

**HUMAN EMBRYONIC ζ -GLOBIN GENE EXPRESSION
IN
MOUSE-HUMAN HYBRID ERYTHROID CELL LINES**

By

HONG YUAN LUO, M.D., M.Sc.

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AUTHOR: Hong Yuan Luo
M.D. (Zhongshan Medical University, China)
M.Sc. (Chinese Academy of Medical Sciences)

SUPERVISOR: Professor David H.K. Chui

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ABSTRACT

Human hemoglobin (Hb) is composed of two pairs of globin chains, each of them contains a heme group. One pair of globin chains is encoded by a gene on the short arm of human chromosome 16 within the α -globin gene cluster and another pair encoded by a gene on the short arm of human chromosome 11 within the β -globin gene cluster. During human embryonic and fetal development, there are orderly changes of Hbs from embryonic to fetal, and later to adult Hbs, which are referred to as hemoglobin switching. These alterations are due to sequential activation of the globin genes in the order of their locations in chromosomes.

Embryonic ζ -globin chains are present in abundance during the first five to six weeks of gestation. Subsequently, ζ -globin chains are supplanted by the expression of α -globin chains (Peschle et al 1985). In infants older than 3 months of age and in normal adults, ζ -globin chains are not detected by sensitive radioimmunoassays. However, exceptions were observed in a subgroup of α -thalassemia-1 carriers with a deletion of two adjacent α -globin genes while the ζ -globin gene in cis to the deletion remains intact, e.g., ($--^{SEA}/$) α -thalassemia-1. In adult carriers with this $--^{SEA}$ deletion, embryonic ζ -globin

chains are persistently present at a low level in their erythrocytes (Chung et al 1984, Chui et al 1986).

In order to understand the mechanism leading to ζ -globin gene expression in the adult carriers with the $--^{SEA}$ deletion, we constructed stable mouse-human somatic cell hybrids with APRT deficient murine erythroleukemia cells bearing human chromosome 16 with either the $--^{SEA}$ deletion or the normal α -globin gene cluster. The results showed that the human ζ -globin gene was constitutively expressed at higher levels in hybrids containing human chromosome 16 with $--^{SEA}$ deletion than that in hybrids with the normal human chromosome 16. These observations indicate that the DNA sequences within the deletion may have a role in regulating ζ -globin gene expression. Moreover, expression of the human ζ -globin gene in cis to the deletion was readily induced by DMSO and HMBA but not butyrate, in parallel with the induction of endogenous mouse α -globin gene expression. These observations are consistent with the hypothesis that ζ -globin gene expression in the adult carriers is linked to the ($--^{SEA}$) deletional human chromosome 16. The ζ -globin gene in cis to the $--^{SEA}$ deletion can be activated by adult regulatory factors in the mouse erythroleukemia cells. It is conceivable that the ζ -globin gene is normally suppressed in the adult erythroid cells by the DNA sequences within the $--^{SEA}$ deletion.

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LIST OF ABBREVIATIONS

AA	alanosine, adenine
AAO	alanosine, adenine, ouabain
APRT	adenine phosphoribosyl transferase
ATP	adenine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BFU-e	burst forming unit-erythroid
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	Curie
cpm	counts per minute
DAP	2,6-diaminopurine
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
Hb	hemoglobin
Hb A	adult hemoglobin
Hb F	fetal hemoglobin
HMBA	hexamethylene bisacetamide
HPFH	hereditary persistence of fetal hemoglobin
HS	hypersensitive site
HVR	hyper-variable region
IMDM	Iscove modified Dulbecco's medium
IPTG	isopropylthio- β -D-galactoside
kb	kilobase
L	litre
LCR	locus control region
M	molar
M β ₂ -MG	mouse β ₂ -microglobulin
MEL	mouse erythroleukemia
ml	millilitre
mM	millimole
MoAb	monoclonal antibody
NET	nitroblue tetrazolium
ng	nanogram

nt	nucleotide
O.D.	optical density
PEG	polyethylene glycol
RIA	radioimmunal assay
RNA	ribonucleic acid
rpm	revolutions per minute
SBIA	slot blot immunobinding assay
SSE	stage selective element
TCA	trichloroacetic acid
Tris	(hydroxymethyl) aminomethane
V	volt
X-GAL	5-bromo-4-chloro-3-indolyl- β -D-galactoside
$^{\circ}$ C	degree centigrade
μ g	microgram
μ l	microlitre

Chapter 1

INTRODUCTION

Hemoglobin (Hb), an oxygen-carrying protein, comprises about 90% of total cellular protein in red blood cells of vertebrates and some invertebrates (Collins & Weissman 1984, Honig & Adams III 1986). It has been well characterized in structure, function, synthesis, evolution, as well as the effect of mutations on molecular behaviour, which has contributed to the advance of biology, genetics, and diagnosis and treatment of diseases (Stamatoyannopoulos & Nienhuis 1987, Steinberg & Benz 1991).

This introduction is primarily concerned with a literature review of molecular biology of hemoglobin and current understanding of developmental regulation of globin gene expression. This is followed by discussion of studies on a naturally occurring deletional α -thalassemia mutation which contributes to understanding of embryonic ζ -globin gene regulation.

1.1. Hemoglobin Molecules

The Hb tetramer consists of two pairs of α - and β -like globin chains with a molecular weight of 64,500. Each globin chain is associated with a heme molecule (Collins & Weissman 1984, Honig & Adams III 1986). The normal α -globin chain contains 141 amino acid residues and the β -globin 146. There is considerable homology in primary amino acid sequences and protein structure among these globins. About 80% of the native globin chain forms the α helix. The tertiary structure of Hb was determined by high-resolution X-ray crystallographic analysis (Perutz and Lehmann 1968, Perutz 1970). It showed that the Hb tetramer is a spheroid with four hemes lying in clefts situated on the surface of the molecule. The structure and composition of Hb greatly influences its function in cooperative behaviour in oxygen affinity and stability (Perutz & Lehmann 1968, Perutz 1970, Bauer et al 1975).

1.2. Globin Gene Structure

The globin gene is a multigene family that is made up of two clusters located on separate chromosomes in humans with an α -like globin gene cluster on chromosome 16 and a β -like globin gene cluster on chromosome 11 (Deisseroth et al 1977, 1978b). The globin genes on both clusters are ordered in the

5' to 3' direction and generally parallel their developmental order of expression (Fig.1). There is coordinated expression of α -like and β -like globin genes throughout development so that nearly equal amounts of each protein that constitutes the tetramer are made.

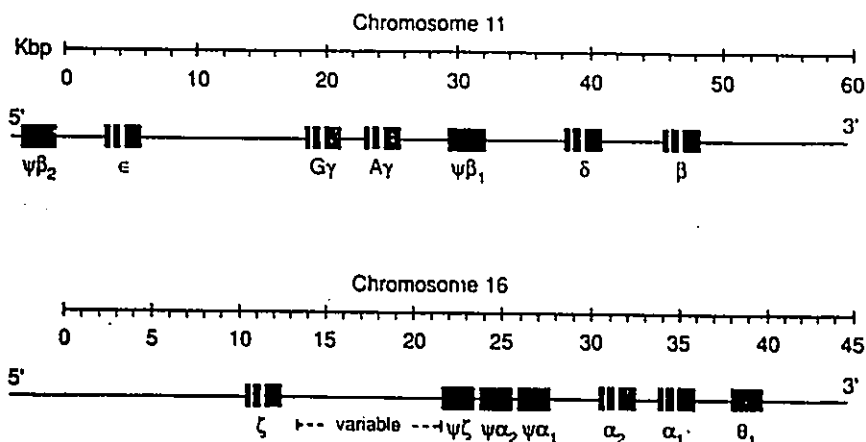


Figure 1: Maps of the β -like and α -like globin gene clusters located on human chromosome 11 and chromosome 16 respectively. Within each gene cluster pseudogenes are remnants of previously expressed globin genes that have become inactive as a result of mutation. Active genes are shown in boxes filled with clear introns; pseudogenes are shown in solid boxes. The distance between the functional ζ - and pseudo ζ -globin genes is variable owing to the presence of repeated elements. (From Steinberg and Benz 1991).

The human α -globin gene cluster, spanning about 30 kb of DNA on the short arm of chromosome 16, includes four functional genes and three pseudogenes, in an order of 5' ζ - $\psi\zeta$ - $\psi\alpha 2$ - $\psi\alpha 1$ - $\alpha 2$ - $\alpha 1$ - θ -3' (Higgs et al 1989). The embryonic ζ -globin gene is expressed very early in embryogenesis and partakes in the formation of embryonic Hbs (Proudfoot et al 1982). The α -globin genes are duplicated. Their encoded amino acid sequences are identical (Michelson & Orkin 1983, Liebhaber et al 1980, Foldi et al 1980). There are minor differences within the second intervening sequences and the 3' flanking regions of the α -globin genes that allow transcripts from each gene to be identified (Liebhaber & Kan 1981, Orkin & Goff 1981). The gene 3' to the $\alpha 1$ -globin gene is the θ -globin gene (Marks et al 1986). Transcripts of the θ -globin gene are found in fetal and adult erythroid tissues in parallel with α -globin mRNA, but at low levels (1% to 5% of α -globin mRNA) (Ley et al 1989, Albitar et al 1989). The relationship between θ - and α -globin gene expression is reminiscent of the relationship between δ - and β -globin gene expression, but θ -globin has not been identified *in vivo*.

The β -globin gene cluster, spanning about 50 kb of DNA on the short arm of chromosome 11, includes five functional genes and two pseudogenes in an order of 5' $\psi\beta_2$ - ϵ -G γ -A γ - $\psi\beta_1$ - δ - β -3' (Fig.1). The embryonic ϵ -globin gene is transcribed only during embryogenesis. The duplicated two fetal γ -globin genes

are identical except at codon 136, where the γ gene codes for glutamic acid and the δ gene for alanine. The δ - and β -globin gene code for adult Hbs. The δ - and β -globin gene are probably the result of duplication. While the β -globin gene has become the predominant adult globin, the δ -globin gene has undergone mutations in several critical regions, so that its expression is greatly curtailed and its product, Hb A₂ ($\alpha_2\delta_2$), has become functionally insignificant by virtue of its very low level in erythrocytes.

All globin genes have the same general structure, which include three exons coding for the amino acid sequences of globins and two introns as intervening sequences that are cleaved from the primary mRNA transcripts. The coding sequences of the globin genes are highly conserved. By contrast, the corresponding introns vary in length and show considerable divergence. They are probably derived from a single ancestral gene by the process of duplication approximately 500 million years ago (Collins & Weissman 1984, Honig & Adams III 1986). Since then they have been diverging from each other within their gene clusters. This reflects on the facts that the α -globin gene cluster is GpC rich (60%) with nonmethylated GpC-rich islands, associated with a constitutive open chromatin structure and early replicating, and contains many Alu family repeats (Higgs et al 1989, 1992, Nicholls et al 1987, Proudfoot 1982). The isolated α -globin

gene is transcribed at reasonable rates in many different cell types in transient assays and has no response to several erythroid specific inducers (Chamay et al 1984). In contrast, the β -globin gene cluster has a normal nucleotide composition (GpC = 39.5%), contains no GpC-rich islands and replicates late in nonhematopoietic cells. Whether these variations reflect a fundamental difference in the regulatory mechanisms of their transcription awaits further investigation. It is most likely that these structural variations between two globin gene clusters are not functionally important in regulating globin gene expression, but rather that some conserved structures exist in both clusters, which are more important for coordinated regulation of expression of these two unlinked globin gene clusters.

1.3. Ontogeny of Erythropoiesis and Hemoglobin

Development of the erythroid system is characterized by parallel switches of the sites of erythropoiesis, morphology of the erythroid cells, and hemoglobin composition in humans as well as all mammals. Erythropoiesis is initiated in the human yolk sac blood island at about 18 days of gestation (Le Douarin 1984, Gilmour 1941). Embryonic erythroid cells are megaloblastic and retain their nuclei at terminal stage of maturation. With the establishment of circulation at 25 days

of gestation, primitive erythroblasts begin to circulate. They synthesize three embryonic Hbs: Hb Gower 1 ($\zeta_2\epsilon_2$), Hb Gower 2 ($\alpha_2\epsilon_2$) and Hb Portland ($\zeta_2\gamma_2$) (Gilmour 1941, Hesseldahl & Larsen 1971).

By 5 to 6 weeks of gestation, the site of erythropoiesis switches to the liver and the definitive non-nucleated erythrocytes containing mainly fetal Hb (Hb F, $\alpha_2\gamma_2$) appear in the circulation. The liver remains the major site of erythropoiesis between 7 to 13 weeks of gestation and up until 20 weeks of gestation, at which time the bone marrow begins to contribute the majority of red blood cell production. Shortly before birth, the bone marrow erythroid cells switch from synthesis of predominant Hb F to produce primarily Hb A ($\alpha_2\beta_2$). What controls these switches in erythropoietic sites is not currently understood. Experiments in several species suggested that switching erythropoietic sites was due to migration of stem cells from yolk sac to liver to bone marrow during development rather than due to generation de novo in the organs (Migliaccio et al 1986, Toles et al 1989, Moore and Metcalf 1970, Stamatoyannopoulos et al 1987b, Papayannopoulou et al 1991a).

The most widely studied changes during red cell ontogeny are the switches in globin types. The Hb composition of erythrocytes varies with the stage of human development to suit the adaption of embryonic and adult globins to their

respective embryonic and adult environments (Perutz 1970). The Hbs specific to the earliest embryonic stage (5 to 6 weeks of gestation) of humans are Hb Gower 1 ($\zeta_2\epsilon_2$) and Gower 2 ($\alpha_2\epsilon_2$). The Hb Portland I ($\zeta_2\gamma_2$) is present in embryos prior to 7 to 8 weeks of gestation and disappears after 11 weeks of gestation (Stamatoyannopoulos et al 1987b, Papayannopoulou & Abkowitz 1991a). These embryonic Hbs with high oxygen binding affinity are important for oxygen uptake by embryos (Bauer 1975, Clegg & Gagnon 1981). These three types of embryonic Hbs are synthesized in succession due to sequential expression of the α - and β -like globin genes during embryonic development (Peschle et al 1985). Since the amount of Gower 1 ($\zeta_2\epsilon_2$) is highest in smaller embryos. Switches from ζ - to α -globin gene production and ϵ - to γ -globin gene production are initiated during the embryonic phase of erythropoiesis but not complete until fetal erythropoiesis is well established (Fig.2).

By 9 weeks of gestation, erythroid precursors within the fetal liver co-express embryonic and fetal globins. The predominant type of Hb synthesized during fetal liver erythropoiesis is Hb F (Stamatoyannopoulos 1987b, Ley et al 1989). Two γ -globin genes are unequally expressed during fetal development. The ratio of $^{\circ}\gamma$: $^{\wedge}\gamma$ is 3:1 at the fetal stage and falls to 2:3 after birth. Hb F gradually declines after birth so that there is less than 1% of Hb F in adults.

HEMOGLOBINS (EMBRYONIC)	HEMOGLOBINS (% AT BIRTH)	HEMOGLOBINS (% IN ADULTS)
GOWER 1 $\zeta_2\epsilon_2$	HbF $\alpha_2\gamma_2$ (75)	HbA $\alpha_2\beta_2$ (97)
PORTLAND 1 $\zeta_2\gamma_2$	HbA $\alpha_2\beta_2$ (25)	HbA ₂ $\alpha_2\zeta_2$ (2.5)
GOWER 2 $\alpha_2\epsilon_2$		HbF $\alpha_2\gamma_2$ (< 1)

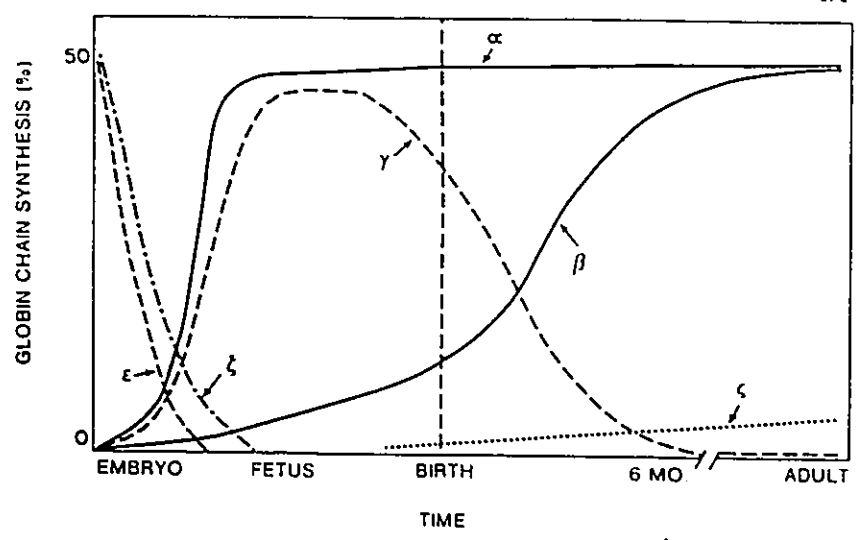


Figure 2: Hemoglobin switching during human embryonic, fetal and adult development. The ζ - and ϵ -globin genes are transcribed during embryonic development and soon replaced by the fetal γ - and adult α -globin genes. At birth, Hb F forms about 75% and Hb A 25% of the total. Transcription of the γ -globin gene begins to fall prior to birth, and by 6 months of age this gene is expressed only at very low levels. Expression of the δ -globin gene begins near birth. In adults Hb A makes up about 97%, Hb A₂ about 2.5%, and Hb F less than 1% of the total. (From Steinberg and Benz 1991).

It should be noted that the embryonic globins are not restricted to primitive erythrocytes but instead continuously exist at low levels beyond the embryonic stage. The levels of ζ - and ϵ -globin mRNA in both cord and adult blood is about 3% to 5% of α -globin mRNA (Albitar et al 1989). These data are consistent with the finding of embryonic Hbs and ζ -globin chains in fetuses at the late stage of gestation and newborns (Chui et al 1989, Weatherall et al 1970), as well as in the burst forming unit-erythroid (BFU-e) cells obtained from normal adults (Bhaumik 1991). The ζ -globin chain is undetectable in infants older than 3 months of age and in normal adults (Chung et al 1984, Chui et al 1986, 1989). The ϵ -globin chain is detectable as late as 20 weeks of gestation, but not beyond (Zhao et al 1988).

The synthesis of adult Hb A ($\alpha_2\beta_2$) begins in the embryo at 7-8 weeks as a minor component throughout the fetal stage (Pataryas 1972, Stamatoyannopoulos 1987b). Despite a remarkable level of structural identity in the $\alpha 1$ - and $\alpha 2$ -globin genes, relative expression of these two genes is developmentally regulated. The levels of two α -globin mRNAs are equal in the embryo (Albitar et al 1992) and subsequently shift to dominant expression of the $\alpha 2$ -globin gene at week 8 to become a major α -globin in normal adults ($\alpha 2:\alpha 1 = 60:40$) (Orkin & Goff 1981). The β -globin synthesis steadily increases from about the thirtieth week gestation onwards, but it

becomes predominant only after birth. The δ -globin gene is inefficiently expressed. Thus, only low levels of Hb A₂ (<3.5% of total Hbs) are present in normal adults.

It should be noted that the embryonic Hbs are increased in some pathological conditions. There are greater amounts of embryonic and fetal Hbs in newborns with chromosomal abnormality of D₁ trisomy (Huehns et al 1964), in infants with hydrops fetalis syndrome due to deletion of all α -globin genes (Weatherall et al 1970), and in one child with congenital anemia (Tang et al 1992). The embryonic and fetal Hb levels are also increased in patients with Juvenile chronic myeloid leukemia (JCML), in which the ϵ - and ζ -globin producing cells are characterized as fetal cells (Weatherall et al 1975, Papayannopoulou et al 1991b). The embryonic Hbs are produced abundantly in erythroleukemia cell lines established from patients with leukemias (Rutherford et al 1979, Papayannopoulou et al 1987). The studies of globin gene expression in these disease states have provided notable information on globin gene regulation.

1.4. Regulation of Globin Gene Expression

The mechanisms responsible for selective expression of different genes at different times during development have been intensively studied over the last two decades. A better

understanding of these mechanisms might also impact on a clinical goal: amelioration of the human hemoglobinopathies. Three major approaches have been particularly useful in these investigations. The first one is to characterize naturally occurring germ-line mutations within the globin gene locus, since comparison of gene expression of mutated and normal alleles can provide insight into the events involved in globin gene regulation. The second approach makes use of erythroleukemia cell lines and embryonic stem cells as models for *in vitro* study of globin gene regulation. Recently, transgenic mice have been used as an *in vivo* experimental system to investigate the role of *cis*-acting DNA regulatory sequences in achieving tissue-specific and developmentally regulated gene expression. Accumulating evidence suggested that developmental stage and tissue specific expression of the globin genes is achieved through at least three regulatory elements. These include the proximal DNA regulatory sequences flanking the coding sequences of the globin genes (Raich et al 1992, Foley et al 1992), the distal major regulatory sequences of the locus control region (LCR) upstream of the β - and α -globin gene clusters (Tuan et al 1984, Grosveld et al 1987, Higgs et al 1990, Vyas et al 1992b) and the erythroid specific and stage specific trans-acting factors (Deisseroth & Lyding 1987, Yu et al 1990, Watt et al 1990, Gallarda et al 1989, Ramseyer et al 1989, Baron and Maniatis 1986, 1991).

1.4.1. Proximal DNA Regulatory Sequences

Several critical elements flanking or within the globin genes are important for regulating globin gene expression. These include promoter, enhancer and silencer.

Promoter

Promoter is a group of transcriptional control DNA sequences that are clustered around the initiation site of mRNA transcription (cap site) in almost every gene. Promoters of the globin genes commonly contain multiple genetic elements, including TATA, CCAAT, CACCC, and GATA consensus sequences (Lin et al 1987, Yu et al 1991, Myers et al 1986, Mellon et al 1981). The TATA box (at about -30 bp) functions to position the start site for RNA synthesis. The CCAAT (at about -80 bp) and CACCC (at about -100 bp) are additional promoter elements which regulate the frequency of transcriptional initiation. It appears that each element functions cooperatively or independently to activate transcription (Myers et al 1986). In the absence of activators, the gene is transcribed at a low basal level (Guarente et al 1992). This is achieved by a core promoter sequence centred at the TATA box which is bound by a general transcription factor TFIID and subsequently by other general transcription factors and RNA polymerase II to form a pre-

initiation complex (Sawadogo & Roeder 1985, Buratowski et al 1989).

