8	17.2	2.6	18.3	81.7	2.1
	S>L	8.9	91.1	1.2	57.4	42.6	1.1
14.5	L	74.3	25.7	1.6	38.7	61.3	4.8
	S	1.6	98.4	0.9	61.6	38.4	2.2
15.5	L>S	54.3	45.7	9.2	45.0	55.0	6.7
	S	0.5	99.5	0.3	59.5	40.5	0.9
16.5	L=S	39.0	61.0	5.3	54.4	45.6	2.4
	S	1.2	98.8	0.8	61.0	39.0	0.8
17.5	S>L	31.7	68.3	1.1	56.5	43.5	1.4
	S	0.5	99.5	0.5	60.7	39.3	0.4
Adult	S	0.0	100.0	0.0	62.6	37.4	1.8

[a]At each day of gestation, data are tabulated for the elutriation fractions containing the largest quantities of embryonic and adult hemoglobins, respectively.

[b]L designates large cells (> 10 μm in diameter) and S designates small cells (< 10 μm in diameter).

Figure 5.2. Percent of adult hemoglobin as β-sminor in erythrocytes of $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice for selected elutriation fractions.

Percentages were calculated on the bases that 100 percent hemoglobin = β-sminor + β-s2major. For each day of gestation, and for the adult, data are plotted for the elutriation fraction containing erythrocytes with the largest quantity of embryonic hemoglobin (●———●), versus the elutriation fraction containing erythrocytes with the smallest quantity of embryonic hemoglobin (● - - - ●). Error bars are the standard error of the mean.

