IRON METABOLISM AND THE REGULATION OF HEME AND FERRITIN SYNTHESIS IN DIFFERENTIATING MURINE ERYTHROLEUKEMIA CELLS

by

Jennifer Dallas Laskey

A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Physiology, McGill University
Montreal, Canada

Jennifer Laskey November 1987

Abbreviated title, less than 70 characters:

Iron metabolism in differentiating MEL cells

ABSTRACT

The main focus of this research has been to examine changes which occur in iron metabolism, in particular with respect to heme and ferritin synthesis, during erythroid differentiation of MEL cells. Transferrin receptor expression, iron uptake and heme synthesis increased following dimethylsulfoxide induced differentiation of MEL cells. Studies investigating the regulation of heme synthesis in induced and uninduced MEL cells support the hypothesis that some step(s) in the pathway of iron from extracellular transferrin to protoporphyrin, rather than ALA synthase, limits and controls the rate of heme and possibly hemoglobin synthesis in erythroid cells. Results suggest that erythroid differentiation is accompanied by the induction of a new iron pathway which shunts increasing amounts of iron to mitochondria for heme synthesis. Furthermore, iron in ferritin is not used for heme synthesis when extracellular iron sources are available.

H and L ferritin subunit mRNA levels increased after induction of MEL cells. Heme administration stimulated H and L ferritin mRNA accumulation in both induced and uninduced cells, while inhibition of heme synthesis during differentiation partially inhibited the accumulation of these mRNAs. These results suggest that the induction of ferritin mRNAs during MEL differentiation may be mediated by heme. Iron administration stimulated the accumulation of ferritin protein but did not alter ferritin mRNA levels, suggesting that iron regulates ferritin synthesis at the level of translation in MEL cells.

The role of transferrin in supporting cell proliferation was also examined. The results indicate that its sole function is to supply cells with iron.

RESUMÉ

Le but de ce travail fut d'examiner les changesments que subit le metabolism du fer et plus particulière les variations au niveau de la synthèse du hème et de la ferritine durant la diffèrenciation érythroïde des cellules MEL. Lorsque les cellules MEL sont indvites à se différencier en les exposant au diméthylsulfoxide, des augmentations au niveau de l'expression du récepteur de la transferrine, de la synthèse du hème et du transport dur fer sont observées. Des études comparant la régulation de la synthèse du hème dans les cellules MEL induites à des cellules non induites supportent l'hypothèse que certaine(s) étape(s) du cheminement du fer, notamment celles entre la transferrine extracellulaire et al protoporphyrine, et non l'etape de la ALA synthase, limite(nt) et contôle(nt) le taux de synthèse du heme et même possiblement de l'hemoglobine dans les cellules érythroïdes. Les résultats suggèrement que la différenciation érythroïde est accompagnée de l'induction d'un nouveas cheminement du fer qui détourne les quantités crissantes de fer vers les mitochondries pour la synthèse du hème. De plus, le fer associé à la ferritine n'est pas utilisé pour la synthèse de hème quand des sources extracellulaire de fer sont disponibles.

Après l'induction des cellules MEL, le niveau d'ARN messager des sous-unités H et L de la protéine ferritine augmente. L'addition de hème

stimule l'accumulation des ARN messagers des sous-unités H et L indépendamment de l'état d'induction de la cellule tandis qu'une inhibition de la synthèse du hème durant la différenciation cellulaire inhibe partiellement l'accumulation de ces ARNs messages. Ces résultats sugèrent que l'induction des ARNs messagers de la ferritine durant la diiférenciation des cellules MEL pourrait etre causée par le hème. L'administration de fer stimule l'accumulation de la protéine de ferritine sans toutefois changer les niveaux du message de la ferritine, suggérant ainsi que le fer régulerait la synthèse de la ferritine au niveau de la transduction dans les cellules MEL.

Le role potentiel de la transferrine dans le support à la prolifération cellulaire fut également étudié. Les résultats obtenus indiquent que l'unique fonctions de cette protéine est de fournir du fer à la cellule.

To my parents

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Prem Ponka, my research director, for his support, encouragement, and advice throughout this work.

I am also grateful to Dr. Herb Schulman for his support, helpful discussion and reading of this manuscript.

I would like to express my gratitude and appreciation to the members of our laboratory, in particular to Cheryl Partridge, Ania Wilczynska, Susanne Caudry, and Eva Holleshack for their technical assistance, advice and support.

I would also like to recognize those whose collaboration was important in this work namely, Dr. W. French Anderson who supplied the globin cDNA probes and Dr. H. Munro who supplied the H and L ferritin subunit cDNA probes.

I am also grateful to the Fonds F.C.A.R. (pour la formation de chercheurs et de l'aide a la recherche), and the Fonds F.R.S.Q. (fonds de la recherche en sante du Quebec) for their financial support during my graduate study.

I would also like to thank Bruce for his support and encouragement throughout this work.

PREFACE

In accordance with the guidelines concerning thesis prepartion, I have taken the option of writing the experimental portion of this thesis in the form of original papers suitable for publication. This option is provided by section 7 in the Guidelines Concerning Thesis Preparation, which reads as follows:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (experimental and design data as well as descriptions of equipment) must be provided in sufficient detail to allow clear and precise judgement to be made of the importance and originality of the research reported. Abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstracts, introduction and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted."