The GATA motif is conserved in promoters and enhancers of a variety of globin and non-globin erythroid specific genes in humans and other mammals (Watt et al 1990, Yu et al 1990, Gong et al 1991, Martin et al 1989, Orkin 1992, Evans et al 1988). The GATA sequence has a functional role in stimulating erythroid tissue specific transcription of globin genes in cooperation with proximal CACCC boxes. It exerts its effect through an erythroid specific DNA binding protein GATA-1 (Tsai et al 1989).

Promoters of the individual globin genes contain sufficient information for determining developmental stage of preferential expression (Chada et al 1986). The DNA elements that determine developmental stage specificity of the globin genes are termed stage selective elements (SSE). The SSE have been identified in regions 5' and 3' to the human β -globin gene (Kollias et al 1986, 1987, Behringer et al 1987), within the γ -globin gene promoter (Trudel et al 1987, Kollias et al 1986), and within the chicken β -globin gene promoter (Choi et al 1988, 1992). There are others remaining to be discovered. However, the SSE alone are unable to produce high level expression without the LCR.

Enhancer

Enhancer is located thousands of base pairs from the promoter at 5' or 3' to the globin genes. It acts in cis to potentiate transcription from the promoter of the globin gene to drive high level transcription in a tissue-specific and an orientation-independent fashion. The enhancer elements have been identified in the region 3' of the human β - and $A\gamma$ -globin genes (Behringer et al 1987, Bodine et al 1987). It is interesting that the $A\gamma$ -globin gene enhancer also mediates negative regulation of γ -globin gene expression during megakaryocytic differentiation of the human erythroleukemia cell line K562 (Lumelsky et al 1991). The enhancer is organized much like the promoter, that is, it is composed of multiple overlapping conserved DNA elements for binding different transcriptional activators, many of which show a cell-specific pattern of distribution (Dyran 1989). The minimal human 3'- β -globin gene enhancer contains multiple GATA-motifs binding an erythroid-specific GATA-1 factor (Wall et al 1988, Talbot et al 1990). The enhancer acts in a developmental specific manner (Kollias et al 1987), but the corresponding trans-acting factors for stage specific expression of the globin genes have not been identified.

The question of presence of an enhancer element in the α -globin gene cluster remains more open (Watt et al 1990). The α -globin gene is expressed constitutively at a high level upon

gene transfer into either erythroid or non-erythroid cells in an enhancer-independent fashion (Chamay et al 1984). The β -globin gene, on the other hand, is dependent on the presence of a linked viral enhancer for its efficient expression upon transfer into heterologous cells (Chamay et al 1984). Recent studies provided evidence supporting the fact that a positive regulatory element responsible for constitutive expression of the α -globin gene is located within the α -globin gene. This positive regulatory element functions in a position and orientation-dependent manner. It contains a DNA sequence for binding an ubiquitous Sp1 transcription factor (Brickner et al 1991).

Silencer

A silencer sequence is involved in developmental control of the globin genes by suppression of gene expression when bound by regulatory proteins. The silencer sequences within the promoter region of the human ζ -globin gene (-167 to -148 bp) (Vyas et al 1992a) and the ϵ -globin gene (-290 to -400 bp) (Cao et al 1989, Raich et al 1992) have been identified to play a role in suppression of these genes in post-embryonic erythroblasts. The mutagenized human ϵ -globin gene constructs with deletion of the silencer region resulted in developmentally aberrant expression of the ϵ -globin gene in definitive erythroid cells in transgenic mice or transiently

transfected HeLa and K562 cells (Raich et al 1992). However, quantitative analysis of relative levels of human ϵ -globin gene expression in primitive versus definitive erythroid cells in transgenic mice suggested that the bulk of the ϵ -globin gene developmental regulation is independent of this element.

It is interesting that suppression of chicken embryonic ϵ -globin gene expression in definitive erythroid cells is specifically associated with sequences between -112 and -12 bp within the chicken adult β -globin gene promoter. Deletion or point mutations in this region concomitantly resulted in an increase in expression of the embryonic ϵ -globin gene in definitive erythroid cells (Choi & Engel 1988). It was shown that the mutations disrupted the binding sites for an adult stage-specific transcription factor NF-E4 which binds purine-rich sequences (Foley & Engel 1992).

It was reported that a negative element located within the human α -globin gene (259 bp at 5' end) suppressed the human β - and γ -globin genes linked in cis to the α -globin gene in transient expression assays (Atweh et al 1988). Its effect was position and orientation independent. However, it needs to be examined whether such suppression is a direct effect of a silencer or an indirect effect due to competition between the linked genes for interacting with a shared enhancer.

1.4.2. Distal Locus Control Region (LCR)

The LCR is a cis-acting regulatory element located far upstream of the β - and α -globin gene clusters. The existence of such regulatory sequences was suggested by observations that deletions removing the sequences upstream of the globin gene clusters resulted in the failure of transcriptional activity in any of the remaining linked globin genes, therefore leading to β - or α -thalassemia phenotypes (Taramelli et al 1986, Kioussis et al 1983, Curtin et al 1985, Driscoll et al 1989, Liebhaber et al 1990, Hatton et al 1990, Wilker et al 1990).

The LCR of the β -globin gene cluster (β -LCR) was mapped to a region 6-18 kb upstream from the embryonic ϵ -globin gene. It is marked by a set of four major DNase I hypersensitive sites (HS) (Tuan et al 1984, Grosveld et al 1987). As a result of deletions, the entire β -globin locus became DNase I resistant and did not form characteristic distinct hypersensitive sites (Forrester et al 1990). The 5' hypersensitive site-2 (5'HS2) is an important regulatory element within the β -LCR. It functions as an inducible enhancer in erythroid cells and accounts for 40-50% of the full effect of the β -LCR (Grosveld et al 1987, Forrester et al 1987, Townes et al 1990, Talbot & Grosvelt 1991, Ney et al 1990)). The other three HS domains in the β -LCR did not function as an inducible enhancer

when they were analyzed individually in transient expression assays, only if integrated into genomic DNA. These observations suggested that their activities were associated with the chromatin structure (Tuan et al 1989, Talbot et al 1989, Philipsen et al 1990). The β -LCR can also drive high level expression of the α -globin gene and other heterologous genes (Ryan et al 1989, Kim et al 1992).

The LCR domains are full of DNA-binding sequences for well known trans-acting factors. The 5'HS2 contains an NF-E2 dimer site, a GATA-1 dimer site and sites for the ubiquitous H-BP and J-BP factors (Ellis et al 1993, Philipsen et al 1990). Comparison of the sequences within the 5'HS2 of the human β -LCR in sickle cell anemia patients with different Hb F levels suggested that genetic variation which creates binding sites for erythroid and ubiquitous factors within the 5'HS2 region of the β -LCR may contribute to higher levels of Hb F in the patients (Oner et al 1992).

The LCR in the human α -like globin gene cluster, termed HS-40, is located 40 kb upstream of the ζ -globin gene (Higgs et al 1990). The importance of HS-40 *in vivo* was confirmed by observations of three α -thalassemia individuals with deletion of this region, e.g., $(\alpha\alpha)^{RA}$ (Hatton et al 1990), $(\alpha\alpha)^{TI}$ (Wilker et al 1990) and $(\alpha\alpha)^{IJ}$ (Liebhaber et al 1990). These deletions overlap the HS-40 region and inactivate the structurally normal α -globin genes that lie in cis. The long-

range genomic organization and chromatin structure around the α -gene cluster is different from the β -gene cluster. The HS-40 lies within an intron of a gene which is activated in both erythroid and non-erythroid cells. The α -globin gene cluster is dispersed within a region of chromatin that is open constitutively in both erythroid and non-erythroid cells (Higgs et al 1990, Vyas et al 1992b).

A number of experimental observations are consistent with a model in which the LCR confers high-level, position-independent and erythroid-specific expression to all genes in the cluster. However, the LCR alone is not sufficient to play any determinative role in developmental expression of the linked globin genes. It was suggested that a primary role of the LCR is to organize the entire globin gene cluster into an open chromatin domain to allow the DNA regulatory sequences within the region of open chromatin accessible to trans-acting factors and free to interact with each other (Felsenfeld 1992, Orkin et al 1990, Townes and Behringer 1990). It has been proposed that sequences in and around the globin genes could form a stem-loop structure (Lavett 1984). This specific secondary structure in the globin gene was considered as a factor to interact with stage-specific factors and determine developmental specificity of the genes. The LCR may be responsible for organizing and maintaining this structure if the LCR and the promoter are placed in intimate proximity, by

appropriate chromosome folding into a loop (Orkin 1990).

1.4.3. Trans-acting factors

The transcriptional effect of DNA regulatory elements is mediated through binding of sequence-specific nuclear proteins (Mitchell and Tjian 1989). There is evidence that the nuclear proteins that interact with TATA, CCAAT and CACCC motifs are not tissue-specific (Plumb et al 1986, Jones et al 1987, Schule et al 1988, 1991). The GATA-1, previously termed nuclear factor-erythroid 1 (NF-E1), Eryf 1 or globin factor-1 (GF-1), is the only one that has been well characterized to be an erythroid specific factor (Wall et al 1988, Tsai et al 1989, Martin & Orkin 1990). The GATA-1 is a zinc finger protein that has a highly conserved binding domain to a consensus GATA motif present in the regulatory elements of the majority of erythroid-expressed globin and non-globin genes to participate widely in gene expression throughout erythroid development (Martin & Orkin 1990, Chiba et al 1991). Activation of transcription is dependent on a domain located outside the finger region. The GATA motif is often found close to either CACCC (Sp1 binding site), CCAAT motif or AP-1/NFE-2. The context in which the motif is placed is crucial to its function (Plumb 1989, deBoer 1988), suggesting its functional

dependence on other adjacent cis-elements (Frampton et al 1989).

The GATA-1 also binds to and activates its own promoter (Tsai et al 1991). Through gene targeting in embryonic stem (ES) cells it has been established that GATA-1 is essential for erythroid development (Pevny et al 1991). The GATA-1 mutant ES cells failed to differentiate into erythroid cells *in vivo* and *in vitro*, and did not express any embryonic globin mRNAs. However, hematopoietic cells lacking GATA-1 did give rise to mature myeloid cells (Simon et al 1992, Pevny et al 1991). Therefore, the progressive increase in GATA-1 throughout cellular maturation indicates its role in erythroid differentiation. It was suggested that GATA-1 acts as a more general factor that works in combination with other highly regulated factors that are more stage specific in regulating the globin genes during the differentiation of the erythroid lineage (Orkin 1992).

While binding sites for several ubiquitous and erythroid specific transacting factors have been identified in the promoter, enhancer and LCR, evidence for developmental stage specific transacting factors in humans remains largely unknown. An adult erythroid specific protein, NF-E4, has been reported to bind to a purine-rich motif present in the chicken β -globin gene promoter and the 3' enhancer. The NF-E4 mediated

the switch from ϵ - to β -globin gene expression in chicken definitive erythrocytes (Gallarda et al 1989).

There is increasing evidence for cooperative protein-protein interactions between proteins binding to cis-acting elements (deBoer et al 1988, Orkin 1992). In the human ζ -globin gene promoter, there are mainly two clusters of multiple nuclear factor binding sites. The proximal cluster spans the DNA sequence from -110 to -60 and consists of binding sites for CP2 and GATA-1, as well as the Sp1-binding site which overlaps both the CP2 and the GATA-1 binding sites. Competition between proteins for binding to a given site has been described. The GATA-1 binding is erythroid specific and has higher competitive affinity for other factors (Yu et al 1990). In the distal promoter region at -250 to -220, another GATA-1 binding site overlaps the Sp1 binding site. Such a structure of mutual exclusion of different nuclear factors at overlapping binding sites of the promoter are conserved in mouse, goat and rabbit (Cheng et al 1988, Yu et al 1990). This suggests that it is essential for the regulation of ζ -globin gene expression. However, it has not been proven that these elements are necessary to provide specificity for ζ -globin gene expression *in vivo*.

Developmental modulation of regulatory factors binding to positive or negative regulatory sequences was demonstrated in the chicken β -globin gene (Jackson et al 1989). A

palindromic sequence (Pal) inhibited and a CACCC site stimulated gene expression. At early stages of embryonic development, the CACCC site was occupied and the Pal site was vacant, but as development progresses, the Pal site was filled gradually and the CACCC site lost its bound protein (Jackson et al 1989). PAL protein is an erythroid specific protein and is transcriptionally controlled in erythroid development (Orkin 1990).

Evidence in several widely used experimental models demonstrated the existence of developmental stage-specific trans-acting factors for regulating globin gene expression across vertebrate classes (mammalia to amphibia). Thus, mechanisms operating developmental Hb switching are highly conserved (Ramseyer et al 1989). The switches from the human embryonic or fetal globins to the adult globins were observed in the stable interspecific hybrid cells (Deisseroth 1978, Papayannopoulou et al 1986). In the transient heterokaryons, the nuclei remain separate and distinct after fusion of adult mouse erythroleukemia (MEL) cells with various mouse or human cells (Baron and Maniatis 1986). This resulted in activation of many stage-specific globin genes in the non-erythroid or erythroid cell nucleus, indicating the presence of trans-acting factors capable of exerting a dominant effect on expression within the introduced nucleus (Baron and Maniatis 1986, 1991). Similar results were observed in the

heterokaryons formed between xenopus erythroblasts from different developmental stages (Ramseyer et al 1989). Recently, transgenic mice have become a very useful experimental method to study the developmental regulation of the human globin genes.

1.4.4. Other Elements

Most of the DNA in living cells is bound by histone and non-histone proteins into a nucleoprotein complex called chromatin. An appropriate chromatin structure is an intrinsic part of effective and efficient gene regulation (Weintraub & Groudine 1976, Forrester et al 1990). The chromatin structure of the transcribed genes differs from that of the inactive genes, being less compacted and more accessible to the transcription apparatus. This is marked by erythroid and developmental specific DNase I hypersensitive sites (HS) that reflect the absence of a canonical nucleosome (Elgin 1988, Benezra et al 1986). The HS sites have now been mapped at a number of specific positions of known function, including promoters, enhancers and LCR in the α - and β -globin gene clusters (Tuan et al 1984, Higgs et al 1990, Groudine et al 1983, Yagi et al 1986).

The human γ - and β -globin genes are known to switch only when integrated into the genome of transgenic mice, but

switching did not occur in a transient *in vitro* system (Enver et al 1990). This indicates that chromatin conformation is critical for the hierarchical control of gene expression and this is mediated at least in part by the LCR (Forrester 1990, Orkin 1990). Evidence has been obtained for proteins capable of inducing HS structure near the chick β -globin gene (Emerson et al 1984). However, changes in chromatin structure appear to be necessary but not sufficient for gene activation. The entire α - and β -globin gene clusters are sensitive to DNase I digestion in erythroid cells throughout all developmental stages (Yagi et al 1986, Tuan et al 1989). This suggests that the active chromatin is a tissue-specific but not stage-specific feature of the globin gene, although the globin genes that are transcriptionally active are more sensitive to DNase I digestion than the silent globin genes (McGhee et al 1981, Stadler et al 1980, Groudine et al 1983).

DNA methylation plays some role in the regulation of gene expression (Felsenfeld et al 1982, Frank et al 1991). The correlation of gene expression and DNA methylation was well documented in the γ -globin gene (Enver et al 1988a, 1988b). The γ -globin gene was extensively methylated in non-erythroid cells and hypomethylated in erythroid cells. The degree of methylation in GpC-rich regions flanking the globin genes may in part influence gene expression (Van der Ploeg & Flavel 1980, Busslinger et al 1983).

Several other structural features of DNA have been implicated as important for gene regulation. The relative order and distance of the genes from the LCR and enhancer influences the transcription, with the more proximal gene having an advantage (Hanscombe et al 1991, Antoniou & Grosveld 1990, Lindenbaum 1990). This is reminiscent of the effect observed when multiple genes were linked to a single enhancer (Wasylyk et al 1983). Targeted insertion of a heterogeneous gene proximal to the LCR on chromosome 11 resulted in the inactivation of the downstream β -globin gene (Kim et al 1992).

1.5. Models of Globin Gene Switching

To delineate the molecular mechanisms regulating differential expression of the globin genes during development, several *in vitro* and *in vivo* approaches have been used. Transplantation experiments in sheep and mice have failed to provide convincing support for effects of environmental factors on the Hb switching process, although some modulation on the rate of switching was seen in these experiments. These results suggested that globin switching occurs as an intrinsic part of a developmental process in the hemopoietic stem cells, which are dependent on gestational age but not on the environment (Wood et al 1985). Recently developmental regulation of globin gene expression was studied

intensely in chicken and transgenic mice. Accumulating evidence supports the hypothesis that developmental regulation of globin genes was achieved by complex interaction of the DNA regulatory elements, including the distal LCR and the proximal promoter and enhancer. This interaction was mediated by tissue- and stage-specific transacting factors (Orkin 1990). Currently, two general models on globin switching have been proposed to explain the developmental regulation of the globin genes.

1.5.1. Competition Model

A competition model has been put forward suggesting that developmental regulation of globin gene switching is a result of mutually exclusive competition between the promoters of the globin genes for interaction with the shared LCR and enhancer in cis (Behringer et al 1990, Choi & Engel 1988, Engel et al 1989) (Fig.3). This hypothesis is supported by the facts that stage specific expression of the human γ - and β -globin transgenes in mice was maintained only when both genes were linked to the β -LCR in the right order. The β -globin gene was competitively suppressed at the fetal stage by the γ -globin gene and was activated only when the γ -globin gene was

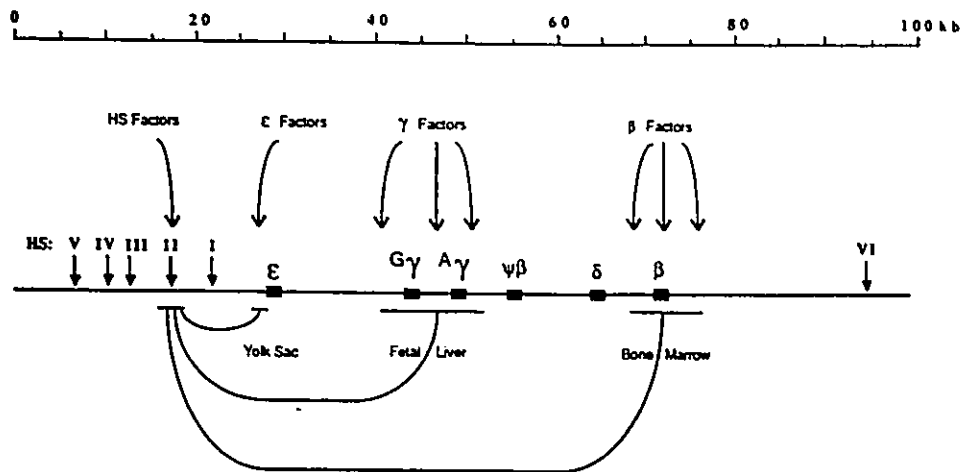


Figure 3: General model for human hemoglobin switching. In this model correct temporal regulation results from competition of individual gene family members for interaction with the β -LCR. Promoter and proximal enhancer binding factors synthesized in yolk sac, fetal liver, and bone marrow influence these competitive interactions either positively or negatively and subsequently determine developmental specificity. (From Behringer et al 1990).

silenced and the LCR became available to interact with the β -globin gene promoter at the adult stage. When either of the genes alone was linked to the β -LCR, the linked gene was constitutively activated at all stages and altered its pattern of temporally regulated expression (Enver et al 1990). Further evidence was observed in the chicken embryonic ϵ - to adult β -globin gene switching. By inserting an additional LCR-like sequence into the cluster, both genes were transcribed without temporal control (Choi et al 1988, Engel et al 1989).

In the competition model, at least three elements are considered to participate in temporal regulation of globin gene expression. The first one is the distal regulatory element LCR, which directs high-level expression of any genes linked in cis in a tissue-specific but developmental stage independent fashion. The second one is the DNA sequences immediately flanking the genes, which recognize the stage-specific signals and activate globin gene transcription during a specific developmental stage. The third one is the stage-specific transacting factors, which act at the specific erythroid developmental stage by DNA-protein or protein-protein interactions. The outcome of competition between genes for activation is determined by the competitive affinity of individual globin genes to interact with the LCR. This would determine developmental specificity of the genes, preferentially allowing the globin gene with the highest

competitive affinity to interact with the transcription apparatus that is stable throughout erythroid development. It is likely that the stage-specific trans-acting factor is a major rate limiting element for globin switching. At present, we know little about such regulatory factors in humans.

However, the mutually exclusive interaction between LCR and globin genes results in expression of only one gene at any give moment. It can not explain the fact that both human α -globin genes are expressed at high levels in adult erythrocytes or the two γ -globin genes in the fetal erythrocytes. In transgenic mice with the transgenes of the human α - and β -globin genes being linked in cis to the β -LCR (LCR- β - α), both α - and β -globin genes were expressed at high levels comparable to the endogenous mouse adult globin genes with an α/β ratio of 0.6 (Hanscombe et al 1989). This result implies that if two linked globin genes have nearly equal competitive affinity to effect the interaction with the LCR, both genes could be expressed.

1.5.2. Non-competition Model

This hypothesis has arisen based on observations that the human embryonic and adult globin genes did not require to be linked in cis to achieve developmental stage-specific expression but were autonomously regulated. The human ϵ - and

ζ -globin genes were expressed almost exclusively at the embryonic stage in transgenic mice (Raich et al 1990, Spangler et al 1990, Albitar et al 1991, Pondel et al 1992). The mechanism underlying the autonomous regulation is unknown. It was suggested that the proximal silencer element is necessary to suppress the human embryonic ϵ -globin gene at the adult stage, which assists with other mechanisms to achieve appropriate developmental stage specificity (Cao et al 1989, Lindenbaum et al 1990, Raich et al 1990, Shih et al 1990, Dillon & Grosveld 1991, Lamb et al 1989).

The autonomous regulation model alone does not adequately explain the fact that human ζ -globin chains are present in fetuses and adults with two α -globin gene deletion of ($--^{SRA}/$) genotype (Chung et al 1984, Chui et al 1989). Although transgenic mice allow an approach for working out the possible mechanisms operating fetal to adult Hb switching, the constructs and the experimental conditions are often quite different from those found as individual genes, especially embryonic globin genes in their natural setting in humans. This raises concerns about interpreting the results of studying developmental regulation of the human embryonic Hb switching during murine ontogeny. It has been observed that the human fetal γ -globin gene was expressed abundantly throughout the mouse embryonic phase of erythropoiesis in transgenic mice, coordinating with its homology mouse $\beta h1$

embryonic globin gene expression (Chada et al 1986). These observations suggested that the human γ -globin gene and the mouse embryonic globin genes may share trans-acting factors conserved during evolution. However, mice may lack the factors homologous to the human embryonic factors which effect high level expression of the human embryonic globin genes. Recently, it was shown that expression of the human ϵ -globin transgene in mice was quite different from that observed during normal human embryonic development (Strouboulis et al 1992). The human ϵ -globin gene was expressed at much lower levels than its homolog murine $\epsilon\gamma$ -globin gene, but the human γ -globin gene was expressed abundantly in early mouse embryos. Therefore, the human embryonic globin gene may be regulated by regulatory factors evolved since the divergence of rodents and primates (Peschle et al 1985). It would be informative to study developmental regulation of the human embryonic globin genes using a model comparable to human Hb ontogeny. Moreover, it is important that the constructs contain all information necessary to specify correct gene expression, and the order of the genes and spacial relationship between the LCR and globin genes are not disrupted. All these factors have been shown to be important for globin gene regulation (Kim et al 1992).