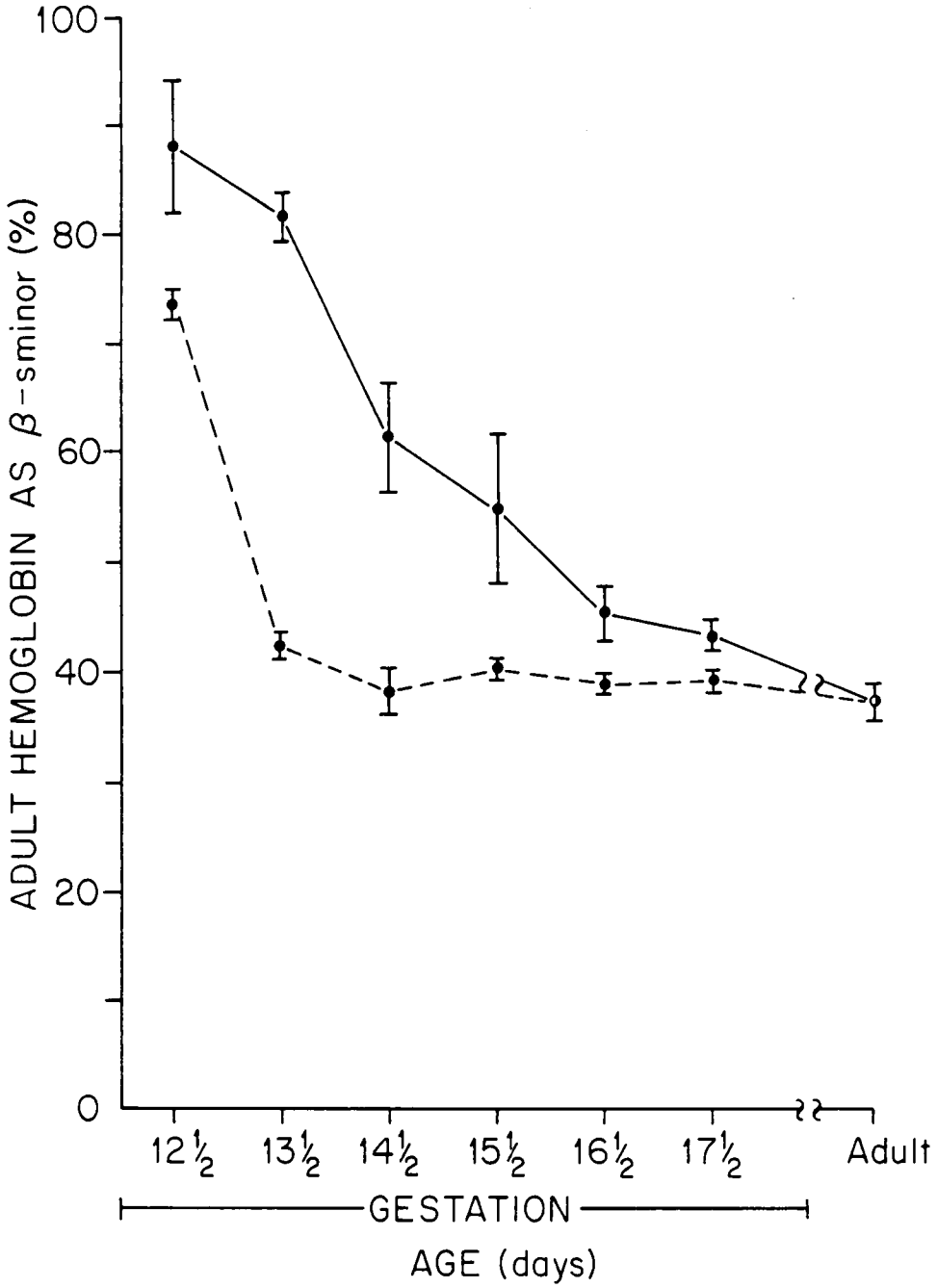

100
80
60
40
20
0
ADULT HEMOGLOBIN AS β-sminor (%)
12½
13½
14½
15½
16½
17½
Adult
GESTATION
AGE (days)

Figure 5.2

Discussion and Conclusions

It has been previously demonstrated that by day 13 of gestation, adult hemoglobin accounts for approximately 10 percent of the total hemoglobin content in yolk sac-derived nucleated erythrocytes (Chui et al., 1979), and that, in the mixed population of erythrocytes taken from fetuses at 13.5 days of gestation, 62 percent of the adult hemoglobin is β-sminor (Figure 2.4 on page 23). When the two erythrocyte populations from a 13.5 day fetus are separated via elutriation, the fractions with 98.8 percent of the larger, nucleated cells contained 81.7 percent β-sminor hemoglobin, and the fractions enriched for the smaller, non-nucleated cells contained 42.6 percent β-sminor hemoglobin (Figure 5.2). A similar difference occurred at 14.5 days of gestation, when the mixed population of erythrocytes contained 44 percent β-sminor hemoglobin (Chapter II), but separation of the subpopulations of red cells revealed that the large (yolk sac-derived) erythrocytes contained 61.3 percent β-sminor hemoglobin and the small (fetal liver-derived) red cells contained only 38.4 percent β-sminor hemoglobin. Consistently, the fractions with the larger nucleated erythrocytes had more embryonic and more β-sminor hemoglobin, and the fractions with the smaller, non-nucleated erythrocytes (and less than 2 percent embryonic hemoglobin) had more β-s2major hemoglobin (Figure 5.2).

The differential expression of the two adult β-globin genes in the subpopulations of red cells reflects the cells' origin. The two kinds of erythrocytes differ in several respects. One basic

difference is that the yolk sac-derived erythrocytes have a higher hemoglobin content than the fetal liver-derived red cells (Steiner and Vogel, 1973). Since the rate of hemoglobin synthesis is similar in both populations of cells, Steiner (1973) proposed that the difference in the final concentration of hemoglobin is due to different cell generation times. Russell and Bernstein (1966) reported that erythropoiesis in the fetal liver produces a population of cells that divide asynchronous whereas yolk sac-derived erythrocytes differentiate in a relatively synchrous fashion.

There may be a programmed expression of unique regulatory genes in progenitor cells (Stamatoyannopoulos et al., 1981), and the two erythrons may differ in perturbation of chromatin structure (Groudine et al., 1981) or methylation of DNA (DeSimone et al., 1982). In a mixed population of yolk sac- and fetal liver-derived erythrocytes, data in Chapter IV demonstrated that the $\beta 2^{sminor}$ gene reaches a high level of transcription by 14.5 days of gestation; in contrast, the level of β-s2major RNA continues to increase during the later period of gestation (Wawrzyniak and Popp, 1986). In yolk sac-derived erythrocytes the embryonic globin genes are expressed approximately 3 to 4 days before the adult globin genes, but expression of the adult globin genes begins at the same time in both the yolk sac- and fetal liver-derived erythrocytes. Thus, the intrinsic regulation of the adult β-globin genes during the initiation of hemoglobin synthesis is very different for the two red cell populations.