1.5.3. Feedback Model

Although the primary sequences of globins have been conserved in widely divergent organisms, genetic changes in regulation of gene expression in the globin gene family have been extensively amplified and diversified during evolution to fit the needs of organisms (Honig & Adams 1986). Feedback regulation is a model for several simple organisms to regulate globin production by environmental oxygen. For example, an insect midge has globin in its fast-growing larval stage, but loses it after metamorphosis (Ingram 1981). The water flea reacts to anoxia by synthesizing globin, but decreases again to the low starting levels when oxygen is once more abundant (Dickerson and Geis 1983). In the vertebrate, the regulation of Hb falls under an elaborate mechanism of transcriptional control in response to oxygen demand by adjusting the amounts of erythropoiesis and programming the Hb switches during development. By using a "phylogenetic footprinting" approach to systematically compare genetic variation across species, it should be possible to obtain evidence of continuous evolutionary changes in gene regulation from the molecular point of view (Orkin 1990). Also, it may provide important clues to identify conserved motifs which might account for the recruitment of embryonic globin gene expression in embryonic stage-specific fashion in humans.

1.6. Mutations Altering Developmental Specificity of Globin Gene Expression

Much of our understanding of globin gene expression has come from the study of naturally occurring mutations which alter developmental specificity of globin gene expression. These mutations provide clues as to the elements involved in gene regulation. A group of human globin gene developmental mutations that are collectively known as hereditary persistence of fetal Hb (HPFH), has been identified and studied (Weatherall & Clegg 1976, Kazazian 1974, Wood et al 1979, Stamatoyannopoulos & Nienhuis 1987). The molecular basis of HPFH is heterogeneous, including various deletions downstream of the γ -globin gene (Feingold & Forget 1989, Jagadeeswaran et al 1982), single base substitutions (Superti-Furga et al 1988) or small deletions in the γ -globin gene promoter (Ilman et al 1988). The molecular mechanisms underlying these disorders of fetal globin gene regulation are thought to be different for deletion and nondeletion HPFH mutations (Stamatoyannopoulos & Nienhuis 1987).

In nondeletion HPFH, point mutations in the γ -globin gene promoter region (i.e., G to A at -117 or T-C at -175) are a cause of increased γ -globin gene expression. This was proven in several experimental systems, including somatic cell hybrids (Papayannopoulou et al 1986), heterokaryons (Baron &

Maniatis 1991), and a transient assay in mouse erythroleukemia cells (Berry et al 1992). The promoter region (-195 to -170) of the γ -globin gene has two AGATA motifs and the conserved ectomere (ATGCAAAT) that separates them. The point mutation affected binding of the transacting factors to the γ -globin gene promoter region, such as GATA-1 (Martin et al 1989, 1990, Berry et al 1992) and Sp1 (Sykes & Kaufman 1990). This suggested that γ -globin gene regulation is achieved by the combined action of several transcription factors (Schule et al 1991, Walters et al 1992). Mutations may change DNA-protein interaction, which leads to increased competitive affinity of the γ -gene promoter to interact with the LCR for activation of transcription (Berry et al 1992). HPFH is always associated with a concomitant decrease in β -globin gene expression.

In deletion HPFH, large deletions remove the adult globin genes (δ and β) (Kazazian 1974, Stamatoyannopoulos & Nienhuis 1987a). The sequences with enhancer activity or hypersensitivity to DNase I are brought into the vicinity of the γ -globin genes as a result of deletion and disrupt normal γ -globin gene regulation. This is consistent with the competition model. In the absence of the competing β -globin gene, the remaining γ -globin genes are expressed in adults.

There are a number of deletional mutations leading to the syndrome of α -thalassemia, termed by their geographical distribution, such as (--^{SEA}/), (--^{BRIT}/), (--^{MED}/), (--^{SA}/) and

(--^{SPAN}/) α -thalassemias. These deletions span 20-30 kb. The 5' break-points of these deletions lie between the ζ - and $\psi\zeta$ -globin genes, and the 3' breakpoints of these deletions lie between the $\alpha 1$ -globin gene and the 3' hypervariable region (3' HVR). The embryonic ζ -globin gene, located upstream of the deletions, remains intact in these deletions (Higgs et al 1989, Nicholls et al 1987) (Fig.4). But in subgroup of these deletions, expression of ζ -globin gene is altered during human Hb ontogeny. Normally, the ζ -globin synthesis is restricted primarily to the primitive erythroblasts in embryos before 7 weeks of gestation (Peschle et al 1985) and is not detected in adults (Chung et al 1984, Chui et al 1986). It was found that ζ -globin chains are continuously synthesized in large amounts in fetuses homozygous for the (--^{SEA}/--^{SEA}) deletion, and in minute amounts in adults heterozygous for the (--^{SEA}/) deletion (Chung et al 1984, Chui et al 1986). The adult carriers of the (--^{SPAN}/) and the (--^{MED}/) deletions also have low but detectable levels of ζ -globin chains in their circulating erythrocytes (Tang et al 1992).

The (--^{SEA}/) α -thalassemia-1 is most prevalent in Southeast Asia. The 5' breakpoint of the --^{SEA} deletion is located 12 kb downstream of the ζ -globin gene and the 3' breakpoint lies within an Alu sequence, 1 kb upstream of the 3' HVR (Fig. 4). Analysis of the polymorphic markers, upstream of the common --^{SEA} breakpoint, suggested that the mutation

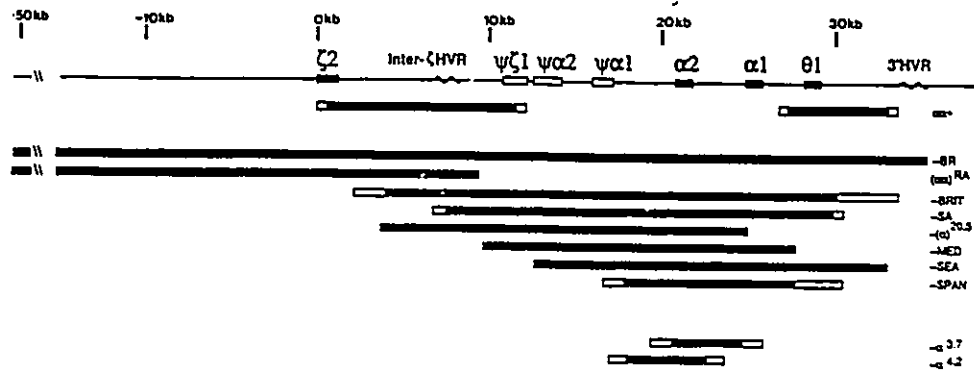


Figure 4: Extent of deletions from the α -globin gene cluster in α -thalassemia patients studied in this investigation. With the exception of the deletion marked $\alpha\alpha^*$, all other deletions are associated with either severely reduced or abolished α -globin gene expression. The globin genes are represented as solid boxes, pseudogenes are open boxes, and hypervariable regions as zig-zag lines. The extent of each deletion is represented as a solid box, and the uncertainty of the breakpoints is indicated by the open boxes. (From Tang et al 1992).

arose once during evolution (Higgs et al 1989). The fetuses homozygous for the ($-\text{SEA}/-\text{SEA}$) deletion, also known as Hb Bart's hydrops fetalis syndrome die before or soon after birth (Weatherall et al 1970). Moreover, there is considerable maternal morbidity associated with this syndrome in the third trimester of pregnancy (Liang et al 1985). Survival of the fetuses to late pregnancy may be attributed to continued expression of the ζ -globin gene beyond the embryonic stage (Weatherall et al 1970). Hb Portland ($\zeta_2\gamma_2$) accounts for 20% of total Hbs in fetuses with Hb Bart's hydrops fetalis syndrome. The infants heterozygous for the ($-\text{SEA}/$) deletion have 2% ζ -globin chains at birth (Chui et al 1989). Adults who are heterozygous for the ($-\text{SEA}/$) deletion have minute amount of ζ -globin chains (0.2% of total Hb) in their erythrocytes (Chui et al 1986, Luo et al 1988).

1.7. The Objective of This Thesis

A monoclonal antibody based immunoassay was developed for screening individuals who are carriers of the ($-\text{SEA}/$) deletion, and to identify couples at risk of conceiving fetuses with Hb Bart's hydrops fetalis syndrome due to homozygous ($-\text{SEA}/-\text{SEA}$) deletion (Luo et al 1989, Ireland et al 1993).

In order to understand mechanisms underlying the disorder of ζ -globin gene expression and to gain insight into embryonic ζ -globin gene regulation, I have constructed mouse-human somatic cell hybrids to examine the events involved in expression of the human embryonic ζ -globin gene, which is in its normal or deletional chromosome setting, in the murine adult erythroid cellular environment. This approach offers the following unique advantages:

1. When mouse erythroleukemia (MEL) cells, which are deficient in adenine phosphoribosyl transferase (APRT), are fused with human cells, only the mouse-human hybrid cells containing the functional human APRT gene are permitted to grow in the selective medium by a complementary mechanism (Deisseroth and Hendrick 1979). While other human chromosomes are gradually lost during culture, human chromosome 16 is selectively retained in the hybrid cells because the human APRT gene is located on chromosome 16q24. Thus, the human α -globin gene cluster with its DNA regulatory sequences on chromosome 16p13 is likely to be preserved in its natural chromosome setting (Morley et al 1991).
2. Genetically identical hemizygote hybrid cells bearing a single human chromosome 16 with the required genotype were readily obtained in large amounts for comparative

studies of human ζ -globin gene expression in various hybrid cell lines under similar conditions *in vitro*.

3. The MEL cells exhibit erythroid commitment and maturation. They provide an adult erythroid environment in which expression of the human chromosomal β - and α -globin genes is coordinately regulated with the endogenous mouse adult globin genes when induced to erythroid differentiation by chemical compounds such as dimethyl sulfoxide (DMSO) and hexamethylene bisacetamide (HMBA) (Zeitlin & Weatherall 1983, Deisseroth & Hendrick 1978a, Chen et al 1982, Fraser & Curtis 1987). Moreover, when human fetal liver erythroid cells that produce Hb F were fused with MEL cells, the hybrids correctly switched to synthesize adult globins as the culture time advanced (Melis et al 1987, Enver et al 1991). These results suggest that mouse erythroid regulatory factors can operate across species to activate the human adult globin genes.

Human ζ -globin mRNA was not detected previously in various interspecific hybrid cells derived from human erythroid or non-erythroid cells (Chiang et al 1984, Anagnou et al 1985, Chamay et al 1984). This may be due to low abundance of ζ -globin gene expression in hybrid cells. In this regard, the challenge of this study was to apply a highly sensitive

assay to quantitate levels of ζ -globin gene expression in various hybrid cell lines.

The studies in this thesis were directed at:

1. Developing a simple and sensitive immunoassay for detecting ζ -globin chains in peripheral blood of the adult carriers with ($--^{SEA}/$) α -thalassemia-1;
2. Constructing MEL x human hybrid cells, containing either normal or ($--^{SEA}$) deletional human chromosome 16 derived from mononuclear cells of normal adults and adult carriers with the ($--^{SEA}/$) deletion, fetal liver hepatic erythroblasts with homozygote ($--^{SEA}/--^{SEA}$) deletion, and human erythroleukemia cell line K562;
3. Quantitative analysis of human ζ -globin mRNA in various hybrids capable of undergoing differentiation in culture when induced with HMBA, DMSO and butyrate;
4. Comparison of human ζ -globin gene expression in hybrid cells with or without the $--^{SEA}$ type α -globin gene deletion in cis;
5. Analysis of correlation of gene expression between the transferred human globin genes and endogenous mouse globin genes during induced erythroid differentiation in order to gain further understanding of human ζ -globin gene regulation.

Chapter 2

EXPERIMENTAL PROCEDURES

2.1. Slot Blot Immunobinding Assay (SBIA)

The anticoagulated peripheral blood 0.1 ml was washed with phosphate buffered saline (PBS) (NaCl 8 g, KCl 0.2 g, Na₂HPO₄ 1.44 g, KH₂PO₄ 0.24 g in 1 litre distilled water, pH 7.4, autoclaved at 120°C for 20 minutes). The cell pellet was lysed in 0.1 ml of water. The Hb concentration was measured by spectrophotometry (Drabkin & Austin 1935). The hemolysate was diluted by Drabkin solution (Sigma, No.525-2) and optical density (O.D.) was measured by absorbance at 540 nm. The amount of Hb present in the hemolysate was calculated from the following equation:

$$[\text{Hb mg/ml}] = 1.46 \times \text{O.D.540} \times \text{dilution factor.}$$

The hemolysate was diluted to 50 µg Hb per ml in TBS [Tris-HCl 10 mmol (pH 8.0), NaCl 150 mmol]. One µg Hb in 20 µl of TBS was blotted by means of a Minifold II Slot-Blotter (Schleicher & Schuell) onto a nitrocellulose membrane pre-wetted with TBS. The membrane was allowed to air dry for 2 hours or overnight. The membrane with blotted samples can be stored in a plastic bag at room temperature for months. The

dried membrane was immersed in TBS with 1% Tween 20 for 30 minutes to block the unbound area and immediately followed by incubation with monoclonal anti- ζ -globin antibody for 30 minutes with gentle agitation. The monoclonal antibody 8E8 was conjugated with alkaline phosphatase (Boehringer Mannheim GmbH, W.-Germany, 1097075) by a protocol as recommended by the manufactures. Just before use, the conjugated antibody (0.1 mg/ml) was diluted 1:100 with TBS containing 1% bovine serum albumin (BSA) and 0.1 % Tween 20. The membrane was washed three times with TBS-0.3% Tween 20 for 15 minutes. Color was developed by adding the substrate solution onto the membrane and incubating at 37°C for 5 to 10 minutes. The substrate solution was prepared freshly each time by sequentially adding 66 μ l nitroblue tetrazolium (NBT) (50 mg/ml in 70% dimethylformamide) and 33 μ l 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml in dimethylformamide) into 10 ml substrate buffer [Tris-HCl 100 mmol (pH 9.5), NaCl 100 mmol, MgCl₂ 5 mmol]. When the desired color intensity was achieved, the membrane was rinsed with water to stop the reaction and allowed to air dry.

In each assay, series of 2-fold diluted Hb fractions with known amount of ζ -globin chains were used as standards. The proportion of ζ -globin in unknown hemolysates can be estimated by visually matching the color intensity with the standards. Alternatively, the color intensity of each slot was

scanned and quantitated by densitometry. The peak height in absorbance units was used for quantitation analysis by a computer analysis program. The results obtained by visual estimation are comparable to those by densitometry (Luo et al 1989).

2.2. Cell Culture

Mouse Erythroleukemia (MEL) Cell Line-179

The MEL-179 (gift of A. Deisseroth) is a tetraploid mouse erythroleukemia cell line, which is deficient in adenine phosphoribosyl transferase (APRT) (Deisseroth et al 1979). The cells were cultured in medium RPMI 1640 with 10% fetal calf serum (FCS) (Gibco), Penicillin 50 units/ml and Streptomycin 50 $\mu\text{g}/\text{ml}$ (Gibco, No.600-5070AG), and 2,6-diaminopurine (DAP) (Sigma, No. D-1627) at 37°C with 5% CO₂ and 100% humidity. DAP was used to select for APRT deficient MEL cells. DAP was prepared as 100 x stock solution and kept at -20°C for a month. Initially, 20 $\mu\text{g}/\text{ml}$ of DAP was used in culture medium. It was gradually raised to and maintained at 100 $\mu\text{g}/\text{ml}$ until the cells became adapted. The MEL-179 cells was subcloned by dilution cloning method. A subclone MEL-10 was chosen for the cell fusion experiment, because it grew well in 100 $\mu\text{g}/\text{ml}$ DAP and its ¹⁴C-adenine incorporation rate was less than 2% of the wild type MEL cells.

Incorporation of ^{14}C -adenine into MEL cells was done as described (Deisseroth et al 1978). 5×10^5 cells were cultured in 1 ml F-12 medium containing 1 μCi 8- ^{14}C Adenine (ICN Radiochemicals, specific activity 56 mCi/mmol) for 24 hours. After washed with PBS twice, the cell pellet was lysed in 0.2 ml H_2O , followed by adding 5 ml 10% ice-cold trichloroacetic acid (TCA) to the cell lysate. The TCA-precipitable ^{14}C -labelled material was collected by filtering the solution through 2.4 cm Whatman GF/C glass-filter disc. The filter was washed with 10% TCA 5 times and 95% ethanol once. After air dry, the filter was immersed into a vial with 5 ml scintillation fluid Aquassure (New England Nuclear) and the radioactivity was counted by β -counter at ^{14}C channel. Incorporation of ^{14}C -adenine into the DAP resistant MEL-179 (APRT-) subclone cells was about 2% of that seen with an equivalent number of APRT+ MEL cells sensitive to DAP.

Human Erythroid Leukemia Cell K562

K562 cells were maintained in RPMI 1640 medium with 10% FCS. Sensitivity of K562 cells to Ouabain was tested before they were used to construct hybrid cells. K562 cells die in medium containing 1 μM ouabain, but MEL cells grow well in the same medium. For analysis of globin mRNA, K562 cells were induced with 40 μM hemin for 3 days.

Mouse-Human Somatic Hybrid Cells

The mouse-human hybrid cells were maintained in AA selection medium. The AA selection medium contains 3.9 $\mu\text{g/ml}$ of adenine (Sigma, No.A-8626) and 5 $\mu\text{g/ml}$ of alanosine (NCI, NSC-153353, Bethesda, Maryland, USA), 20 mM L-glutamine (Gibco, No. 320-5030AG), 10% FCS and penicillin-streptomycin in RPMI 1640 medium. Adenine was prepared as 200 x stock by dissolving 67.6 mg adenine in 50 ml distilled H_2O and sterilized by filtration through a 0.22 μM filter, stored in 10-ml aliquots at -20°C . Alanosine was prepared as 100x stock solution and kept at 4°C for maximum two weeks. Because of karyotypic instability, chromosome loss may occur during continuous culture of hybrid cells. Thus, careful monitoring of the presence of human chromosome 16 is required.

The MEL x K562 hybrid cells were grown in AAO medium in the first month after cell fusion. The AAO medium contains 1 μM ouabain (Sigma, No.O-3125) in AA medium to kill unfused K562 cells and MEL cells. After subcloning, hybrid cells were maintained in AA medium.

2.3. Human Cell Preparation

Mononuclear Cells

The anti-coagulated peripheral blood (30-40 ml) from normal adults or adult carriers heterozygous for ($--^{SEA}/$)

deletion was centrifuged at 300g for 10 minutes at room temperature. The buffy coat was collected into a 50 ml tube with PBS 20 ml and then carefully layered onto a fresh 50 ml tube containing 25 ml Ficoll-Paque (Pharmacia, 10-A-001-07) and centrifuged at 400g for 25 minutes at room temperature (Deisseroth et al 1979). The mononuclear cells at the interface were collected and washed 3 times in the cold PBS with 1% BSA and kept in ice.

Fetal Hepatic Erythroblasts

Human fetal liver tissues were obtained from 20 to 28 week abortuses in Thailand and Hongkong (obtained from Drs. S.Fucharoen and A. Ghosh). The fetuses were diagnosed as hydrops fetalis syndrome due to homozygous ($--^{SEA}/--^{SEA}$) deletion. The fetal liver specimens were obtained from the abortuses at postmortem examination. The specimens were cut into 2 cm³ and kept in 100 ml RPMI 1640 medium with 10% FCS at 4°C and delivered to Canada within 2 days. Upon arrival in our laboratory, the specimens were washed with medium containing antibiotics and chopped into fine pieces in cold medium. After gently shaken the fined tissue in 40 ml of cold medium with 10% FCS. The single cell suspension was collected after the tube was left to stand on ice for 60 minutes (Papayannopoulou et al 1986, Melis et al 1987). Viability of the cells was tested by trypan blue staining. Cell preparation with

viability over 80% was considered to be used for cell fusion experiment. Smears of the cell suspension were prepared and fixed with 100% methanol for 5 minutes for histological staining with Giemsa and May-Grunwald (BDH Inc) as well as immuno-fluorescence staining with the anti- ζ -globin MoAb conjugated with fluorescein isothiocyanate (FITC). In general, fetal liver erythroblasts account for 50% of the nuclear cells (Fig.5) and they were stained positively with the anti- ζ -globin MoAb conjugated with FITC (data not shown).

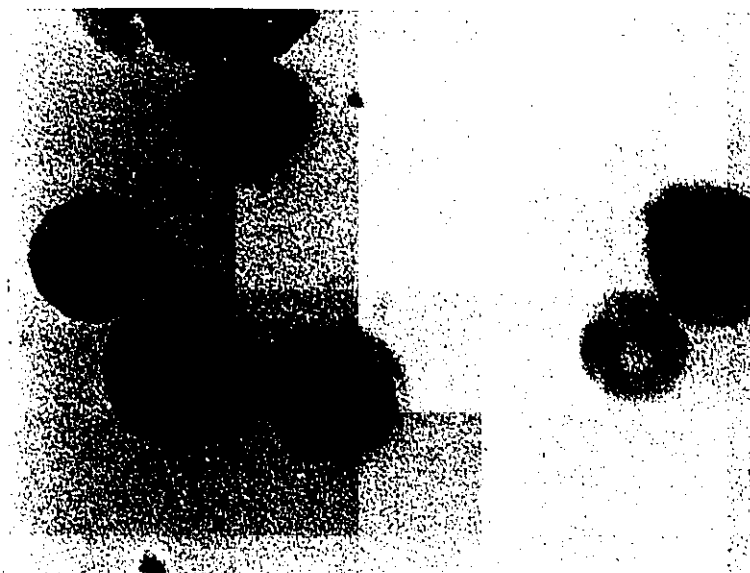


Figure 5: A smear of human fetal hepatic erythro-blasts from a mid-gestation fetus with homozygous ($--^{SEA}/--^{SEA}$) α -thalassemia was stained with Giemsa and May-Grunwald.