At 12.5 through 14.5 days of gestation, the ratio of β-sminor to β-s2major hemoglobin is higher in the nucleated erythrocytes obtained from the younger fetuses, and during this period the ratio changes in favor of larger quantities of β-s2major hemoglobin (Figure 5.2). In contrast, throughout the period of 13.5 to 17.5 days of gestation, the smaller, non-nucleated erythrocytes contained approximately 40 percent β-sminor hemoglobin, which was similar to the 37 percent β-sminor hemoglobin found in erythrocytes of normal adult mice analyzed by the procedure used for this study. In short, the factors that regulate the relative amounts of the two types of adult hemoglobins synthesized in these fetal liver-derived red cells persist in the bone marrow-derived red cells of adult mice.

The ratio of 37 percent β-sminor : 63 percent β-s2major hemoglobin reported in Figure 5.2 for adult mice analyzed in this study appears different from the 30 : 70 ratio previously reported in Chapter II. This discrepancy is due to the scanning densitometric analysis of the Ponceau S stained hemoglobin samples in the present study versus the analysis of unstained hemoglobin samples in the earlier study. Ponceau S staining was used for this study in order to quantitate the small amounts of hemoglobin in erythrocytes of some of the fractions obtained after two cycles of elutriation.

The Wright's staining of various fractions, followed by quantitation of nucleated versus non-nucleated erythrocytes, was used to determine the degree of separation that occurred during elutriation. Elutriation was more efficient at purifying the large nucleated

erythrocytes during earlier periods of gestation; i.e., 12.5 through 14.5 days of gestation (Table 5.1). This is due in part to the initial preponderance of large red cells, and in part to the relatively larger size of the newly-formed nucleated cells. Although the purity of the nucleated cell fractions was lower for later periods of gestation, an enrichment was still achieved. In the later periods of gestation, some of the non-nucleated cells were as large as the nucleated cells, which suggests that either they are yolk sac-derived erythrocytes that have lost their nucleus (Bethlenfalvay and Block, 1970), or that some fetal liver-derived red cells are larger than previously reported. Steiner and Vogel (1973) reported that on day 13, 0.7 percent of the total yolk sac cells are non-nucleated, and on days 14 and 15, 7.6 and 34.6 percent, respectively, of the yolk sac-derived erythrocytes are non-nucleated. Therefore, these large cells are probably yolk sac-derived.

The $\beta 1^{major}$ and $\beta 2^{minor}$ genes of the $\underline{Hbb}^{s2}$, $\underline{Hbb}^{s}$, $\underline{Hbb}^{d}$ and $\underline{Hbb}^{p}$ haplotypes are functionally homologous (Alter and Goff, 1980; Whitney, 1977; Wawrzyniak et al., 1985; Wawrzyniak and Popp, 1985), and structural homology of these genes at the level of the DNA has been reported (Erhart et al., 1985). Therefore, this study suggests that, also for hemoglobin haplotypes other than the $\underline{Hbb}^{s2}$ studied here, the $\beta 2^{minor}$ globin gene may be expressed at a higher level in yolk sac-derived erythrocytes of 12.5 day than of 14.5 day fetuses. Comparative studies are difficult because the β-dminor hemoglobin in fetuses of the $\underline{Hbb}^{d}$ haplotype electrophoreses to the same position as

EII embryonic hemoglobin, and in mice of the $\underline{Hbb}^{s}$ haplotype the β-smajor and β-sminor globins have identical amino acid sequences (Weaver et al., 1981).

Erythropoiesis in the mouse is a dynamic process and the erythrocytes that differentiate in various erythropoietic tissues differ in terms of size, hemoglobin concentration, nucleation and the types of globin produced. This study demonstrates that the same $\beta 2^{sminor}$ and $\beta 1^{s2major}$ adult globin genes are expressed differently in the embryonic and adult erythrons in $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice.

CHAPTER VI

SUMMARY AND IMPLICATIONS

Erythroid differentiation in mice is a coordinated series of cellular and molecular events involving changes in red cell populations and hemoglobin switching; both of these reflect regulatory processes occurring at the level of the DNA. The mouse, like most vertebrates, has four primary sites of erythropoiesis, which produce erythrocytes that differ in size, hemoglobin concentration, nucleation and the type of hemoglobin synthesized. The first functional erythron is located in the yolk sac blood islands. Between 8 and 14 days of gestation the yolk sac blood islands give rise to primitive nucleated erythrocytes that are large, 12.6 μm in diameter (Steiner and Vogel, 1973), and synthesize mostly embryonic hemoglobins (Fantoni et al., 1967). A small quantity of adult hemoglobin is produced in these red cells between 12 and 14 days of gestation (Chui et al., 1979). These yolk sac-derived red cells lose their cytoplasmic ribosomes by 15 days of gestation and thus their ability to synthesize protein (Kovach et al., 1967). These primitive nucleated red cells are replaced by a different population of red cells that differentiate in the second site of erythropoiesis, the fetal liver. Fetal liver-derived erythrocytes are smaller, 8.7 μm in diameter (Steiner and Vogel, 1973), non-nucleated and synthesize only adult hemoglobin (Wong et al., 1983). The fetal liver-derived red cells begin to appear in the circulation by 12 days of gestation, and