2.4. Construction of MEL x Human Cell Hybrids.

The basic protocol for fusion of mouse and human cells is similar to previously published reports (Davidson et al 1976, Deisseroth et al 1979, Papayannopoulou et al 1986, Melis et al 1987). The optimal conditions for cell fusion were tested prior to fusion experiment was carried out, i.e., concentration of polyethylene glycol (PEG), the time of exposure of cells to PEG, and the method for PEG removal. Briefly, the MEL cells were washed 3 times with cold RPMI 1640 medium without serum. The cells were mixed in a ratio of 1:1-2 (MEL : human cells) and pelleted by centrifugation for 5 minutes at 300g. The supernatant was removed and 1 ml of 50% PEG 1500 (Boehringer Mannheim, No. 779512) was slowly added to 10^8 cells in 1 minute and gently mixed. After 1 minute incubation at 37°C, the suspension was diluted by slowly adding 30 ml serum-free RPMI 1640 medium within 5 minutes with swirling (1 ml for the first minute, 2 ml for the second minute, 4 ml for the third minute, 8 ml for the fourth minute and the rest for the last minute). After centrifugation to remove supernatant, the cells were cultured in 100 ml IMDM medium with 20% FCS in flasks for 24 hours. After replacing with AA selection medium, the cells were plated into 96-well plates (3×10^5 cells/0.2 ml/well).

Two to 4 weeks later, the cell colonies can be observed as circular patches of cell aggregates. The cell clones were picked out and transferred to the 6-well plates with 3 ml AA medium per well. The cell clones were initially screened for the human Alu sequences by a DNA slot blot assay. After subcloning by limiting dilution, the positive clones were subjected to Southern blot analysis to identify the human α -globin genotype. The established hybrid cell lines were frozen in aliquot of 90% FCS and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

2.5. DNA Slot Blot Assay

In order to quickly identify the mouse-human hybrid cells from over a hundred cell colonies, a simple and sensitive DNA slot blot assay was first performed. This method only needs little amount of DNA (about 10^5 cells) and it is not necessary to measure the DNA concentration. The culture cells were collected and lysed in 200 μ l lysis buffer [Tris-HCl 50 mM (pH 8.0), ethylene diaminetetraacetic acid (EDTA) 10 mM, NaCl 50 mM, 0.5% sarcosyl, proteinase K 100 mg per ml] at 55°C overnight. After extraction with phenol/ chloroform, DNA was denatured and dotted onto a nitrocellulose or nylon membrane (Rubin & Kan 1985). Alternatively, the cell pellet was lysed in 0.5 ml of 0.4 M NaOH and 10 mM EDTA, heated at

95°C for 20 minutes, then applied to the Zeta-Probe membrane (Bio-RAD) (Reed & Matthaei 1990). The membrane was hybridized to the random primer labelled human Alu probe in 10 ml hybridization solution (7% sodium dodecyl sulfate (SDS), 10% PEG and 1.5 x SSPE) at 65°C for 4 hours or overnight (Waye et al 1989). After hybridization the membrane was washed with 2x SSC solution (20x ssc: 175.3 g NaCl, 88.2 g sodium citrate, pH 7.0, H₂O to 1 litre) with 0.5% SDS at 65°C for 10 to 20 minutes and then exposed to an X-ray film. The cell clones positive for the human Alu sequences were subjected to Southern blot analysis of the human α -globin gene cluster.

2.6. Southern Blot Analysis

DNA was prepared from hybrid cells by proteinase K digestion and phenol/chloroform extraction (Maniatis et al 1982). DNA (20 μ g) was digested with BamH I using conditions recommended by the manufacturer. The digested DNA fragments were fractionated by electrophoresis on 0.7% agarose gel, and transferred onto nitrocellulose or nylon membrane by the method of Southern (Southern 1975, Maniatis et al 1982). The membrane was hybridized to the random primer labelled probes: the human α -globin cDNA probe is a 1.6 kb Mbo II fragment of JW 101 plasmid; the human ζ -globin cDNA probe is a 2.0 kb Hinf I fragment of the plasmid p ζ ; and the human chromosome 16

centromere specific probe is pSE16-2 (Greig et al 1989). After hybridization at 65°C overnight, the membrane was washed with high stringency buffer (0.1 x SSC, 0.1% SDS at 65°C) for 20-30 minutes and exposed to an X-ray film with intensifying screen at -70°C. The --^{SEA} deletion was characterized by a 20 Kb fragment instead of a normal 11 Kb fragment when probed with human ζ -globin cDNA, and lacking a 14 kb fragment when probed with human α -globin cDNA (Chui et al 1986). Human chromosome 16 is characterized as a major 1.7 kb EcoR V fragment probed with pSE16-2 (Greig et al 1989).

2.7. Chromosomal Analysis

Six hybrid cell lines growing in log-phase were exposed to 0.02% colcemid (Gibco) for 30 minutes at 37°C. The metaphase spreads were prepared after treating the cultures to hypotonic solution and fixative. These slides were then exposed to trypsin and Giemsa stain so as to generate GTG banding patterns to identify human chromosome 16 in the hybrid cells (Seabright 1971).

2.8. Induction of Erythroid Differentiation

The cells growing in log-phase were induced with 1% dimethyl sulphoxide (DMSO), 5 mM hexamethylene bis-acetamide

(HMBA) (Sigma, No.H-6260), or 1-2 mM butyrate (sodium butyrate, Sigma, No.B-5887). After exposed to inducers for various days, cells were collected for RNA extraction. About 1/10 of induced cells were washed and re-cultured in fresh medium without inducers for additional 5 days before RNA extraction. Total cell number for each induction experiment was calculated and cell viability was determined by trypan blue.

2.9. RNA Preparation

Total cellular RNA was extracted from $1-2 \times 10^8$ cultured cells using guanidinium/cesium chloride method (Maniatis et al 1982). Briefly, the cell pellet was washed with cold PBS once and lysed in 7 ml guanidinium solution (4 M guanidine thiocyanate, 0.1 M β -mercaptoethanol, 25 mM sodium citrate, pH 7.0) and vortexed vigorously for one minute. The cell lysate was forced to pass through a 18 gauge needle about 10 times to shear DNA, then gently layered over 2.7 ml of cesium chloride solution (5.7 M cesium chloride, 100 mM EDTA) in a polyallomer centrifuge tube (14 x 89 mm, Beckman, 331372). The centrifugation was performed at 26,000 rpm for 20 hours at 20°C using a SW41 or SW40 rotor (Beckman). The supernatant was aspirated carefully. RNA pellet at bottom of the tube was dissolved in 1 ml TES solution [Tris-HCl 10 mM

(pH 7.4), EDTA 1 mM, 0.5% SDS] and stored at -70°C . The concentration of RNA was measured by spectrophotometer at 260 nm wavelength and calculated from the following equation:

$$\text{RNA } \mu\text{g/ml} = 40 \times \text{O.D.}_{260} \times \text{dilution factor.}$$

2.10. Oligo-dT Column Chromatography

The poly(A) rich mRNA was prepared from total RNA by a single cycle of oligo-dT cellulose chromatography. One gram of oligo-dT cellulose type 7 (Pharmacia, 27-5543-02) was washed sequentially with H_2O once, 0.1 N NaOH once and H_2O 3-4 times by spinning at 300g for 5 minutes until pH <8. It was suspended in 10 ml binding buffer [Tris-HCl 10 mM (pH 7.4), EDTA 1 mM, NaCl 0.5 M]. One ml of 10% oligo-dT cellulose was aliquoted into a 2 ml Eppendorf tube. After centrifuging at 3000 rpm for 4 minutes in an Eppendorf centrifuge, the supernatant was removed and 1 mg of RNA in 1.2 ml binding buffer was added to the oligo-dT cellulose pellet and mixed for 2 hours at room temperature. After washing the gel with 400 μl low salt buffer [Tris-HCl 10 mM (pH 7.4), EDTA 1 mM , NaCl 0.1 M] for 3 times, the bound mRNA was eluted with 250 μl elution buffer [Tris-HCl 10 mM (pH 7.4), EDTA 1 mM] at 60°C 4 times. The RNA in the eluate was precipitated by adding 0.1 ml 6x binding buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 3.0 M NaCl], 10 μl glycogen (20 mg/ml), 2.5 ml cold 100% ethanol and

keeping at -20°C overnight. The RNA was collected by centrifugation at 11,000 rpm for 15 minutes at 4°C . The pellet was washed with 70% ethanol once, dried by air, then dissolved in sterilized deionized distilled H_2O and stored at -70°C . In general, mRNA could be enriched 5 to 10-fold after a single cycle of oligo-dT column selection.

2.11. S1 Nuclease Protection Assay

2.11.1. Probe Preparation

Five probes were used for S1 nuclease protection assay (Table 1): (1) a human ζ -globin cDNA probe, a 0.52 Kb *Ava* I /*Pvu* II digested fragment of pEGM-16H ζ plasmid, labelled at the 3' end and giving a protected fragment of 320 nt; (2) a mouse ζ -globin gene probe, a 0.179 Kb *Ava* II/*Xba* I digested fragment of Sp ζ -M ζ plasmid (a gift of T. Enver), labelled at the 5' end and giving a protected fragment of 151 nt (Baron and Maniatis 1986); (3) a human α 1-globin cDNA probe, a 0.55 Kb fragment of JW 101 plasmid digested with *Hind* III, labelled at the 3' end and giving a protected fragments of 268 nt and 172 nt for the human α 1- and α 2-globin gene transcripts respectively. The shorter length of the α 2-globin mRNA protected fragment is due to the presence of nucleotide sequence differences between α 1 and α 2 globin mRNAs at 3'

untranslated region (Orkin et al 1981). (4) a mouse α -globin gene probe, a 0.45 Kb *BstE* II/*Sac* I digested fragment of plasmid NP42, labelled at 3' end and giving a protected fragment of 200 nt (Helder et al 1987); (5) a mouse β_2 -microglobulin ($M\beta_2$ -MG) gene probe (gift of T. Ley), a 1.9 kb *EcoR* I digested fragment, labelled at 5' end and giving a protected fragment of 208 nt (Potter et al 1987).

Table 1. DNA Probes Used in S1 Nuclease Protection Assay

Plasmid	Description of Probes (Kb)	Enzyme Digestion	³² p- Labelling	Protected Fragment (nt)
pGEM-H ζ -16	Human ζ -globin cDNA (0.52)	Ava I/ Pvu II	3' end Klenow	320
pSP64-M ζ	Mouse ζ -globin gene -46 to 151 (0.197)	Ava II/ Xba I	5' end T4 Kinase	151
JW 101	Human α -globin cDNA (0.55)	Hind III	3' end Klenow	α 1 268 α 2 172
NP42	Mouse α -globin gene exon 3 (0.45)	<i>BstE</i> II/ <i>Sac</i> I	3' end Klenow	200
pB2mdIII.B	Mouse β_2 -microglobulin gene exon 2 (1.9)	<i>EcoR</i> I	5' end T4 Kinase	208

2.11.2. Probe Labelling

3' end-Labelling

The probes specific for human ζ -, human α - and mouse α -globin mRNAs were digested with appropriated restriction endonucleases (Tab.1) and labelled by fill-in 3' end with [α - 32 P] deoxycytidine triphosphates (dCTP) (specific activity 6000 Ci/mmole, Amersham, PB 10475) by Klenow DNA polymerase as described (Maniatis et al 1982). Briefly, 5-10 μ g of DNA probes were mixed with the following solution to final volume of 20 μ l :

2 μ l of solution A [1.25 M Tris-HCl (pH 8.0), 0.125 M MgCl₂, 1.8% β -mecaptoethanol, 0.5 mM of each dATP, dGTP and dTTP];

1 μ l of bovine serum albumin (BSA) (1 mg/ml);

5 μ l of [α - 32 P] dCTP;

3 units of klenow DNA polymerase.

After incubating for one hour at 37°C, DNA was precipitated by 2.5 volume 100% ethanol and 0.1 volume of 3 N NaOAc at -70°C for one hour, followed by centrifugation at 14,000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol twice and allowed to air dry.

5' end-Labeling

The probes specific for mouse ζ -globin and M β_2 -MG mRNAs were prepared by digesting with appropriate restriction endonucleases, dephosphorylating with calf intestinal phosphatase, and labelling with [γ - 32 P] ATP (adenosine triphosphate) (specific activity 5000 Ci/mmol, Amersham, PB.10218) by T4 polynucleotide kinase. 1 or 2 μ g of DNA probe was mixed with 5 μ l of T4 kinase buffer [10 x: 0.6 M Tris-HCl (pH 7.8), 0.1 M MgCl₂, 0.15 β -mercaptoethanol], 5 μ l of [γ - 32 P] ATP, 10 units of T4 polynucleotide kinase, and H₂O to a final volume of 50 μ l. The mixture was incubated at 37°C for 2 hours, then applied to 1 ml Sephadex G-50 spun-column and centrifuged at 300g for 5 minutes to remove un-incorporated isotope. The labelled probe in eluate was precipitated by ethanol as before.

Sephadex G-50 column was prepared by equilibrating Sephadex G-50 (Medium, DNA grade, Pharmacia, Sweden, No. 17-0045-02) in TNE buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA] overnight. After replacing with fresh TNE buffer, Sephadex G-50 was autoclaved at 120°C for 10 minutes.

T4 DNA polymerase Labelling

One μ g of 1 kb DNA ladder (BRL) was labelled by T4 DNA polymerase as recommended (BRL). The reaction mixture includes 0.5 μ g of DNA, 4 μ l of reaction buffer [5x: 165 mM Tris

acetate (pH 7.9), 330 mM sodium acetate, 50 mM magnesium acetate, 2.5 mM dithiothreitol (DTT), BSA 0.5 mg/ml], H₂O to 29 μ l and 1 μ l of T4 DNA polymerase. The exonuclease reaction was carried out at 37°C for 2 minutes and stopped by cooling in ice. After sequentially adding 6 μ l of reaction buffer, 2 mM of each dATP, dTTP and dGTP, 5 μ l of 20 μ M dCTP, 10 μ l of [α -³²P]dCTP, the polymerase reaction was carried out at 37°C for 25 minutes, followed by incubation for additional 15 minutes after adding 1 μ l of 2 mM dCTP. The reaction mixture was applied to Sephadex G-50 spun-column and centrifuged at 300g for 5 minutes. The eluate was collected and stored at -20°C.

Single Strand DNA Probe Preparation

The ³²P-labelled double strand DNA probes (except M β 2-MG) were dissolved in 20 μ l gel loading buffer (DMSO 3 ml, 0.5 M EDTA 20 μ l, bromophenol blue 50 mg, xylene cyanal 50 mg, H₂O to 10 ml) and denatured by heating at 90°C for 5 minutes. They were separated to single strands by electrophoresis on a 5% polyacrylamide gel (size of 240 x 80 x 1.5 mm) at 110 volts for 14 hours in 1/2 TBE buffer [5 x TBE: 59 g Tris base, 27.5 g boric acid, 0.5 M EDTA 20 ml (pH 8.0), H₂O to 1 litre]. An antisense strand of a probe was identified by exposure the gel to an X-ray film for 5 minutes. The bands corresponding to the antisense strands of the probes were located and cut off from

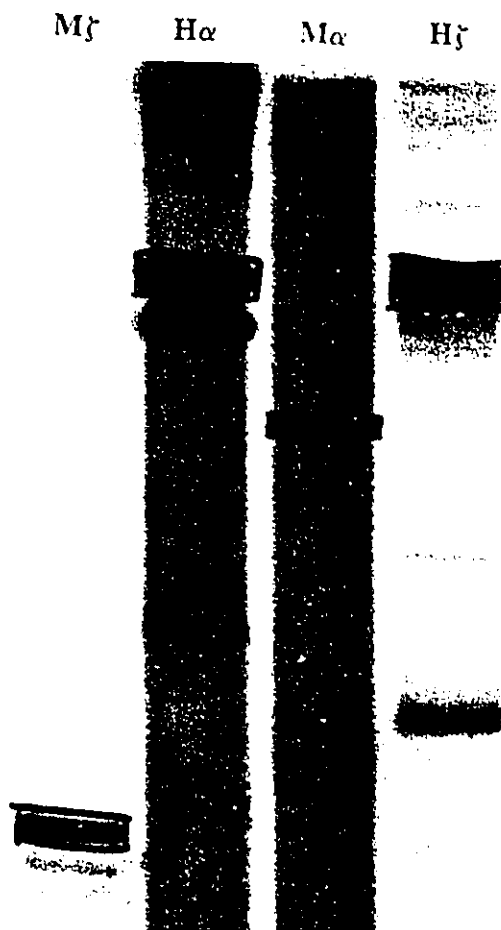


Figure 6. Separation of single strand DNA probes on 5% polyacrylamide gel. The ^{32}P -labelled double strand DNA probes were denatured to single strands and separated by electrophoresis. The bands corresponding to the antisense strands of the probes were marked by black boxes. $\text{M}\zeta$, mouse ζ -globin gene probe; $\text{H}\alpha$, human α -globin gene probe; $\text{M}\alpha$, mouse α -globin gene probe; $\text{H}\zeta$, human ζ -globin gene probe.

the gel (Fig.6). The gel slide was immersed in 2 ml elution buffer (0.5 M NH_4OAc , 10 mM MgOAc , 1 mM EDTA, 0.1% SDS, tRNA 1 $\mu\text{g/ml}$) for 3 hours or overnight. The eluted DNA probes in the solution were precipitated by ethanol and dissolved in 50 μl hybridization solution.

2.11.3. Hybridization and S1 Nuclease Digestion

S1 nuclease assay was carried out essentially as described previously (Anagnou et al 1985). All solutions were sterilized by filtration through 0.2 μm membrane or autoclaving at 120°C for 20 minutes after treatment with 0.1% diethyl pyrocarbonate (DEPC). The 1.5 ml Eppendorf tubes for hybridization reaction were silicolized and autoclaved. For analysis of human and mouse ζ -globin mRNAs, 30 μg of each mRNA sample was hybridized with the specific probe of 10^4 cpm in 20 μl of hybridization cocktail [5x: 3 M NaCl, 0.1 M Tris-HCl (pH 7.5), 5 mM EDTA]. For analysis of human and mouse α -globin mRNAs, 0.2 μg of each mRNA sample in 10 μl of the hybridization cocktail was used in each reaction. After heated at 90°C for 8 minutes, the hybridization reaction was performed at 45°C overnight. 1 μg of each mRNA sample was hybridized with the $\text{M}\beta_2$ -MG probe of 10^5 cpm in 10 μl 80% formamide hybridization cocktail [80% deionized formamide, 0.5 M Pipes (pH 6.4), 4 M NaCl, 50 mM EDTA] at 51°C

overnight. All assays were performed in probe excess. After hybridization, 40-80 units of S1 nuclease (Pharmacia, 270-920-01) in cold S1 buffer [10x S1 buffer: 280 mM NaCl, 30 mM NaOAc (pH 4.5), 4.5 mM Zn₂SO₄] was added to each sample to a final volume of 150 μ l and incubated at 37°C for 40 minutes. The S1 nuclease digestion was stopped by adding 2.5 volume 100% cold ethanol and kept at -70°C for 1 hour. After centrifugation, the pellet was dried and dissolved in 10 μ l loading buffer (80% formamide, 0.5% bromophenol blue and xylene cyanol) for electrophoresis on a urea polyacrylamide gel.

2.11.4. Urea Polyacrylamide Gel

For double gels (size of 160 x 80 x 1 mm), 45 ml of gel solution was prepared by mixing 4.5 ml 40% acrylamide stock solution (acrylamide 38 g, N'N'-methylenebisacrylamide 2 g, H₂O to 100 ml, sterilization by filtration through a filter with 0.45 μ M pore size), 39 ml urea mixture (8 M urea, 1x TBE), 0.4 ml ammonium persulfate, and 15 μ l Temed. The gel solution was poured into a gel apparatus and allowed to polymerize for 1 hour. The gel was pre-run for 1 hour at 250 volts in 1x TBE buffer. The samples in 10 μ l loading buffer were heated at 90°C for 3 minutes and loaded onto the gel. Electrophoresis was carried out at 250 volts for 2 hours until

the dye bromophenol blue run off the gel. The gel was dried and exposed to an X-ray film (Kodak, XAR 5) using the intensifying screens at -70°C for 3-4 weeks to observe the ζ -globin mRNA protected fragments, 1-3 days for the α -globin mRNA signals.

2.12. Subcloning of the Human ζ -Globin cDNA

In attempt to quantitate human ζ -globin mRNA in hybrid cells, the human ζ -globin cDNA was subcloned into pGEM-4Z vector (Promega) for synthesis of large amount of human ζ -globin mRNA *in vitro*.

Construction of Recombinant DNA Clones

The human ζ -globin cDNA fragment of 0.5 kb was isolated from a P ζ plasmid digested with *Pst* I (gift of A. Deisseroth). The pGEM-4Z vector (Promega) was digested with *Pst* I and dephosphorylated with alkaline phosphatase. The molar of vector and the human ζ -globin cDNA insert were in a ratio of 1:3. The ligation reaction was carried out by T4 DNA ligase in 15 μl at 4 $^{\circ}\text{C}$ overnight. T4 DNA ligase buffer (10x) contains 0.4 M Tris-HCl (pH 7.5), 0.1 M MgCl_2 , 100 mM DTT, 10 mM ATP, BSA 500 $\mu\text{g/ml}$.

Transformation of Competent *E. coli* JM 109

The constructs were transformed into *E. coli* JM 109 (Maniatis et al 1982). Briefly, a single colony of JM 109 on a minimal glucose plate was inoculated into 3 ml YT medium (8 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl, in 1 litre distilled H₂O, pH 7.2-7.4) and cultured at 37°C overnight. The culture was diluted 1:150 and cultured at 37°C in a shaker until A₆₀₀ is 0.4 (3 hours). To prepare the competent cells, the culture was chilled on ice for 10 minutes. After centrifugation at 400g for 10 minutes at 4°C, the cells were resuspended in 50 ml cold CaCl₂ solution [50 mM CaCl₂, 10 mM Tris-HCl (pH 8.0)] and kept in ice for 20 minutes. The cells were collected by centrifugation and re-suspended gently in 6 ml cold CaCl₂ solution. 0.3 ml of CaCl₂ treated cells was mixed with 0.2 µg DNA constructs and incubated for 30 minutes at 4°C, followed by heat shock at 42°C for 2 minutes. The cells were incubated in 1 ml LB broth (Luria-Bertay broth: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl in 1 litre distilled H₂O, pH 7.5, autoclaved at 120°C for 15 minutes) at 37°C for 1 hour. The cells were re-suspended into 0.2 ml LB, 10 µl IPTG (100 mM isopropylthio-β-D-galactoside in H₂O) and 50 µl X-Gal (2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethylformamide) and applied 0.1 ml to each LB agar plate (30 ml of 15% Bacto-agar LB broth with 50 µg/ml ampicillin in each 100 mm Falcon

petri dish). The cells were spread to cover the surface of the plate and incubated at 37°C overnight.

Identification of Transformants with the Recombinant DNA

The transformants with the recombinant human ζ -globin cDNA plasmid were first identified as white cell colonies because insertion inactivated lacZ α peptide. They were picked and grown in 5 ml LB at 37°C overnight for restriction enzyme analysis. Minipreparation of plasmid DNA was done (Maniatis et al 1982). 1 ml cell culture was transferred into 1.5 ml tube and the cells were collected by centrifugation. The cell pellet was resuspended in 0.1 ml lysis buffer [25 mM Tris-HCl, (pH 7.5), 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme] and incubated on ice for 30 minutes. The cells were lysed in 200 μ l of alkaline SDS (0.2 N NaOH and 1% SDS) for 5-10 minutes, followed by adding 150 μ l 3 M potassium acetate (pH 4.8) and incubating for 60 minutes on ice. After spinning in Eppendorf centrifuge for 5 minutes, the supernatant was extracted with phenol/chloroform and DNA was precipitated with ethanol twice. The DNA pellet was dissolved in 40 μ l of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and analyzed by restriction enzyme digestion and gel electrophoresis.

Plasmid DNA Preparation

The transformants were cultured in 250 ml LB medium with ampicillin at 37°C with shaking overnight. The cells were collected by centrifugation at 5000g for 15 minutes at 4°C. The cell pellet was re-suspended in 6 ml of lysis buffer and incubated in ice for 20 minutes, followed by adding 12 ml alkaline SDS solution and kept in ice for 10 minutes. After adding 7.5 ml of 3 M sodium acetate (pH 4.6) the cell lysate was centrifuged at 10,000g for 15 minutes at 4°C. The supernatant containing the plasmid DNA was removed to a fresh tube and DNA was precipitated by ethanol (Maniatis et al 1982).

2.13. Quantitation of Human ζ -Globin mRNA

Preparation of RNA Standard

In order to obtain large amount of human ζ -globin mRNA as a standard for quantitation, the pGEM-H ζ -16 plasmid was linearized with *Hind* III and transcribed by SP6 RNA polymerase using the Promega *in vitro* transcription kit. The synthesized RNA was treated with RNase-free DNase RQ1 (1 unit/ μ g DNA) at 37°C for 10 minutes to remove the DNA template, and then purified by Sephadex G-50 spun-column to remove nucleotides. The flow-through RNA was extracted with phenol/chloroform and precipitated with ethanol. The RNA pellet was dissolved in TE

buffer and the concentration of RNA was measured by spectrophotometry at 260 nm wavelength. However, the measurement of absorbance to determine absolute RNA quantities may be subject to artifacts due to contamination of undigested DNA template and unincorporated nucleotides. To eliminate this possibility, the purity of the synthesized ζ -globin mRNA was documented by electrophoresis on 2% agarose gel together with known amount of rabbit globin mRNA (BRL, 8103SB). The agarose gel (2% agarose in 1x TBE with 0.4 μ g ethidium bromide) was pre-run for 1 hour and run for 1.5 hours at 50 volts at 4°C. The signals stained with ethidium bromide were recorded by photography and the density of each band was scanned by densitometer UltroScan XL (Bio-RAD).

Calculation of mRNA copy number

The synthesized human ζ -globin mRNA is 560 nt in length. It has molecular weight of 1.9×10^5 (560 nt x 340). The copy numbers of the synthesized ζ -globin mRNA molecule per ng RNA was calculated by the following formula:

$$\frac{6 \times 10^{23}}{1.9 \times 10^5 \times 10^9} = 3.2 \times 10^9 \text{ copies/ng.}$$

The synthesized human ζ -globin mRNA was diluted in H₂O with 1 μ g/ μ l tRNA. The aliquots were stored at -70°C and used as a standard for each S1 nuclease assay. Density of the

protected fragments on the X-ray films correspond to *in vitro* synthesized ζ -globin mRNA (330 nt) and *in vivo* synthesized ζ -globin mRNA (320 nt) were scanned by densitometer. The amount of ζ -globin mRNA molecules in each RNA sample was calculated by computer Minitab regression program.

In order to provide comparable data for ζ -globin mRNA levels in various hybrid cell lines, mRNA samples were normalized by mouse β_2 -microglobulin mRNA levels to correct variations which could arise from different recoveries during RNA preparation and oligo-dT selection. There was little variation on the M β_2 -MG mRNA levels between various hybrid cell lines with or without induction. As internal control of induction, mouse and human α -globin mRNAs were also analyzed for each sample by S1 nuclease assay.

Two parameters are required for calculating the concentration of human ζ -globin mRNA: (1) total ζ -globin mRNA copies in each hybridization reaction, which were estimated by the synthesized human ζ -globin mRNA standard; (2) amount of total cellular RNA in each reaction, which was calculated by the known RNA standard. Then, the concentration of ζ -globin mRNA in each sample was calculated by the following equation:

$$\text{mRNA copies}/\mu\text{g RNA} = \frac{\zeta\text{-globin mRNA copies in a reaction}}{\text{Amount of total cellular RNA } (\mu\text{g})}$$

Chapter 3

RESULTS

3.1. Human ζ -Globin Chains in α -Thalassemias

A simple and sensitive method, slot blot immunobinding assay (SBIA), was developed to screen and diagnose carriers of ($--^{SEA}/$) deletion. This method can detect as little as 0.04% of ζ -globin chains in total Hb (Fig.7).

I	% of ζ Globin Chains	II	Genotype
—	2.8%	—	$-\alpha^{3.7}/-^{SEA}$
—	1.4%	—	$\alpha^{CS}\alpha/-^{SEA}$
—	0.7%	—	$\alpha\alpha/-^{SEA}$
—	0.35%		$-\alpha^{3.7}/-\alpha^{3.7}$
—	0.18%		$-\alpha^{3.7}/\alpha^{CS}\alpha$
—	0.09%		$-\alpha^{3.7}/\alpha\alpha$
—	0.04%		$\alpha^{CS}\alpha/\alpha\alpha$
—	0%		$\alpha\alpha/\alpha\alpha$

Figure 7: SBIA for human embryonic ζ -globin chains. 1 μ g of hemolysate was blotted to nitrocellulose membrane and reacted with anti- ζ -globin MoAb 8E8 conjugated with alkaline phosphatase. Color was developed by incubation with NBT and BCIP. Column I, 1 ug of hemolysates with known amounts of ζ -globin chains; column II, 1 ug of hemolysates from adults with known α -globin genotypes. Note that only those blood samples from individuals who were carriers of the ($--^{SEA}/$) deletion were positive for ζ -globin chains. (From Luo et al 1989).

Hemolysates from 131 individuals were analyzed by SBIA. The results are comparable with previous observations by liquid phase radioimmunoassay (RIA) using rabbit polyclonal anti- ζ -globin antibodies (Luo et al 1988). The results of 80 individuals with known ζ - α -globin genotypes are summarized in Table 2.

Table 2. ζ -Globin Chains in Carriers of α -Thalassemias

α -Globin Genotype	No. of Subjects	ζ -globin in Hemolysate Mean \pm SD (%)	Range of ζ -globin in Hemolysate (%)
$\alpha\alpha/\alpha\alpha$	14	Undetectable	Undetectable
$-\alpha^{3.7}/\alpha\alpha$	10	Undetectable	Undetectable
$-\alpha^{3.7}/-\alpha^{3.7}$	6	Undetectable	Undetectable
$\alpha\alpha/--SEA$	20	0.20 ± 0.09	0.06 - 0.43
$\alpha^{CS}\alpha/--SEA$	4	0.31 ± 0.23	0.19 - 0.66
$-\alpha^{3.7}/--SEA$	6	0.27 ± 0.05	0.19 - 0.33
$--SPN/-\alpha^{3.7}$	2	0.12, 0.14	
$--MED/-\alpha^{3.7}$	4	0.04 ± 0.02	0.04 - 0.06
$--MED/\alpha\alpha$	2	Undetectable 0.06	
$--BRIT/\alpha\alpha$	6	Undetectable	
$--BRIT/-\alpha^{3.7}$	2	Undetectable 0.06	
$--BR/\alpha\alpha$	1	Undetectable	
$(\alpha\alpha)^{20.5}/\alpha\alpha$	2	Undetectable	
$--SA/\alpha\alpha$	1	Undetectable	

(From Luo et al 1989, Tang et al 1992).

In 30 individuals whose genotypes were either normal, heterozygous for the one rightward α -globin gene deletion ($-\alpha^{3.7}/\alpha\alpha$), or homozygous for the rightward α -globin gene deletion ($-\alpha^{3.7}/-\alpha^{3.7}$), ζ -globin chains were not detected in their hemolysates. On the other hand, in 30 individuals who are carriers of two α -globin gene deletions with the ($--^{SEA}/$) genotype, ζ -globin chains were present in their hemolysates with a mean of 0.3%. The ζ -globin chains were also present in most carriers with two α -globin gene deletions of the $--^{SPAN}$, $--^{MED}$ genotypes, but not detectable in almost all $--^{SA}$ and $--^{BRIT}$ carriers (Tang et al 1992).

3.2. Characteristics of MEL x Human Cell Hybrids

Since MEL revertants (APRT+) can grow in AA selection medium, the DNA slot blot assay was applied to screen hybrid cells for human Alu sequences. This assay needs less than 10^5 cells to generate a positive signal (Fig.8). A total of 30 independent cell colonies positive for the human Alu sequences were obtained from seven cell fusion experiments. All of them also showed the presence of human chromosome 16 centromere specific sequences (Fig.9).

After subcloning, the Alu positive cell clones were subjected to Southern blot analysis to identify human α -globin genotype. Among 30 hybrids, 29 showed the presence of the

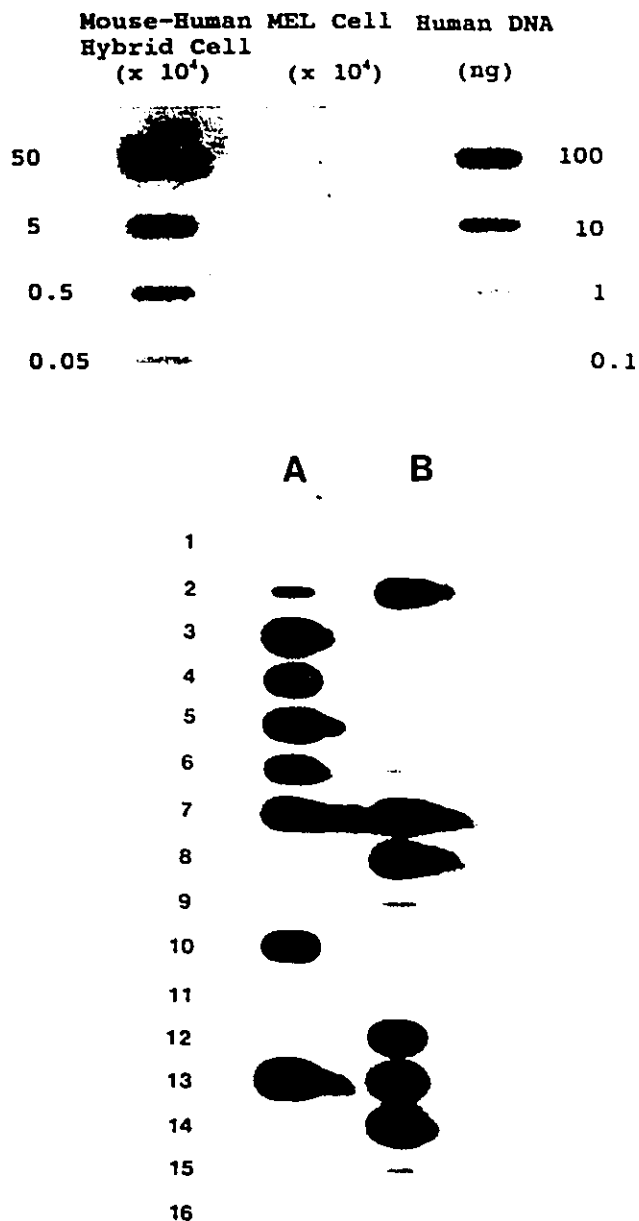


Figure 8: DNA slot blot assay for screening mouse-human hybrid cells.

Upper figure: DNAs extracted from known amount of mouse-human hybrid cells (left column) and MEL cells (middle column), and human DNA (right column) were dotted onto the Zeta-probe membrane and hybridized to the human Alu probe.

Lower figure: DNA extracted from 10⁵-10⁶ cells was dotted onto a nitrocellulose membrane and hybridized with the probe specific for human Alu sequence. A1, a negative control (1 μg DNA from MEL cells); A2, a positive control (1 μg DNA from a human-mouse hybrid cell line). The rest slots are DNAs of testing samples.

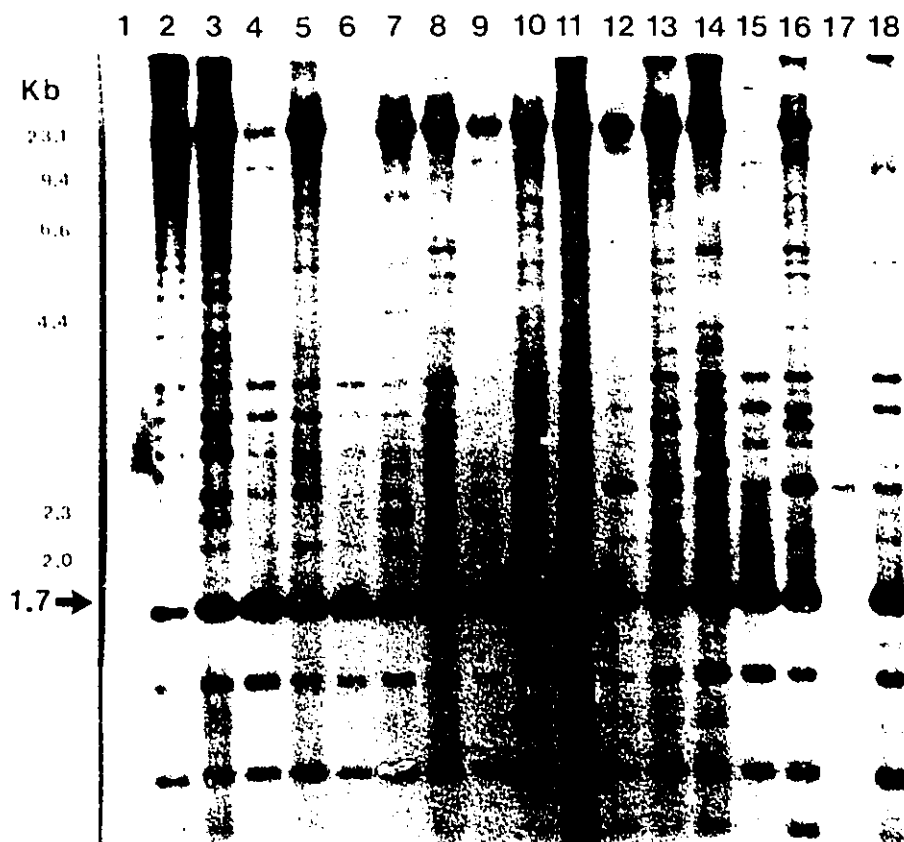


Figure 9: Southern blot analysis of human chromosome 16 centromere specific sequences in mouse-human hybrid cells. 5 μ g of DNA from mouse (lane 1), human (lane 2) and human-mouse hybrid cells (lane 3 to 18) were digested with *EcoR* V, and further hybridization was performed with D16Z3 insert D16SE as probe.

functional human ζ -globin gene, which is indicated by a 5.9 kb *BamH* I fragment probed with the human ζ -globin cDNA probe (Fig.10B). One cell line that showed lack of the 5.9 kb band may have a partial deletion on the short arm of human chromosome 16 because it is positive for the human chromosome 16 centromere specific probe. Ten of 29 hybrids had a normal α -globin gene cluster, which was indicated by a 10 or 11 Kb pseudo ζ -globin gene fragment (Fig.10B). The 19 other hybrids showed a 20 Kb fragment which indicated abolition of the *BamH* I site downstream of the pseudo ζ -globin gene as a result of the two α -globin gene deletion (Fig.10B). This was proven by lack of human α -globin gene signal in these 19 hybrids hybridized with the human α -globin gene probe. The other 10 hybrids showed a normal α -globin gene fragment of 14 Kb (Fig.10A).

Most of the hybrid cells were stable with continuous culture in AA medium over a year, except two of them lost human chromosome 16. The karyotype was done for 6 cell lines. Five of them showed the presence of a single human chromosome 16 (Fig.11). One cell line had no human chromosome 16 although it showed the normal human α -globin gene cluster in Southern blot analysis. This cell line may have undergone chromosomal rearrangement.

A total of 29 hybrid cell lines bearing human chromosome 16 are summarized in Table 3. They are classified

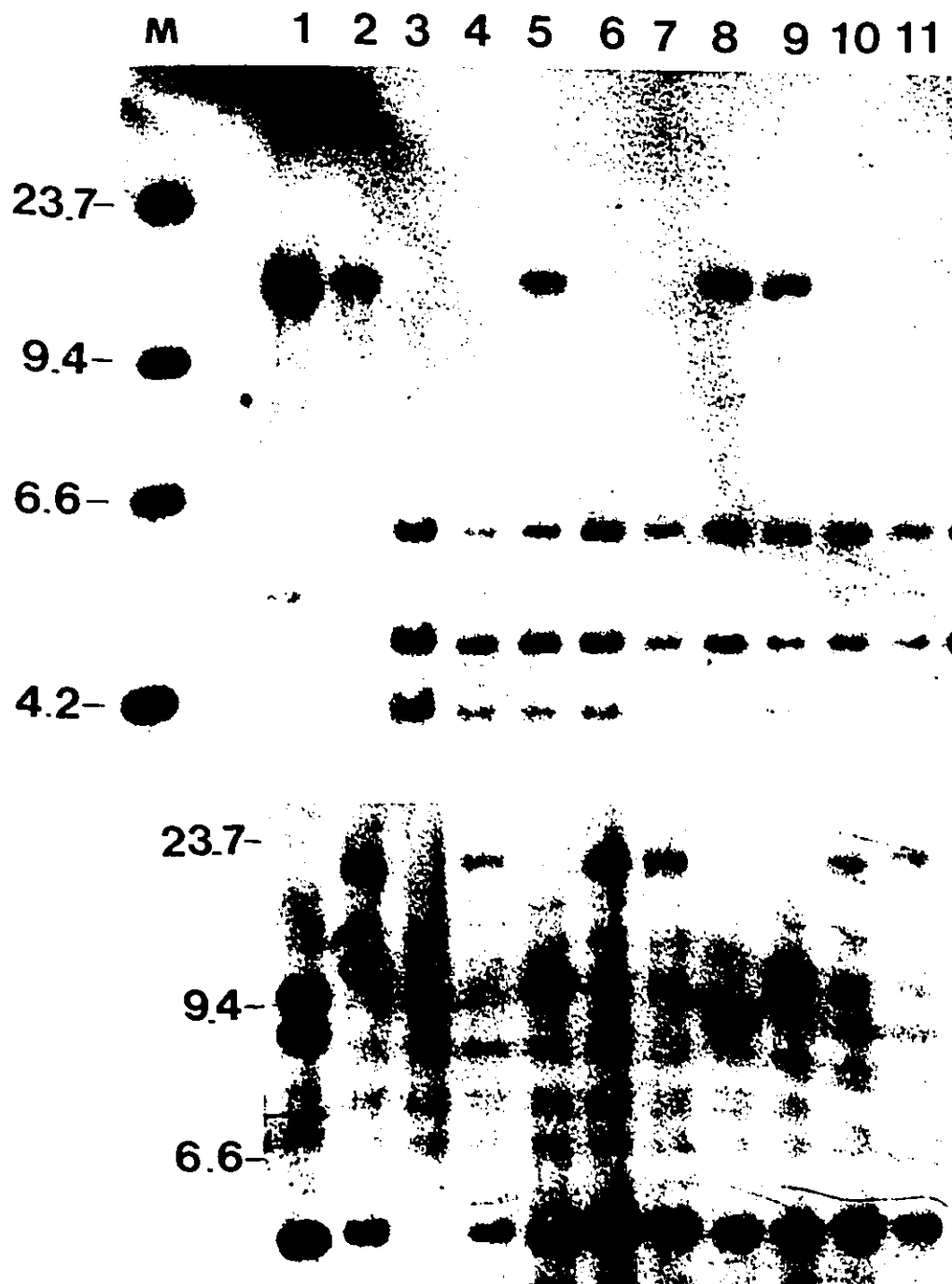


Figure 10: Southern blot analysis of human α -globin genotype of mouse-human hybrid cells. 20 μ g of DNA digested with *Bam*H I was fractionated on a 0.7% agarose gel, blotted onto a nitrocellulose membrane and hybridized with the human α -globin gene probe (A) and the human ζ -globin gene probe (B).

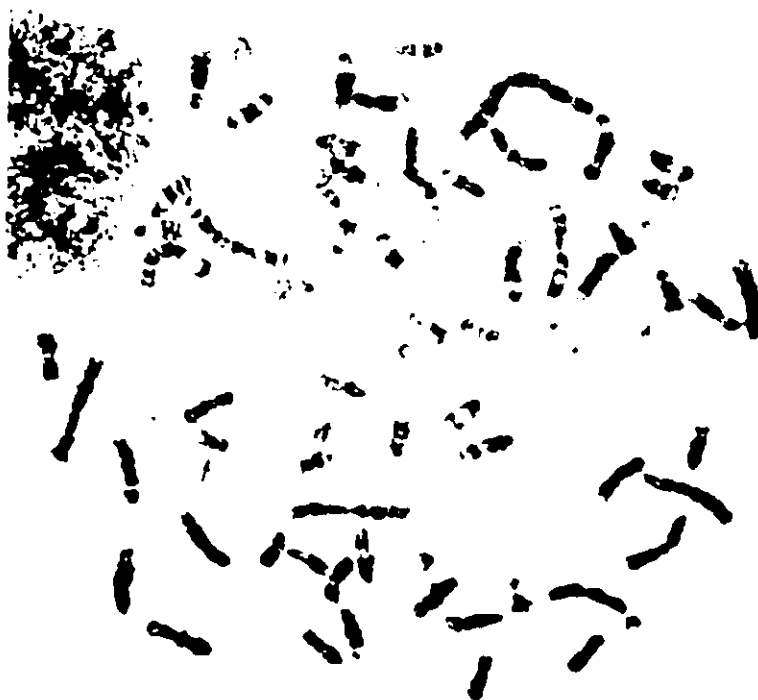


Figure 11: Metaphase spread of hybrid cells derived from fusion of the tetraploid MEL cell with the K562 cell was subject to Giemsa-trypsin (GTG) banding. The human chromosome 16 was indicated by an arrow.

into five groups based on sources of donor cells and genotype of human α -globin gene cluster (Table 3).