the fetal liver persists as a major site of red cell production until near birth (Rifkind et al., 1969). Erythropoiesis in the fetal liver produces a population of cells that divide asynchromous whereas yolk sac-derived erythrocytes differentiate in a relatively synchrous fashion (Russell and Bernstein, 1966). The bone marrow and spleen are the major sites of erythropoiesis in juvenile and adult mice. In these tissues non-nucleated red cells, 6.5 μm in diameter, are produced beginning at 17 days of gestation. Like the fetal liver-derived red cells, bone marrow and splenic erythrocytes synthesize only adult hemoglobins.

Mice have two adult β-globin genes; in mice homozygous for the Hbb^{s2} haplotype, they are called β1^{s2major} and β2^{sminor}. Their β-globin products are easily separated by electrophoresis, which permits the expression of the two genes to be determined throughout fetal and postnatal development. Analysis of hemoglobin in the circulating red cells from 12.5 days of gestation through post-weaning demonstrated that the two genes are independently regulated and differentially expressed during development. At 11.5 days of gestation, β-s2major hemoglobin comprises under 20 percent and β-sminor over 80 percent of the adult β-globin in circulation. The relative level of β-sminor hemoglobin decreases through fetal development; at birth β-sminor hemoglobin represents 34 percent of the β-globin present in circulating red cells. By 6 days after birth the ratio of the two hemoglobins expressed is like that in adult mice: 70 percent β-s2major and 30 percent β-sminor.

The step at which regulation for this differential expression was investigated by analyzing the quantity of the β-sminor and β-s2major RNAs in circulating reticulocytes during development. The quantity of β-sminor RNA in circulating reticulocytes at 14.5 and 17.5 days of gestation is nearly the same as in induced reticulocytes of adult mice. In contrast, the quantity of β-s2major RNA in circulating reticulocytes at 14.5 days of gestation is 23 percent and at 17.5 days of gestation is 66 percent of the amount found in induced reticulocytes of adult mice. The results of the RNA analysis correlates with the observation that the $\beta 2^{sminor}$ gene approaches its normal adult level of expression by 14.5 days of gestation, whereas $\beta 1^{s2major}$ gene expression increases between 14.5 days of gestation and 6 days postnatally. This indicates that transcription is a primary control step for changing the expression of the $\beta 2^{sminor}$ and $\beta 1^{s2major}$ globin genes during development.

To assess further the basis for the differential expression of the two adult β-globin genes, hemoglobins were analyzed in the subpopulations of erythrocytes that were separated by centrifugal elutriation. From 12.5 through 17.5 days of gestation, relatively more β-sminor than β-s2major hemoglobin was found in the yolk sac-derived red cells, than in the fetal liver-derived red cells. That the yolk sac-derived red cells produce more β-sminor hemoglobin than do the fetal liver-derived red cells may be attributed to intrinsic or extrinsic differences between these two cell populations. The yolk sac-derived red cells are larger, synthesize more hemoglobin, and are

usually nucleated. In the yolk sac-derived erythrocyte the embryonic globin genes are expressed three to four days before the adult globin genes, although expression of the adult globin genes begins at the same time in the yolk sac-derived and fetal liver-derived erythrocytes. Thus, the two red cell populations differ markedly with respect to the intrinsic regulation of the adult β-globin genes during the initiation of hemoglobin synthesis.

Another feature of the regulation of the two adult β-globin genes is their expression in response to hematological stress, such as thalassemia, phlebotomy, and macrocytic anemia. In mildly anemic α-thalassemic heterozygotes, the level of β-sminor hemoglobin increases 9 percent, but in asymptomatic β-thalassemic heterozygotes, β-sminor is elevated to a lesser extent (5 percent). The greatest increase in β-sminor hemoglobin (12 percent) occurred in doubly heterozygous α-thalassemic, β-thalassemic mice. The maximum increase in β-sminor hemoglobin in response to phlebotomy was from 30 to 36 percent after bleeding every other day for 30 days. A relative increase in β-sminor hemoglobin (30 to 36 percent) was also detected in $\underline{W}/\underline{W}^{V}$ mice that exhibit a macrocytic anemia. Analysis of the β-sminor and β-s2major RNA levels in reticulocytes of normal and thalassemic mice demonstrates that the increase in β-sminor hemoglobin is accompanied by an increase in the level of β-sminor RNA.

That the levels of β-sminor hemoglobin is elevated both during development and during hematological stress in adult mice may be indicative of increased demands on the erythropoietic system under

the two conditions. Because the DNA sequence is similar over 99.6 percent of the region bordered by the translational start and stop codons of the $\beta 1^{s2major}$ and $\beta 2^{sminor}$ genes, but a greater degree of heterogeneity in their 5' and 3' noncoding regions, it has been concluded that the regulatory sequences for the differential expression of $\beta 1^{s2major}$ and $\beta 2^{sminor}$ genes probably lie outside the coding regions (Erhart et al., 1985). The differential expression of the same two adult β-globin genes in two populations of erythrocytes at the same time in development suggests that an additional regulatory mechanism, other than noncoding nucleotide sequence differences, influences the expression of these two genes. A regulatory mechanism based on functional differences between the two polypeptides is highly unlikely because the $\beta 1^{smajor}$ and $\beta 2^{sminor}$ genes of the $\underline{Hbb}^{s}$ haplotype code for two identical polypeptides chains. The organization of nucleosomes along the DNA may have a role in the independent expression and regulation of these two adult β-globin genes. Benezra et al. (1986) have found two structural states for the 5' area of the $\beta 1^{dmajor}$ globin gene in erythroleukemia cells versus non-erythroid cells. In non-erythroid cells (mouse fibroblast) the nucleosomes were present along the $\beta 1^{dmajor}$ gene from position -3000 to +1500, whereas in both non-induced and induced Friend erythroleukemia cells nucleosome phasing was interrupted from position -200 to +500.

This study indicates that the adult β-globin genes of the $\underline{Hbb}^{d}$ haplotype (which code for β-dmajor and β-dminor hemoglobin), of the $\underline{Hbb}^{s2}$ haplotype (which code for β-s2major and β-sminor hemoglobin),

and presumably of the $\underline{Hbb}^{s}$ haplotype (which code for β-smajor and β-sminor hemoglobin) have homologous functions. By analysis of heteroduplexes (Weaver et al., 1981), the $\beta 1^{smajor}$ and $\beta 1^{dmajor}$, and the $\beta 2^{sminor}$ and $\beta 2^{dminor}$, genes have been shown to be alleles. Where comparable times in gestation have been studied for $\underline{Hbb}^{d}/\underline{Hbb}^{d}$ (Whitney, 1977; Alter and Goff, 1982) and $\underline{Hbb}^{s2}/\underline{Hbb}^{s2}$ mice (Wawrzyniak and Popp, 1985), the independent regulation and expression of the $\beta 1^{major}$ and $\beta 2^{minor}$ genes of the two haplotypes appear to be similar. The $\beta 1^{major}$ gene is expressed at low levels during early periods in gestation, and its expression increases during development. In contrast, the $\beta 2^{minor}$ gene has a high level of expression earlier in gestation. In addition, the β-sminor hemoglobin of the $\underline{Hbb}^{s2}$ haplotype and β-dminor hemoglobin of the $\underline{Hbb}^{d}$ haplotype are similarly elevated when the hematopoietic system is stressed by phlebotomy (Alter et al., 1982; Wawrzyniak et al., 1985), thalassemia (Popp, 1976; Wawrzyniak and Popp, 1985) or congenital macrocytic anemia (Whitney, 1981).