- A. a hybrid containing a normal human chromosome 16 derived from peripheral blood mononuclear cells (MNC) of a normal adult with the genotype of $(\alpha\alpha/\alpha\alpha)$;
- B. a hybrid containing a human chromosome 16 with the normal α -globin gene cluster derived from MNC of an adult carrier with the $(--^{SEA}/\alpha\alpha)$ genotype;
- C. a hybrid containing the $--^{SEA}$ deletional human chromosome 16 derived from MNC of an adult carrier with the $(--^{SEA}/\alpha\alpha)$ genotype;
- D. a hybrid containing the $--^{SEA}$ deletional human chromosome 16 derived from fetal hepatic erythroblasts (HFHE) of 20 to 25 weeks fetuses with homozygous $(--^{SEA}/--^{SEA})$ α -thalassemia;
- E. a hybrid containing a normal human chromosome 16 derived from a human erythroleukemia cell line K562, which has the normal $(\alpha\alpha/\alpha\alpha)$ genotype. The K562 cells synthesize large amount of human embryonic and fetal Hbs but not adult Hb A (Rutherford et al 1979).

Table 3. Characteristics of MEL-Human Cell Hybrids

Groups	Hybrid Cell Lines	Source of Donor Cells	Genotype of Donor Cells	Genotype of Hybrids
A	7-32 †* 17-77 † 17-113 † 17-193 †	Adult A (MNC) Adult B (MNC) Adult B (MNC) Adult B (MNC)	$\alpha\alpha/\alpha\alpha$	$\alpha\alpha$
B	8-55 †* 8-119 † 16-1 †	Adult C (MNC) Adult C (MNC) Adult D (MNC)	$\alpha\alpha/--_{SEA}$	$\alpha\alpha$
C	8-6 † 8-7 8-23 8-24 † 8-125 †*	Adult C (MNC)	$\alpha\alpha/--_{SEA}$	$--_{SEA}$
D	13-42 † 13-37 14-20 † 14-21 † 14-23 †* 14-25 †* 14-29 † 14-81 14-86 14-89 14-91 † 14-95 † 14-96 14-97	Fetus A (HFHE) Fetus A (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE) Fetus B (HFHE)	$--_{SEA}/--_{SEA}$	$--_{SEA}$
E	6d †* 12b † 4b	R562	$\alpha\alpha/\alpha\alpha$	$\alpha\alpha$

*, Karyotyping was done to identify a single copy of human chromosome 16 in a hybrid cell.

†, Human ζ -globin mRNA was quantitated by S1 nuclease protection assay.

3.3. Identification of Recombinant Human ζ -Globin cDNA Clones

Human ζ -globin cDNA was subcloned into pGEM-4Z vector for *in vitro* synthesis of human ζ -globin mRNA. The putative recombinant plasmid DNAs extracted from 20 white cell colonies were digested with Pst I and analyzed by electrophoresis on 1% agarose gel at 55 volts for 2.5 hours. Seven colonies with a 500 bp insert were identified. Southern blot analysis proved that the 500 bp insert was human ζ -globin cDNA (Fig.12). The structure of the recombinant clones was then verified by restriction analysis of plasmid DNA from miniprep. DNAs from clones #5, #12, #15, #17 and #20 generated fragments of 2364, 600 and 250 bp when digested with Pvu II. Thus, the human ζ -globin cDNA were inserted in 3' to 5' direction corresponding to a Sp6 promoter. Clone #16 generated Pvu II digested fragments of 2364, 700 and 200 bp, suggesting insertion in 5' to 3' direction (Fig.13). Clone #16 (pGEM-H ζ -16) was used for *in vitro* RNA synthesis by Sp6 RNA polymerase.

3.4. Quantitation of Human ζ -globin mRNA

Thus far very few studies have been carried out to analyze in detail the human ζ -globin gene expression in adult erythroid cells. This is mainly due to low abundance of human ζ -globin mRNA challenging the sensitivity limit and accuracy

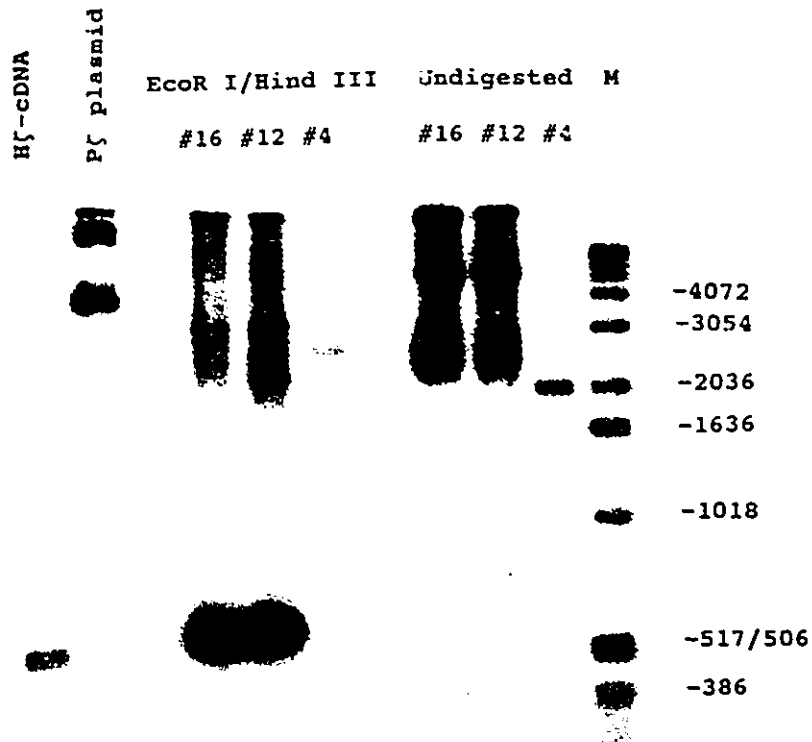


Figure 12: Southern blot analysis of the recombinant DNAs with a human ζ -globin cDNA insert. Plasmid DNAs from mini-preparation were digested with *EcoR* I and *Hind* III and fractionated on a 0.7% agarose gel, blotted onto a nitrocellulose membrane and hybridized with a human ζ -globin cDNA probe. H ζ -cDNA, human ζ -globin cDNA isolated from the P ζ plasmid digested with *Pst* I. #16 and #12, recombinant DNA clones with a single copy of H ζ -cDNA inserted into a pGEM vector. #4, pGEM vector without an insert. M, molecular marker 1 kb DNA ladder.

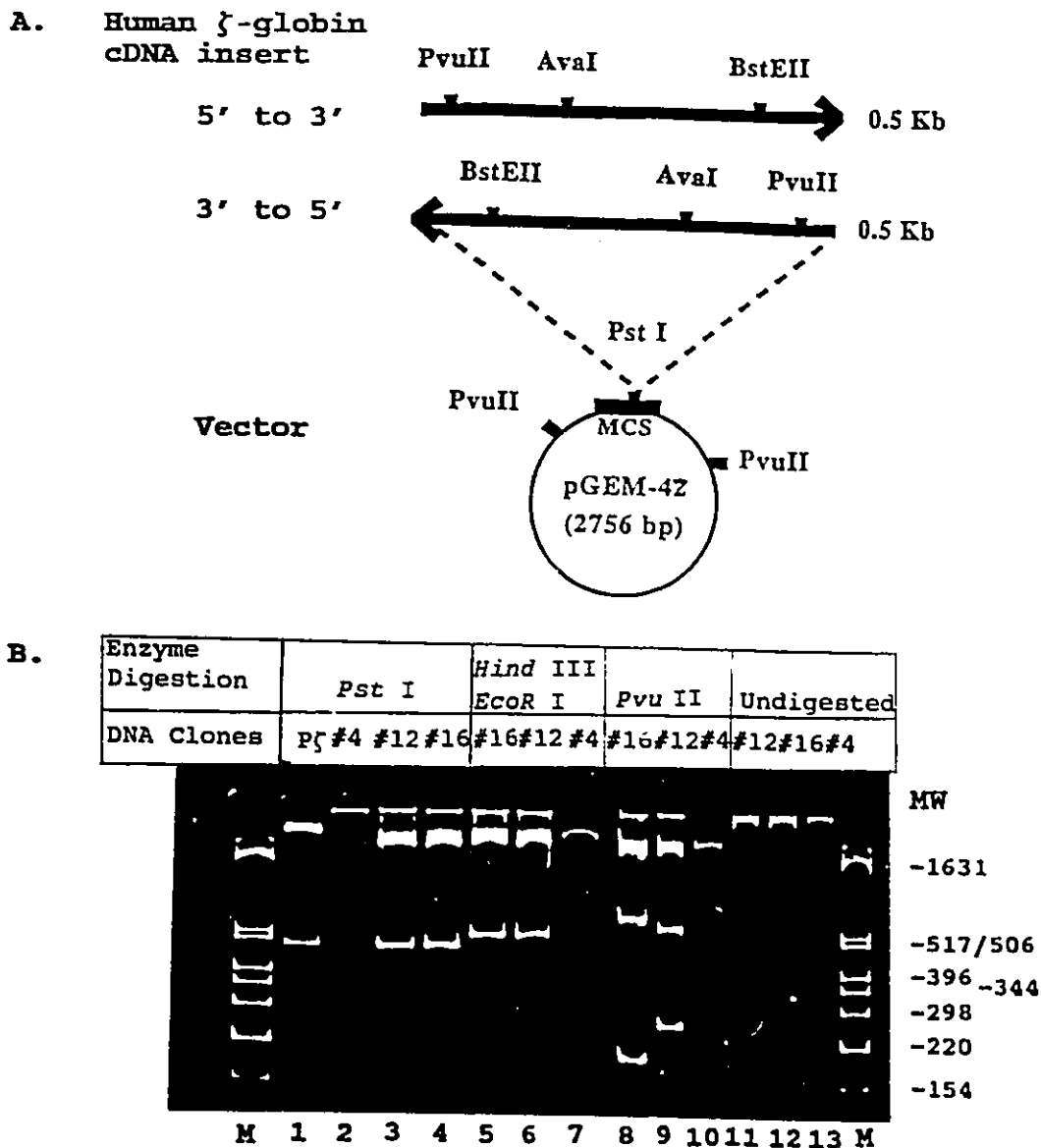


Figure 13: Subcloning of human ζ -globin cDNA into a pGEM vector. **A.** Constructs of recombinant DNA with a human ζ -globin cDNA insert. An arrow indicates direction of the inserted gene. **B.** 5% polyacrylamide gel electrophoresis of DNAs digested with restriction enzyme. Lane 1, a human ζ -globin cDNA insert. Lane 2 to 4, plasmid DNAs digested with *pst* I; lane 5 to 7, with *Hind* III/*EcoR* I; lane 8-10, with *Pvu* II; lane 11-13, without digestion. Lane 2, 7, 10 and 13, a pGEM plasmid; lane 3, 6, 9 and 11, clone #12; lane 4, 5, 8 and 12, clone #16. M, 1 kb DNA marker.

of the detection system. The modified S1 nuclease protection assay in this paper can detect 10^6 mRNA molecules. Moreover, the sensitivity of the method could be increased using poly(A) enriched RNA and prolonged exposure of the film for up to 4 weeks. Therefore, it allows quantitative measurement of gene expression at very low levels.

The *in vitro* synthesized human ζ -globin mRNA provides a standard to estimate the human ζ -globin mRNA copy number. The purity of the synthesized ζ -globin mRNA is over 95% (Fig.14A). A linear correlation between the ζ -globin mRNA copies and intensity of the protected fragments in S1 nuclease protection assay was observed (Fig.14B).

The abundance of human ζ -globin mRNA in the K562 cell line maintained in our laboratory was quantitated by S1 nuclease protection assay (Fig.14B). The constitutive K562 cells contain 1055 copies of human ζ -globin mRNA per cell or 5.0×10^7 copies per μg RNA. After induction with 40 μM hemin for three days, human ζ -globin mRNA was increased to 7655 copies per cell or 5.5×10^8 copies per μg RNA (Table 4). The concentration of ζ -globin mRNA increased approximately ten-fold after induction. The cell content of ζ -globin mRNA increased 7.7-fold. The amount of ζ -globin mRNA in fetal liver erythroblasts from a fetus with homozygous ($--^{SEA}/--^{SEA}$) deletion was approximately 10 times higher than that of

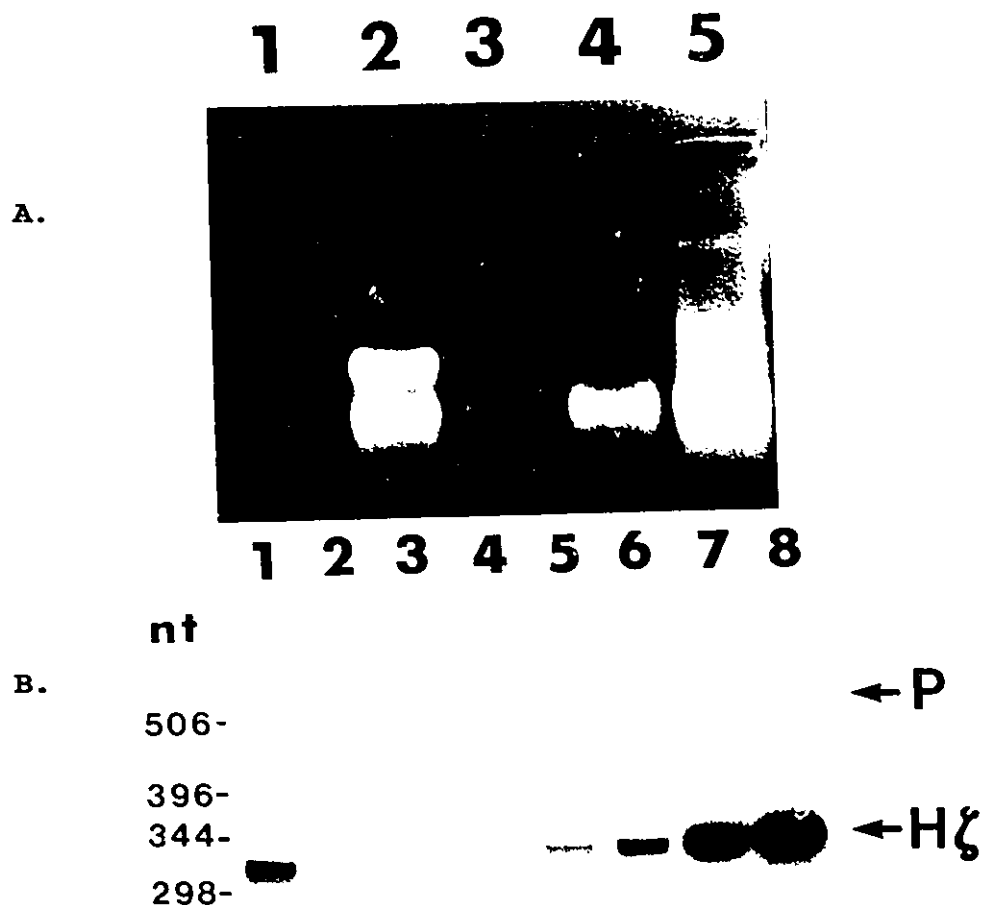


Figure 14: Analysis of the *in vitro* synthesized human ζ -globin mRNA.

A. Purity analysis on 1% agarose gel with ethidium bromide. Lane 1 and 2, the synthesized human ζ -globin mRNA 0.5 and 1 μ l respectively; lane 3 to 5, the rabbit globin mRNA 0.56, 1.12, and 2.25 μ g respectively. **B.** S1 nuclease protection analysis. Lane 1 and 2, 1 μ g cellular RNA from K562 cells with and without Hemin treatment respectively; lane 3 to 8, *in vitro* synthesized human ζ -globin mRNA of 2, 4, 20, 40, 200 and 400 $\times 10^7$ copies respectively. P, input human ζ -globin gene probe; H ζ , human ζ -globin mRNA protected fragments (320 nt for ζ -globin mRNA from K562 cells, 330 nt for the *in vitro* synthesized human ζ -globin mRNA).

constitutive K562 cells (data not shown).

Under our experimental conditions, the amount of human ζ -globin mRNA in K562 cells was 3 to 5 fold higher than the previous report using non-specific probes of globin genes (Tonkonow et al 1982). This could arise from (1) differences in the degree to which each cell line responds to the inducer to stimulate globin gene expression; (2) the specificity of the probe; (3) the accuracy of the standard.

Table 4. Human ζ -globin mRNA in K562 Cells

	RNA pg/cell	ζ -globin mRNA molecules	
		per μ g RNA	per cell
Without induction	21	5.0×10^7	1055
Hemin 40 μ M	14	5.5×10^8	7655

It should be noted that the extent of induction varied among independent cell lines as well as for each induction in the same cell line. Therefore, each hybrid cell line was often induced more than once with different inducers. The induction of the mouse and/or human α -globin genes was used as internal indicators for monitoring induction efficiency. An additional variation in quantitating ζ -globin mRNA could arise from the recovery of mRNA during RNA preparation and oligo-dT affinity chromatography. This was corrected by the levels of mouse β_2 microglobulin mRNA. In contrast with the mouse and human α -globin mRNAs, the M β_2 -MG mRNA levels were not increased when

the hybrid cells were induced to erythroid differentiation. As shown in Fig.15, a representative hybrid cell line with the normal human α -globin gene cluster derived from MNC was induced with DMSO for 0 to 4 days. The levels of human α -globin mRNA increased with induction, while $M\beta_2$ -MG mRNA levels remained relatively constant during induction. Therefore, the levels of $M\beta_2$ -MG mRNA was used as an internal control to normalize the amount of RNA in each sample.

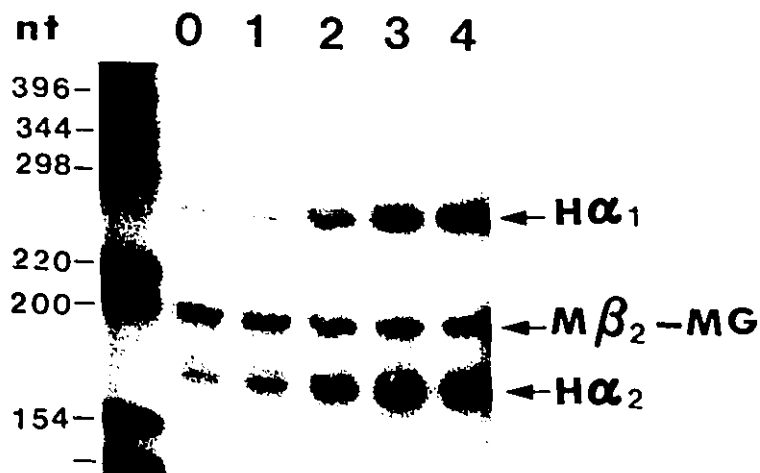


Figure 15: S1 nuclease protection analysis of human α -globin mRNA and mouse β_2 -microglobulin mRNA in the hybrid cell line #8-55 with the normal human α -globin gene cluster. Each of 20 μ g RNA sample, extracted from the cells induced with 1% DMSO for 0, 1, 2, 3 and 4 days, was hybridized with probes specific for human α -globin mRNA and mouse β_2 -microglobulin mRNA. H α_1 and H α_2 , human α_1 - and α_2 -globin mRNA protected fragments of 268 and 172 nt respectively. M β_2 -MG, mouse β_2 microglobulin mRNA protected fragment of 208 nt.

3.5. Human ζ -Globin Gene Expression in Hybrid Cells

MEL x MNC ($\alpha\alpha$ /) hybrids, (Group A and B)

Seven hybrid cell lines, each containing human chromosome 16 with the normal α -globin gene cluster derived from MNC of two normal adults and two carriers were studied. As shown in Fig.16, human ζ -globin mRNA was either undetectable in four different RNA samples or was present at very low levels with a mean of 0.8×10^3 copies/ μ g of total cellular RNA in six other samples. However, the human ζ -globin mRNA was detectable in all of these 7 hybrid cell lines with induction by DMSO or HMBA. In four of them human ζ -globin mRNA levels were slightly increased upon induction. In contrast, the human and mouse α -globin genes were strongly inducible in all hybrid cells. There was no significant difference in the human ζ -globin mRNA levels between hybrids derived from different sources.

These results are consistent with the recent finding that human ζ -globin mRNA was detectable in normal adult reticulocytes by highly sensitive methods (Albitar et al 1989). Previous reports that ζ -globin mRNA was not present in similarly constructed hybrid cells with induction might be due to the sensitivity of the assay method employed (Anagnou et al 1985, Charnay et al 1984).

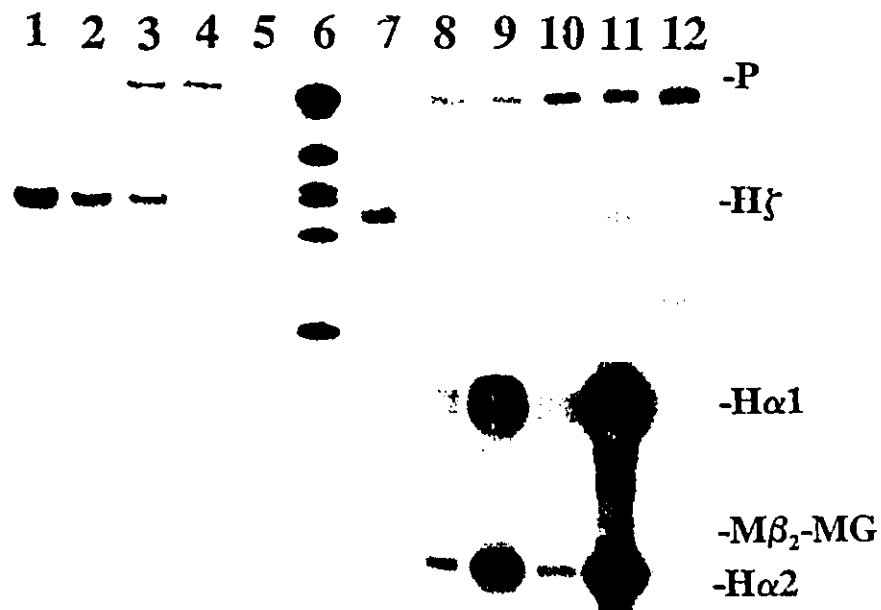


Figure 16: S1 nuclease protection analysis of globin gene expression in #7-32, a hybrid cell line with the normal α -globin gene cluster.