The common functional homology among the $\underline{Hbb}^{d}$, $\underline{Hbb}^{p}$, $\underline{Hbb}^{s2}$ and presumably $\underline{Hbb}^{s}$ haplotypes may be controlled by the homology of the adult globin genes at the DNA sequence level. Comparison of the 5' flanking area of the $\beta 1^{dmajor}$ and $\beta 1^{smajor}$ genes demonstrates a 95 percent homology, and the intervening sequences of these genes are very similar (Erhart et al., 1985). The homology between the 5' flanking and first intervening sequence of the $\beta 2^{dminor}$ and $\beta 2^{sminor}$ genes is also very high even though the genes are not homologous in

their second intervening sequences (Erhart et al., 1985). The 13 kilobases of DNA which separate the $\beta 1^{major}$ and $\beta 2^{minor}$ genes are very different in the $\underline{Hbb}^{d}$ and $\underline{Hbb}^{s}$ haplotypes. These differences involve four or five L1Md elements (a family of long interspersed sequences in Mus domesticus), as well as other repeated and single copy DNA sequences (Burton et al., 1985). Although the sequences are different, the overall distance between the $\beta 1^{major}$ and $\beta 2^{minor}$ genes of the $\underline{Hbb}^{d}$ and $\underline{Hbb}^{s}$ haplotypes remains the same, suggesting that there is a natural selection for a specific spatial distance between these genes. This distance may be important for maintaining the structural and functional homology of the 5' versus the 3' genes of the two haplotypes.

The adult β-globin genes were duplicated prior to speciation of rodents. The two haplotypes, $\underline{Hbb}^{s}$ and $\underline{Hbb}^{d}$, demonstrate different ways in which duplicated gene pairs can evolve. The adult genes in the $\underline{Hbb}^{s}$ haplotype are thought to be identical presently due to a recent gene convergence, whereas the $\underline{Hbb}^{d}$ genes have diverged.

Expression of the family of globin genes during ontogeny in mammals is generally 5' embryonic genes prior to the adult genes which are located 3'. The higher expression of the $\beta 2^{sminor}$ gene prior to the $\beta 1^{s2major}$ gene is an observation which does, however, not stand alone. In the chicken β-globin gene cluster, there is a similar instance where an expressed second embryonic gene resides 3' to the adult β-globin gene (Robinson and Ingram, 1982).

The developmental profile of the β-sminor hemoglobin of mice resembles that of a fetal hemoglobin in other mammals. In the human, hemoglobin F (HbF), a fetal hemoglobin, represents 90-95 percent of the total hemoglobin being synthesized from 8-30 weeks of gestation (Bard, 1975). Between 28 and 34 weeks of gestation, a switch begins from predominantly fetal γ-globin (HbF) to predominantly adult β-globin (HbA) synthesis (Bard et al., 1970). A gradual transition lasts for about 22 weeks, and a final level of less than 1 percent HbF is found in normal adults. An increased synthesis and reappearance of HbF is observed in adults with thalassemia and some other hemoglobinopathies (Cooper and Hoagland, 1972). The use of drugs, such as 5-azacytidine (a cytidine analog), in patients with β+ thalassemia (Ley et al., 1982) and sickle cell anemia (Charache et al., 1983) has been shown to increase the level of HbF. The increase in HbF synthesis was shown to be beneficial to patients with hemoglobinopathies, although further studies are necessary to evaluate the efficiency, long term toxicity and mechanism by which 5-azacytidine exerts its effect before the approach can be used for patients with hemoglobin synthesis disorders.

Mice with the $\underline{Hbb}^{s2}$ haplotype described in this study could be used in future experiments. If an increase in a specific β-globin was detected in response to 5-azacytidine treatment, the system could be used to further analyze whether the gene activation was associated with a decrease in methylation, possibly by inhibition of a DNA-cytosine methyltransferase (Jones, 1985). The increase of a specific

β-globin may also be due to a cytoxic event that would cause nonspecific hematopoietic stress. In addition an erythroleukemia cell line of these mice could be induced with the Friend virus for in vitro studies on the induction and regulation of β-globin synthesis. Both experiments would provide new information to compare β-globin regulation in mice of the different β-globin haplotypes. To assess further the specific non-transcribed sequences which are involved in regulation, an SV40 expression vector system could be used. Different site and size deletions of DNA within the 14 kilobases of DNA which lie between the $\beta 1^{major}$ and $\beta 2^{minor}$ genes (Myers et al., 1986) could be produced and the expression of the two globin genes analyzed in the SV40 expression vector system. There has been no information to suggest that the β-globin genes are rearranged prior to transcription like those of the immunoglobulin genes, although this has not been studied extensively to exclude minor rearrangements. An approach would be to assay for restriction fragment length polymorphisms between DNA from yolk sac-derived and bone marrow-derived red cells and reticulocytes from embryonic and adult mice.

Further understanding of the mechanism(s) involved in murine β-globin switching may give insight into the regulation of other genes during development as well as provide a way for altering the natural expression of genes.

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