Upper Figure Hybridization with the human ζ -globin cDNA probe. Lane 1 to 5, the in vitro synthesized human ζ -globin mRNA of 10 , 5 , 2.5 , 1 , 0.5×10^6 copies respectively. Lane 6, 1 kb DNA ladder; lane 7, 0.1μ g RNA from the constitutive K562 cells. Lane 8 to 12, 30μ g poly-A rich RNA in each reaction from the hybrid cells. Lane 8, without induction; lane 9, DMSO treatment for 3 days; lane 10, 5 days after DMSO induction; lane 11, HMBA treatment for 3 days; lane 12, 5 days after HMBA induction. P, undigested probe. H ζ , human ζ -globin mRNA protected fragment.

lower Figure 1μ g poly-A rich RNA from the same preparation as that used in the upper figure was hybridized with probes specific for mouse β_2 -microglobulin and human α -globin mRNAs. H α 1 and H α 2, human α 1- and α 2-globin mRNA protected fragments.

These results demonstrate that very minute amounts of human ζ -globin mRNA were detected in some hybrid cells containing human chromosome 16 with the normal α -globin gene cluster. The cell content of the ζ -globin mRNA was estimated as approximately 0.1 copy per cell on average. This implies that only a small proportion of the cells contains ζ -globin mRNA at a given time. To determine if there were cellular heterogeneity in the ability to express the human ζ -globin gene in the hybrid cells, subcloning experiments were performed in two of these cell lines. All five subclones tested displayed low levels of human ζ -globin mRNA similar to their parental cell lines (data not shown).

In S1 nuclease protection assay, it was frequently observed a smaller protected band (about 170 nt) in the hybrid cells with normal human chromosome 16 after treatment with inducers (Fig.16, 20). However, this smaller band was not shown in the hybrid cells with the --^{SEA} deletional human chromosome 16, although these cells produced more human ζ -globin mRNA. It is unknown whether this is an artifact of S1 nuclease digestion or it represents a process in post-transcriptional modulation of ζ -globin mRNA.

We considered whether human ζ -globin gene expression in hybrid cells may be due to the fact that the parental MEL cells produce factors that activate murine embryonic globin gene. However, the results demonstrated that induction of the

parental MEL cells by DMSO and HMBA led to a marked elevation in mouse α -globin gene expression, but mouse ζ -globin mRNA was not detectable. In one hybrid cell line, #7-32, mouse ζ -globin mRNA was detected when induced with butyrate (data not shown).

MEL x MNC (--^{SEA}/) hybrids, (Group C)

Three hybrid cell lines, each containing human chromosome 16 with --^{SEA} deletion, derived from MNC of an adult heterozygous for (--^{SEA}/) deletion, were also investigated. Human ζ -globin mRNA was determined to be $5.3 \pm 2.8 \times 10^3$ copies/ μ g RNA in four separate mRNA samples without induction. Induction by either DMSO or HMBA led to moderate increases in human ζ -globin mRNA levels in two of these hybrid cell lines (Fig.17). The third hybrid cell line had a higher level of human ζ -globin mRNA without induction, and induction did not increase its human ζ -globin mRNA.

These data suggest that human ζ -globin gene expression was higher in constitutive hybrid cells containing the --^{SEA} deletional human chromosome 16 than that containing the normal one (group A and B). Induction with either DMSO or HMBA further enhanced human ζ -globin gene expression. However, the levels of human ζ -globin mRNA present in these hybrid cells, even with induction, were relatively low, compared to that of MEL x HFHE (--^{SEA}) hybrid cells (group D). This may be

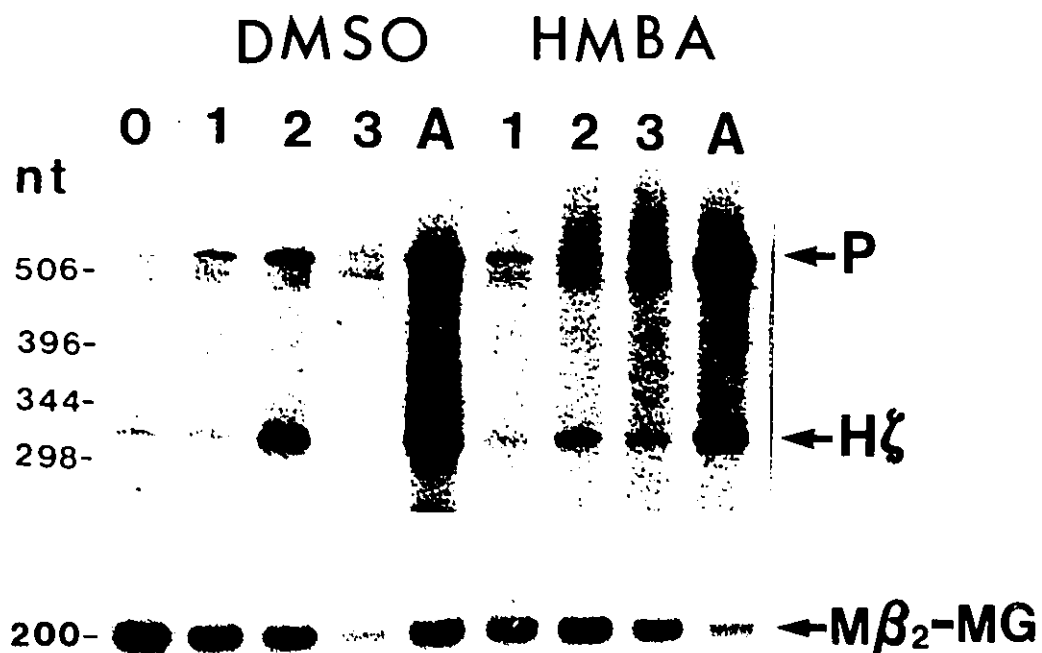


Figure 17: S1 nuclease protection analysis of ζ -globin mRNA from a hybrid cell line #8-6 bearing the $--^{SEA}$ deletional chromosome 16 derived from MNC of a adult carrier.

Upper figure 30 μ g poly-A rich RNA in each reaction was hybridized with a human ζ -globin cDNA probe. Lane 0 to 3, induced with either DMSO or HMBA for 0-3 days; lane A, 5 days after withdrawn DMSO or HMBA.

Lower figure 2 μ g poly-A rich RNA from the same preparation as that used in the upper figure was assayed for $M\beta_2$ -MG mRNA.

explained by the fact that expression of the human α -globin gene in the chromosome mediated gene transfer experiment depends on the functional state of the gene in the original human parental donor cells. Expression of the human α -globin gene derived from non-erythroid cells was much lower than that from the human erythroid cells (Deisseroth et al 1980).

MEL x HFHE ($--^{SEA}/$) hybrids, (Group D)

In order to construct hybrid cells with human erythroid cells containing chromosome 16 with the $--^{SEA}$ deletion, human fetal liver tissues from two mid-gestation fetuses with homozygous ($--^{SEA}/--^{SEA}$) deletion were obtained at postmortem examination. The HFHE were highly active in ζ -globin mRNA and protein synthesis. All 14 hybrid cell lines of MEL-HFHE fusion were shown by Southern blot analysis to contain the human α -globin gene cluster with $--^{SEA}$ deletion. Karyotyping was carried out in two hybrid cell lines, and each was shown to contain one copy of human chromosome 16.

The most interesting observation was that the levels of human ζ -globin mRNA were significantly higher in most hybrid cell lines while they are continuously cultured for over a year. The human ζ -globin mRNA levels in eight hybrid cell lines were carefully quantitated. The constitutive level of human ζ -globin mRNA was $7.8 \pm 0.95 \times 10^3$ copies/ μ g RNA as

assessed in nine different RNA samples. Induction by DMSO or HMBA resulted in elevated human ζ -globin mRNA levels in the hybrid cell lines with means of 27.5 ± 9.78 and $33.9 \pm 9.03 \times 10^3$ copies/ μ g RNA respectively. The presence of mouse ζ -globin mRNA was searched for in these hybrids but not found, except in one instance when induced with 2 mM butyrate (Fig.18). It is interesting that HMBA and DMSO were more efficient inducers of expression of the human ζ -globin gene in cis to the Δ ^{SEA} deletion than butyrate. This was distinct from induction of the endogenous mouse ζ -globin gene as well as the human ζ -globin gene within the normal α -globin gene cluster as observed in this study and previous reports (Burns et al 1988).

Another interesting feature was that human ζ -globin gene expression closely correlated with that of the endogenous mouse α -globin gene during induction, although human ζ -globin gene expression was not as efficient as the mouse α -globin gene (Fig.18, Fig.19). No consistent correlation, however, was found in quantitative expression between endogenous mouse ζ -globin gene and human ζ -globin gene in the hybrid cells treated with HMBA and DMSO. The mouse ζ -globin mRNA was undetectable in most of the hybrid cell lines. Only one hybrid cell line showed increased production of mouse as well as human ζ -globin mRNA when treated with 2 mM butyrate (Fig.18).

One of eight hybrid cells tested had a relatively low

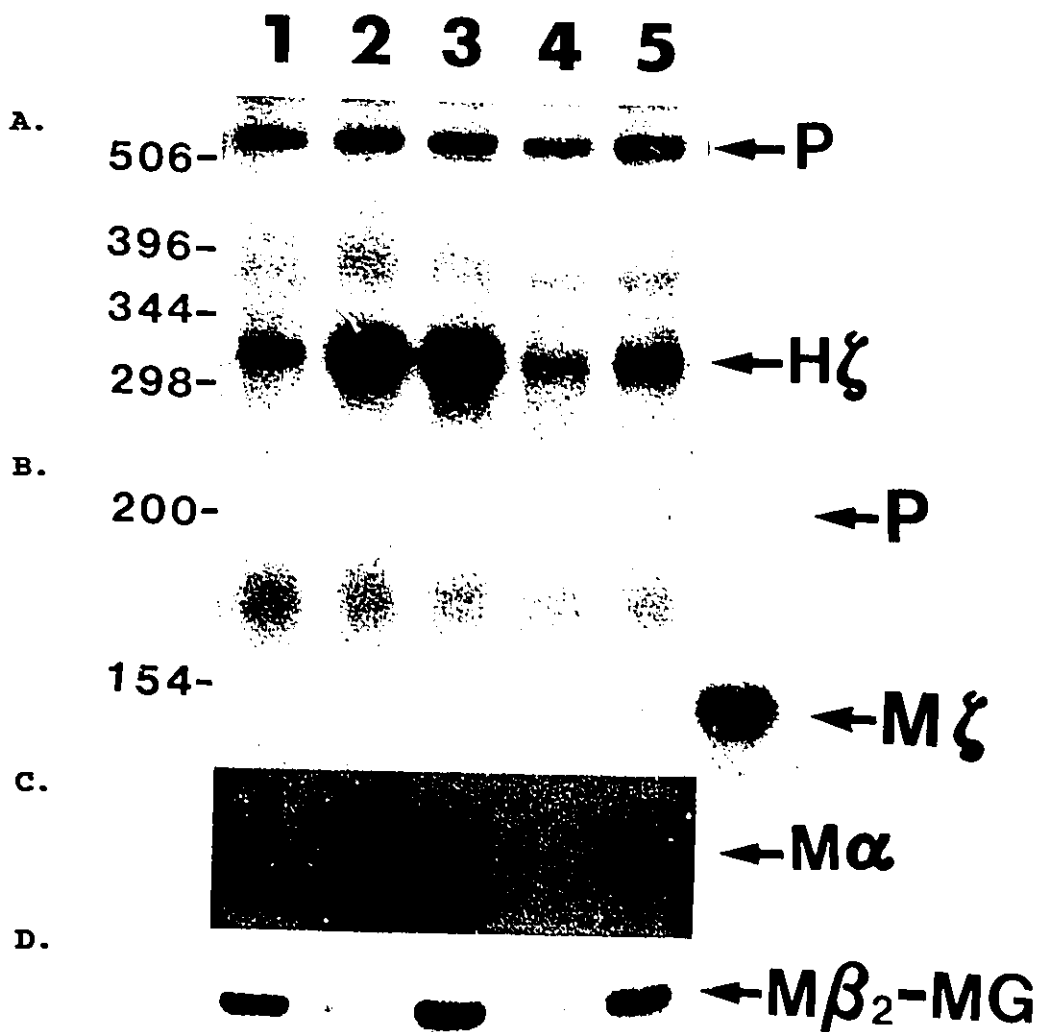


Figure 18: S1 nuclease protection analysis of human and mouse globin gene expression in #25, a hybrid of MEL x HFHE ($--^{SEA}$). A. 30 μ g of each mRNA sample was hybridized with the probe specific for human ζ -globin mRNA. Lane 1, without induction; lane 2 and 3, induced with HMBA and DMSO for 3 days respectively; lane 4 and 5, induced with butyrate 1 and 2 mM for 4 days respectively. P, input probes. H ζ , human ζ -globin mRNA protected fragment. B. 30 μ g of mRNA from the same preparation as lane 1-5 in A was assayed for mouse ζ -globin mRNA. Lane 6, 0.1 μ g cellular RNA extracted from day 10-11 mouse embryonic circulating erythrocytes. P, murine ζ -globin gene probe. M ζ , murine ζ -globin mRNA protected fragment. C. 0.2 μ g mRNA was assayed for mouse α -globin mRNA. D. 0.5 μ g mRNA was assayed for mouse β_2 -microglobulin mRNA. By comparison with known RNA standards, the poly-A rich RNAs in lanes 1-5 represent 2.2, 0.5, 2.7, 0.7, and 2.6 μ g of cellular RNA respectively.

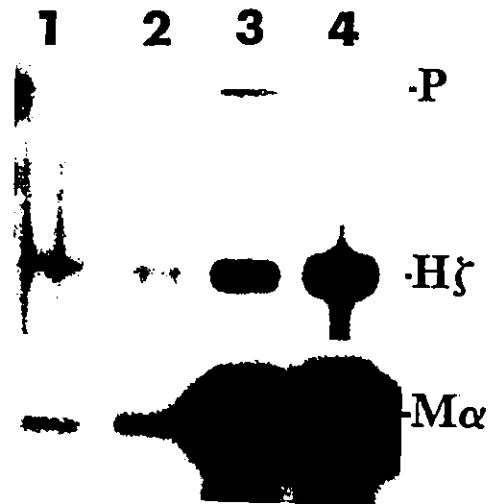


Figure 19: S1 nuclease protection analysis of globin gene expression in #29, a hybrid cell line of MEL x HFHE (---SEA).

Upper figure: 30 μ g poly-A rich RNA was hybridized with human ζ -globin gene probe. Lane 1, no induction. Lane 2 to 4 represent cells induced with 1% DMSO for 1, 2 and 3 days respectively.

Lower figure: 0.2 μ g mRNA from the same preparation as that used in the upper figure was assayed for mouse α -globin mRNA.

level of human ζ -globin mRNA with induction. However, endogenous mouse α -globin gene expression also failed to respond to induction in this hybrid (data not shown). In another hybrid cell line, both mouse α - and human ζ -globin gene expression was strongly inducible 10 months after cell fusion. However, this same cell line failed to respond to any inducers a month later.

MEL x K562 ($\alpha\alpha$ /) hybrids, (Group E)

Human erythroleukemia cell K562 was derived from adult chronic myelogenous leukemia (Lozzio & Lozzio 1975). It synthesizes embryonic and fetal hemoglobins (Andersson et al 1979, Hoffman et al 1980).

Three hybrid cell lines of MEL x K562 were constructed and two of them were studied in detail. Southern blot analysis revealed the normal human α -globin gene cluster of these hybrids. In one hybrid cell line, karyotyping was done and a single copy of human chromosome 16 was present in a hybrid cell (Fig.11). In concert with previous reports, these hybrids had very few copies of human ζ -globin mRNA, with a mean of $3.7 \pm 1.50 \times 10^3$ copies/ μ g RNA in four different RNA samples without induction. Induction with DMSO or HMBA resulted in a minimal elevation of human ζ -globin mRNA (Table 5). On the other hand, human α -globin gene expression in these hybrids

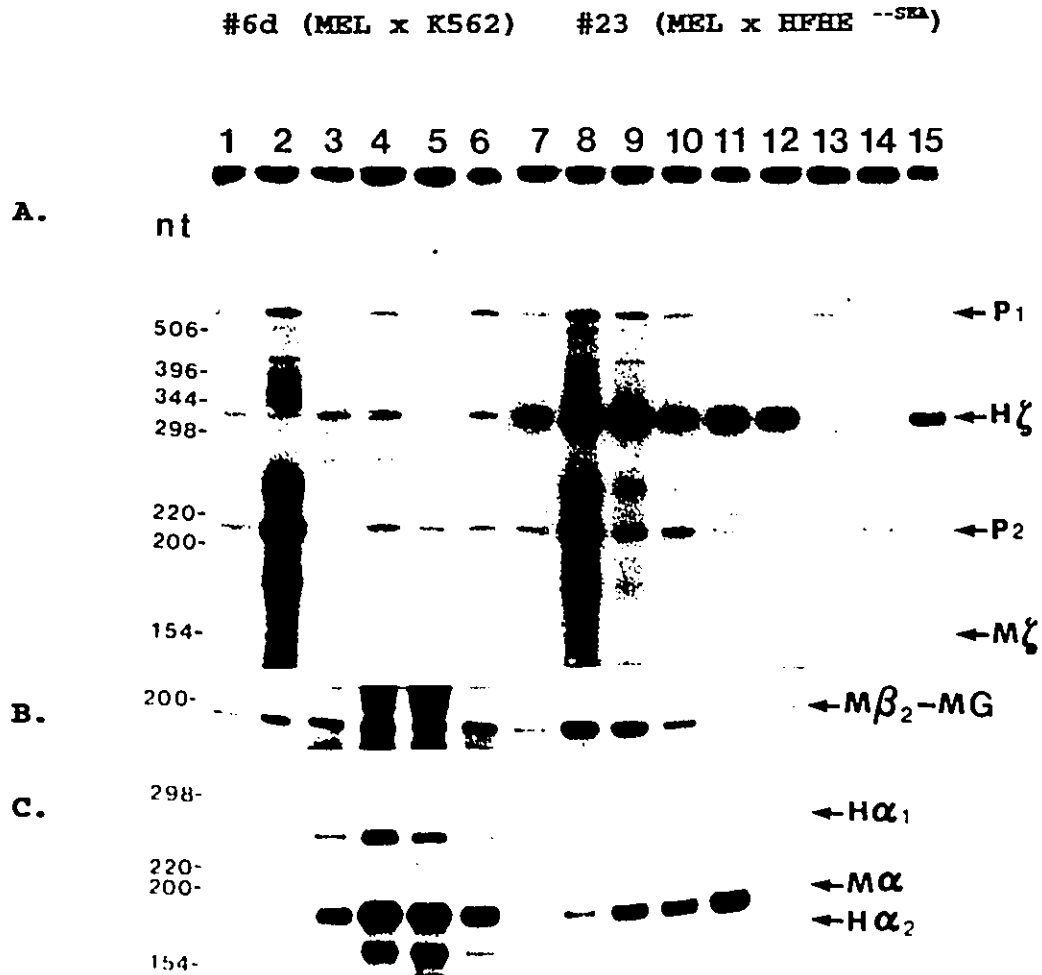


Figure 20: Comparison of human and mouse globin gene expression in cell lines #6d, MEL-K562 fusion, and #23, MEL-HFHE(^{--SEA}/) fusion.

Figure 20A: Autoradiographic signals obtained in S1 nuclease protection assay. A, 30 μg of mRNA from hybrid #6d (lane 1 to 6) and hybrid #23 (lane 7 to 12) were hybridized with human and mouse ζ-globin gene probes. Lane 1 and 7, without induction; lane 2 to 6 and 8 to 12, induced with HMBA for 1, 1.5, 2.5, 3.5 days and after withdrawn HMBA for 5 days; lane 13, without mRNA; lane 14, 10 ng RNA from 10-11 days mouse embryos; lane 15, 25 ng of RNA from constitutive K562 cells. B, 2 μg of the mRNA sample same as A was hybridized with Mβ₂-MG probe. C, 0.13 μg of the same mRNA sample was hybridized with probes specific for the human and mouse α-globin gene. P1, undigested human ζ-cDNA probe; P2, undigested mouse ζ-globin gene probe.

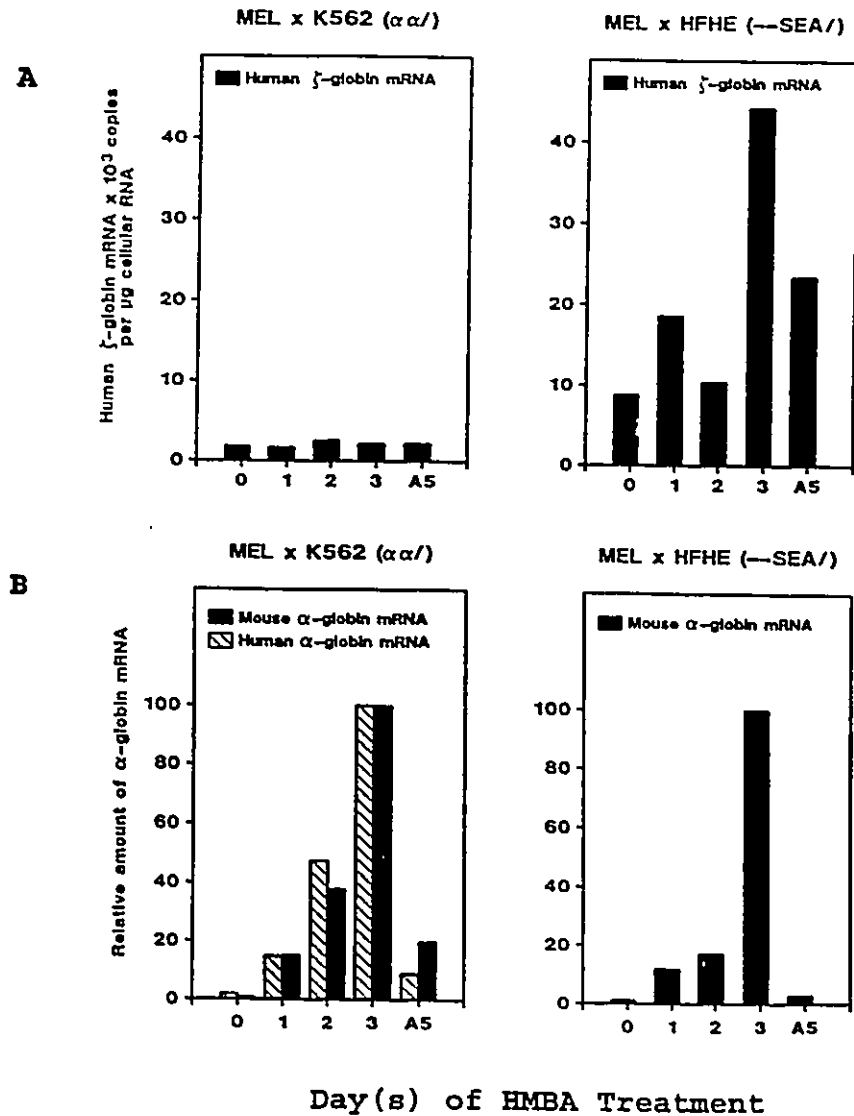


Figure 20B: Summary of the results in (20A).
A. The accumulation of human ζ -globin mRNA was plotted as copies $\times 10^3$ per μg RNA against time course of induction with HMBA. The human ζ -globin mRNA levels were normalized by $M\beta_2$ -MG mRNA.
B. Relative amount of mouse and human α -globin mRNAs (area $\text{mm}^2/\mu\text{g}$ RNA) to the maximal induction was plotted against time of induction. Human α -globin mRNA was undetectable in the hybrids of MEL x HFHE ($--^{SEA}$ /). A5, 5 days after induced with HMBA for 3 days.

was significantly enhanced by DMSO or HMBA treatment, in parallel with the induction of endogenous murine α -globin gene expression (Fig.20). These results are in contrast to the constitutive and inducible expression of the ζ -globin gene in cis to the SEA deletion. The data suggest that whatever reasons causing transcriptional activation of the ζ -globin gene in K562 cells, when the chromosomal ζ -globin gene was transferred into the MEL cells, it was regulated correctly and transcribed at levels similar to that of Group A and B hybrids derived from the adult MNC with the normal α -globin gene cluster. This suggests that the normal structure of the α -globin gene cluster is required for correct regulation of human ζ -globin gene expression in the adult erythroid environment. High levels of human ζ -globin mRNA in the group D hybrids with SEA deletional chromosome cannot be explained by transcriptional activation of the gene prior to the chromosomal gene transfer.

Several other control experiments were also considered: (1) MEL x HFHE ($\alpha\alpha/$) hybrids. The normal HFHE at mid-gestation synthesize very little ζ -globin chains. Furthermore, these samples were not readily available. (2) MEL x human embryonic erythroblasts ($\alpha\alpha/\alpha\alpha$) hybrids. Although the human ζ -globin gene in the primitive erythroblasts is transcriptionally active, it has been limited by the inability to obtain the samples from the embryos before 6 weeks of gestation.

Table 5. Summary of ζ -Globin mRNA Levels in Four Groups of MEL-Human Hybrid Cell Lines

Cell Lines (Genotype) No. of Clones	$\times 10^3$ mRNA copies/ μ g RNA					
	Control Mean \pm SE		HMBA Mean \pm SE		DMSO Mean \pm SE	
MEL \times MNC ($\alpha\alpha$ /) 7	0.48 \pm 0.22 (10)*	p < 0.05	2.42 \pm 0.59 (16)	p < 0.05	3.30 \pm 0.65 (8)	p < 0.01
MEL \times MNC (-- ^{SEA}) 3	5.29 \pm 2.84 (4)		9.71 \pm 2.89 (3)		13.90 \pm 2.42 (7)	
MEL \times K562 ($\alpha\alpha$ /) 2	3.69 \pm 1.50 (4)	p < 0.05	5.70 \pm 3.24 (2)	**	5.20 \pm 0.54 (5)	p < 0.01
MEL \times HPHE (-- ^{SEA}) 8	7.85 \pm 0.95 (9)		33.93 \pm 9.03 (8)		27.53 \pm 9.78 (9)	

The statistical significance was calculated by Minitab statistical Glm program. P value between groups was calculated by Kruskal-Wallis test.

* Number of independent experiments.

** P value was not calculated due to a few samples in one group.

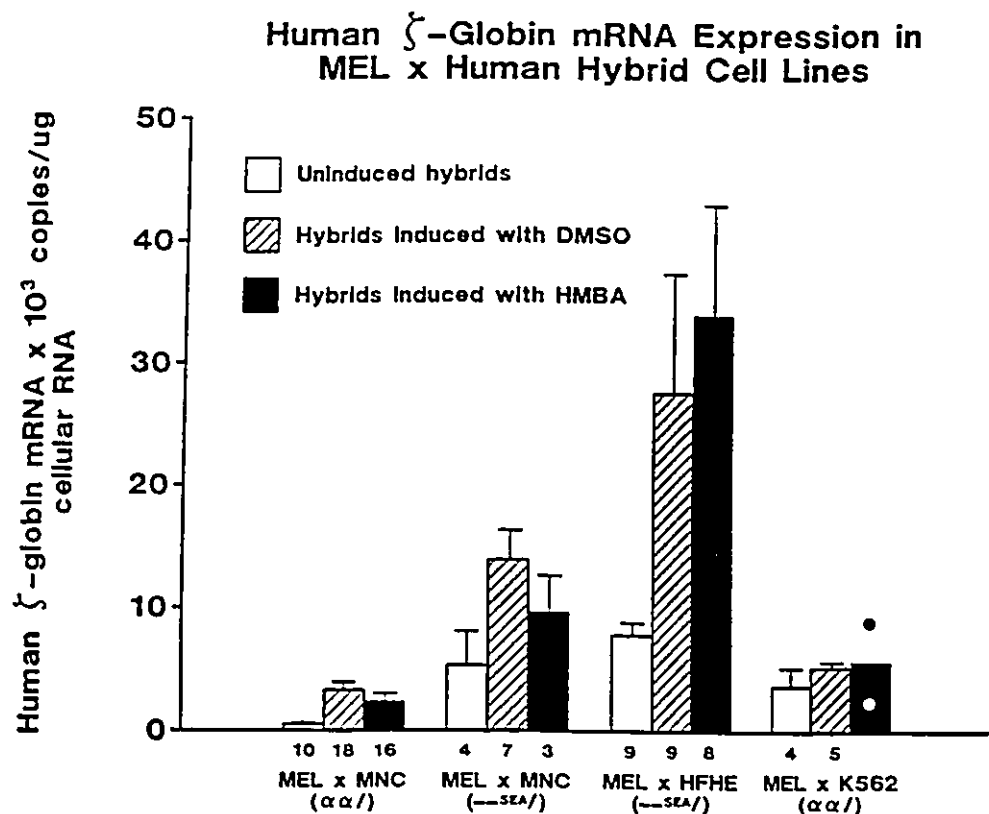


Figure 21. Summary of the human ζ -globin mRNA levels in four different sets of hybrid cell lines. These are seven MEL x MNC(α/α) hybrid cell lines, three MEL x MNC($--SEA/$) hybrid cell lines, eight MEL x HFHE($--SEA/$) hybrid cell lines, and two MEL x K562(α/α) hybrid cell lines.

In each induction experiment, only the highest value of human ζ -globin mRNA levels during the course of induction was included in the tabulation. The data are expressed as mean \pm SEM. The figure under each column represents the number of different induction experiments performed. Note only two HMBA induction experiments were done in MEL x K562 hybrids.

Chapter 4

DISCUSSION

Understanding the mechanisms operating developmental stage specific and erythroid tissue specific expression of the globin gene is a subject presently under active investigation. Recent studies suggest that this is achieved through the interaction of at least three elements. These include the proximal DNA regulatory sequences flanking the coding sequences of the globin genes (Raich et al 1992, Foley et al 1992), the distal major regulatory sequences of the LCR in the β -globin gene cluster and of the HS-40 region in the α -globin gene cluster (Tuan et al 1984, Grosveld et al 1987, Higgs et al 1990, Vyas et al 1992b), and both ubiquitous and erythroid specific transacting factors (Yu et al 1990, Gallarda et al 1989, Ramseyer et al 1989). The present work was undertaken to study the regulation of human embryonic ζ -globin gene expression in interspecific hybrid cells in the presence or absence of downstream α -globin genes due to the --^{SEA} deletion.

4.1. Correlation of ζ -Globin Chains and α -Globin Gene

Deletions

Based on analysis of ζ -globin chains in various naturally occurring mutations in the α -globin gene cluster, it was found that ζ -globin is present at a low level in adult carriers of ($--^{SEA}/$), ($--^{SPN}/$) and ($--^{MED}/$) α -thalassemias. Amount of ζ -globin higher than 0.03% of total Hbs can be detected by immunoassay and used as an indicator for these deletions. A monoclonal antibody-based immunoassay (SBIA) for ζ -globin provided a novel and simple approach for screening and diagnosis of carriers with the ($--^{SEA}/$) deletion. It is especially important for identifying couples at risk of conceiving fetuses with homozygous ($--^{SEA}/--^{SEA}$) α -thalassemia. A clinical trail for the ζ -test was done recently in Hawaii. The sensitivity of the assay was 95% and the specificity for ($--^{SEA}/$) deletion was 100% (Ireland et al)

Not all the natural α -thalassemia deletions involving two α -globin genes are accompanied by constitutive expression of ζ -globin chains in adult carriers. For example, adult carriers of the ($--^{SA}/$), ($--^{BRIT}/$) and ($-(\alpha)^{20.5}/$) deletions do not have detectable ζ -globin chains in their peripheral blood (Tang et al 1992). These observations are suggestive that the DNA sequences located between the 5' breakpoints of ($--^{SEA}/$) and ($--^{SA}/$) deletions may have a positive regulatory role on

embryonic ζ -globin gene expression in adults. Deletions involving this region as well as two α -globin genes abolish ζ -globin production. At present, it is unknown whether this element plays a role in regulating ζ -globin gene expression at the embryonic stage.

4.2. Expression of the Normal Chromosomal ζ -Globin Gene

The present observations together with previous publications are suggestive that MEL cells elaborate adult type erythroid transacting factors intracellularly which can affect expression of the endogenous murine adult α -globin genes, as well as the exogenous human chromosomal α -globin genes in the interspecific hybrid cells. But previously, human ζ -globin mRNA was not detected in the interspecific hybrid cells (Chamay et al 1984, Anagnou et al 1985, Deisseroth & Lyding 1987).

The current studies report the detailed quantitative data of human ζ -globin gene expression in mouse-human hybrid cells. Since human ζ -globin gene expression is minimal in the hybrids bearing the normal human chromosome 16, it is important to develop an accurate assay to quantitate the amount of human ζ -globin mRNA in these hybrids, against which the aberrant expression of the ζ -globin gene on the deletional chromosome 16 can be compared. Our data illustrates several

interesting features of human embryonic ζ -globin gene expression in these hybrid cells, which have not been reported in other studies. First, the data demonstrated that the human embryonic ζ -globin gene under its normal chromosome setting is expressed, albeit at a low level, in the mouse adult erythroid cell environment, regardless of stage or origin of the parental human donor cells. This result is consistent with the recent finding that ζ -globin mRNA was detectable in normal adult bone marrow and peripheral reticulocytes using highly sensitive methods (Albitar et al 1989). This may represent a basal level of human embryonic ζ -globin gene expression.

Second, the human ζ -globin gene is transcribed at low frequency in a stochastic fashion in adult erythrocytes. The estimated amount of human ζ -globin mRNA in the hybrid cells is 0.1 copy per cell on average when induced to erythroid differentiation. However, human ζ -globin mRNA production is unlikely committed to certain distinct cell population. This is indicated by the fact that amounts of human ζ -globin mRNA produced in the subclones were the same as that in the parental hybrid cell lines.

Third, in contrast to the human adult α -globin genes, the human ζ -globin gene is only slightly inducible in some hybrid cells, regardless of its transcriptional activity prior to chromosomal gene transfer. Also, there is no consistent correlation between the human embryonic ζ -globin gene

expression and the mouse embryonic ζ -globin gene expression in these hybrid cell lines.

These results are suggestive that the human ζ -globin gene within the normal α -globin gene cluster was properly regulated in the mouse adult erythroid environment. From the results reported here, it is conceivable that human embryonic ζ -globin gene expression in the interspecific hybrid cells is relevant to that observed in humans. This system could be used to study the events of human ζ -globin gene regulation in an adult erythroid environment.

4.3. Expression of the ζ -Globin Gene in cis to $--^{SEA}$ Deletion

The present studies showed that expression of the human ζ -globin gene was significantly elevated in hybrid cells containing the $--^{SEA}$ deletional human chromosome 16 derived from the HFHE. These results provide direct experimental evidence that the $--^{SEA}$ deletional human chromosome 16 but not the normal one is primarily responsible for constitutive expression of the ζ -globin gene in cis. This is consistent with the clinical observations that ζ -globin chains are present in erythrocytes of adult carriers of $(--^{SEA}/)$ α -thalassemia-1 (Chung et al 1984, Chui et al 1986). It suggests that the DNA sequences within the SEA deletion are important

for normal developmental regulation of ζ -globin gene expression.

The transcriptional activity of the human ζ -globin gene prior to chromosome mediated gene transfer cannot completely explain why the ζ -globin gene is constitutively expressed above basal levels after being transferred into an adult erythroid environment. The human ζ -globin gene derived from K562 cells was transcriptionally active prior to chromosomal transfer, but its expression was switched to the normal basal level once transferred into MEL cells. Therefore, expression of the human ζ -globin gene is properly regulated in the mouse adult erythroid environment as long as the normal structure of the α -globin gene cluster is conserved.

Another observation was that expression of the human ζ -globin gene on the --^{SEA} deletional chromosome was readily induced by DMSO and HMBA but not butyrate. Moreover, induction of human ζ -globin gene expression is correlated with induction of endogenous mouse α -globin gene expression. These results demonstrate that expression of the human ζ -globin gene in cis to the SEA deletion can be augmented, probably at a low efficiency, by the adult erythroid trans-acting factors produced in MEL cells.

Even though the relative amount of ζ -globin mRNA in MEL x HFHE (--^{SEA}) hybrid cells is elevated significantly against the basal level, the absolute amount of ζ -globin mRNA, at the

best, is less than 10 copies per cell on average with induction, which is 0.1% of the parental HPFH with homozygous ($--^{SEA}/--^{SEA}$) deletion. Therefore, the major factors which allow high level human embryonic ζ -globin gene expression are lacking in the MEL cells.

4.4. Possible Mechanisms Regulating Human ζ -Globin Gene Expression.

The molecular basis of human ζ -globin gene expression in ($--^{SEA}/$) α -thalassemia-1 may be in some aspects analogous to the γ -globin production in individuals with HPFH (Feingold et al 1989, Jagadeeswaran et al 1982). However, there is no evidence of an enhancer-like sequence, located 3' of the breakpoints, being brought closer to the ζ -globin gene. This is inferred from the observation that ζ -globin chains are not detected in adult carriers with the ($--^{SA}$) deletion, whose 3'-breakpoint is very close to that of the ($--^{SEA}$) deletion (Tang et al 1992). There is also no evidence that a second mutation in cis to this deletion could account for constitutive expression of the ζ -globin gene in adult carriers. It seems unlikely that double mutations occur in several independent deletions ($--^{SEA}$, $--^{SPN}$ and $--^{MED}$).

It is possible that the DNA sequences within the deletion contain essential elements which normally suppress ζ -

globin gene expression in the adults. It has been reported that the DNA sequence within the α -globin gene acted positively for α -globin gene expression and negatively for the genes linked in cis on the same plasmid transferred into MEL cells (Atweh et al 1988, Brickner et al 1991). On comparison of human ζ -globin gene expression in the presence versus the absence of the α -globin genes in cis, the present studies are consistent with the hypothesis that competition between the human ζ - and α -globin genes for interaction with the globin gene major regulatory region in cis represents one of the regulatory mechanisms during normal human hemoglobin ontogeny. Nevertheless, this does not exclude non-competition mechanisms operating globin gene switching. As a consequence of the SEA deletion, the human ζ -globin gene is expressed at relatively high level compared to the normal basal level. On the other hand, adult carriers of a number of deletional α -thalassemia-1 do not have ζ -globin chain expression in their blood. It is likely that the switch from ζ - to α -globin expression is achieved by the interplay of competition and non-competition mechanisms.

Clinical observations provide further evidence in supporting the competition model. It is well known that in fetuses homozygous for the SEA deletion, human ζ -globin is synthesized in a large amount in fetal liver erythroblasts even in the third trimester of gestation (Weatherall et al

1970). Cord blood of newborns heterozygous for the SEA deletion has approximately ten times more ζ -globin chains than that of normal newborns (Chui et al 1989). Adult carriers of the SEA deletion consistently have a minute amount of ζ -globin chains in their peripheral blood whereas ζ -globin is not detected in normal adults (Chung et al 1984, Chui et al 1986). Taken together, these observations suggest that expression of the ζ -globin gene beyond the embryonic stage could be the result of that ζ -globin gene regulatory sequences in cis to the SEA deletion are less discriminating to fetal and adult stage specific regulatory factors. Therefore, ζ -globin gene could be regulated by the existing factors during erythroid development.

The present studies demonstrate that the induction of human ζ -globin gene expression in cis to the SEA deletion is correlated with the induction of mouse α -globin gene expression. Deletion of the adjacent α -globin genes may bring about changes to the accessibility of the ζ -globin gene regulatory sequences to the HS-40 and transacting factors, which allow the ζ -globin gene to be transcribed at higher levels in adult erythroid environment.

The competition hypothesis was proposed based on studies of human γ - to β -globin gene switching in transgenic mice (Enver et al 1990) and chicken embryonic to adult globin switching (Engel et al 1989). Competition has not been proven

in the human embryonic globin gene switching by the transgenic mouse model. The human ζ -globin gene was expressed only in the murine yolk sac erythroid cells and not in fetal liver erythroblasts, even when the constructs did not contain the human α -globin genes in cis (Spangler et al 1990, Raich et al 1990, Albitar et al 1991, Pondel et al 1992). These results were interpreted to demonstrate the autonomous developmental regulation of human ζ -globin gene expression.

However, caution should be taken not to over interpret the results in mice. The Hb ontogeny of mouse, chick and xenopus is different from that of man, sheep and cattle. During mouse development, as yolk sac erythroblasts are replaced by hepatic erythroblasts, a single switch from embryonic Hb to adult Hb occurs. During human development, however, Hb switching occurs twice. In the first switch, embryonic Hb is replaced by fetal Hb at 6 to 7 weeks gestation. A second switch occurs at about 32 to 34 weeks of gestation. Given that these quite distinct expression patterns for the globin genes are maintained in divergent organisms, regulatory factors have also been altered during evolution to fit the specific needs of the host. There is no corresponding embryonic trans-acting factors in mouse for high level expression of the human embryonic globin gene (Chada et al 1986). Moreover, the regulatory factors produced in K562 cells cannot activate the mouse embryonic ζ -globin gene in transient

heterokaryons (Baron & Maniatis 1986). The human embryonic specific transacting factors might have evolved after the divergence of rodents and humans. Recent studies demonstrate that the expression pattern of the human ϵ -globin gene in transgenic mice was different from the normal pattern observed in human embryos when the transgenes included the β -LCR and entire β -globin gene cluster in its natural organization (Strouboulis et al 1992). The human γ -globin gene was the earliest and most abundantly expressed gene in the mouse embryos. The human embryonic ϵ -globin was never predominantly expressed in mouse embryos and bypassed the embryonic to fetal Hb switching as observed at late embryonic stage in humans. Taken together, these observations indicate that the current interpretation of the transgenic mouse experiments with regard to embryonic ζ -globin gene regulation during human development might need modification.

Ideally, to study developmental regulation of human ζ -globin gene expression should consider the animal model whose Hb switching is comparable to human Hb ontogeny. The current experimental animals (i.e., mouse, chicken and xenopus), having only one Hb switch during Hb ontogeny, cannot operate two Hb switches as observed in humans. Autonomous regulation of embryonic globin genes may simply be due to lack of embryonic stage specific factors regulating high level human ζ -globin gene expression in this experimental model. Thus, the

human ζ -globin gene, regardless of presence or absence of the human α -globin genes in cis, expressed at low levels in transgenic mice.

In addition to the experimental system employed, it should also be noted that the constructs used in most transgenic mice experiments were highly artificial, lacking the normal spatial relationship and other known and yet unknown DNA regulatory regions.

4.5. Model of Human ζ -Globin Gene Switching

Even though the normal pattern of human ζ -globin gene expression during development demonstrates only one switch at late embryonic stage, expression of the embryonic ζ -globin gene in the absence of two α -globin genes due to the SEA deletion exhibits two distinct switches during development. The first switch occurs at the late embryonic stage as observed in normal human embryo (Fig.2). This switch is abolished in fetuses with homozygous ($--^{SEA}/--^{SEA}$) α -thalassemia. The ζ -globin gene is expressed at high levels until the third trimester, which is presumably regulated by fetal specific factors. These observations support the hypothesis that competition between the ζ - and α -globin genes for interaction with the HS-40 and the fetal trans-acting

factors plays an important role in the ζ -globin gene switching in normal embryos.

The second switch occurs at birth. Fetuses with homozygous ($--^{SEA}/--^{SEA}$) deletion cannot survive beyond this switch. The present study demonstrated that the ζ -globin gene was expressed at a high level in the HFHE from those fetuses prior the gene transfer and was constitutively expressed at a low level after being transferred into MEL cells, which is significantly higher than that observed in hybrids with the normal human chromosome 16. The present study demonstrated that the ζ -globin gene is regulated by adult trans-acting factors in MEL cells. The data suggested that the competition between the ζ - and α -globin genes for the adult factor dependent transcription acts as one of the mechanisms in suppression of the ζ -globin gene in normal adults.

Taken together, these results suggest that human embryonic ζ -globin gene expression is modulated by multiple positive and negative regulatory mechanisms during development. Competition between the human α - and ζ -globin genes is one mechanism to account for expression of a particular gene at specific developmental stage. Deletion of α -globin genes could bring competition into play which favours adult trans-acting factor dependent ζ -globin gene expression.

SUMMARY

I have generated murine monoclonal anti-human embryonic ζ -globin chain antibody, with which I developed an immunological assay to detect human ζ -globin chains in adult erythrocytes. This assay can be used to identify couples at risk of conceiving fetuses with homozygous ($--^{SEA}/--^{SEA}$) α -thalassemia-1.

To determine the effect of the SEA deletion on ζ -globin gene expression and potential mechanisms by which it may explain aberrant expression of the ζ -globin gene in the adult erythroid cells, I have generated mouse-human hybrid cell lines with MEL cells containing human chromosome 16 with either the normal α -globin gene cluster or the ($--^{SEA}$) deletional α -globin gene cluster derived from α -thalassemia patients. The data show that the human embryonic ζ -globin gene in cis to the SEA deletion is expressed in MEL cells and can be induced by either DMSO or HMBA. Furthermore, the kinetics of human ζ -globin gene induction is correlated with that of the endogenous murine α -globin gene. These observations are consistent with the hypothesis that ζ -globin gene expression in the adult carriers is linked to the ($--^{SEA}$) deletional human chromosome 16. The ζ -globin gene in cis to the $--^{SEA}$ deletion

can be activated by adult regulatory factors in the mouse erythroleukemia cells. It is conceivable that the ζ -globin gene is normally suppressed in the adult erythroid cells either directly by silencer sequences within the SEA deletion or indirectly by competition between the α -globin genes and the ζ -globin gene for interaction with the α -LCR and adult trans-acting factors.

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