In this thesis, manuscripts of original papers are presented in Chapters 2-6, and are joined by connecting texts. Each paper has its own Abstract, Introduction, Materials and Methods, Results and Discussion section. A common Abstract, Introduction, Discussion, Contribution to Knowledge, and Reference sections are also included.

The work described in Chapter 3-6 have been published or submitted in the following journals.

Chap. 3 Laskey, J.D., Ponka, P., and Schulman, H.M. (1986). Control of heme synthesis during Friend cell differentiation: role of iron and transferrin. J. Cell. Physiol. 129:185-192.

Chap. 4. Laskey, J.D., Ponka, P., and Schulman, H.M. (1987a). Ferritin and ferritin iron metabolism in murine erythroleukemia cells. Biochim. Biophys. Acta submitted, November 1987.

Chap. 5 Laskey, J.D., Ponka, P., and Schulman, H.M. (1987b). Control of ferritin gene expression during MEL cell differentiation: the role of iron and heme. Mol. Cell. Biol. submitted, November 1987.

Chap. 6. Laskey, J.D., Webb, I., Schulman, H.M., and Ponka P. (1987c). Evidence that transferrin supports cell proliferation by supplying iron for DNA synthesis. Exp. Cell Res. (in press).

The work presented in Chapters 2-5 is entirely mine. In Chapter 6, Iain Webb, a summer student in our laboratory, performed the lymphocyte experiments and Lan Vo collaborated in the work performed with Raji cells, specifically the Raji growth curves, and DNA synthesis studies. All manuscripts were prepared by me. The use of the terms "we" and "our" in refers to me and my supervisor.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

LIST OF TABLES

LIST OF ABBREVIATIONS

ALA	:	δ-aminolevulinic acid
BFU-E	:	burst forming unit-erythroid
BME	:	β-mercaptoethanol
cDNA	:	complementary DNA
CFU-E	:	colony forming unit-erythroid
CFU-S	:	colony forming unit spleen
CSF	:	colony stimulating factor
cpm	:	counts per minute
dCTP	:	2'-deoxy-cytidine-5'-triphosphate
DNAse	:	deoxyribonuclease
EDTA	:	ethylenediamine tetraacetic acid
ELISA	:	enzyme linked immunosorbent assay
mRNA	:	messenger RNA
PAGE	:	polyacrylamide gel electrophoresis
PBG	:	porphobilinogen
PBS	:	phosphate buffered saline
PMSF	:	phenylmethyl-sulfonylfluoride
SA	:	succinylacetone (4,6-dioxoheptanoic acid)
S.D.	:	standard deviation
SDS	:	sodium dodecyl sulfate
SIH	:	salicylaldehyde isonicotinoyl hydrazone
solutions:		
SSC	:	1 X SSC is 0.15M NaCl,0.015M sodium citrate, pH=7.0
SSPE	:	1 X SSPE is 0.15M NaCl, 0.01M NaH_2PO_4, 1mM EDTA, pH=7.0
TBE	:	1 X TBE is 90 mM boric acid, 2.5 mM EDTA, 90 mM Tris, pH=8.0
TE	:	1 X TE is 10 mM Tris, 1 mM EDTA, pH=8.0

CHAPTER 1

GENERAL INTRODUCTION

1. HEMATOPOIESIS:

1.1 Introduction

The hematopoietic system has proved to be a useful model for the study of cellular differentiation and transformation. While in man, most tissues consist of differentiated cells with little proliferative or self-renewal capacity, hematopoietic tissue, intestinal mucosa and skin are in dynamic states, continually renewing themselves. In the hematopoietic system for example, about 1×10^{10} erythrocytes come to the end of their lifespan every hour (Whetton and Dexter, 1985). To supply new hematopoietic cells there are a small number of "stem cells" which can both replicate themselves and differentiate. This process of blood cell formation must be regulated, able to respond rapidly to changing physiological needs, such as would occur during blood loss or infection. For this to occur normally, there must be a finely tuned balance between signals for self-renewal and proliferation, and signals for terminal differentiation. Leukemic transformation may represent an imbalance in these signals.

1.2 The hematopoietic stem cell

Blood cell production begins from the pluripotent hematopoietic stem cell. These cells are characterized by their ability to maintain themselves through self-renewal, and their capacity to differentiate into the various blood cell lineages (erythrocyte, granulocyte-monocyte, lymphocyte and platelets), hence the term pluripotent. All mature hematopoietic cells will ultimately be derived from these stem cells. An overview of the various blood elements and how they are formed is presented in Figure 1. Red cells, platelets, granulocytes and monocytes are blood elements that as a group are known as myeloid cells and are

Figure 1. The development of the various blood elements from bone marrow cells. The bone marrow contains a small number of pluripotent stem cells which can both self renew and/or differentiate into committed stem cells. The committed stem cells will in turn differentiate along specific cell lineages. The identifiable morphological changes associated with blood cell development are depicted. Cells below the horizontal line, with the exception of the late normoblast may be found in normal peripheral blood. From W.F. Ganong (1983): Review of Medical Physiology 11th edition, p415.

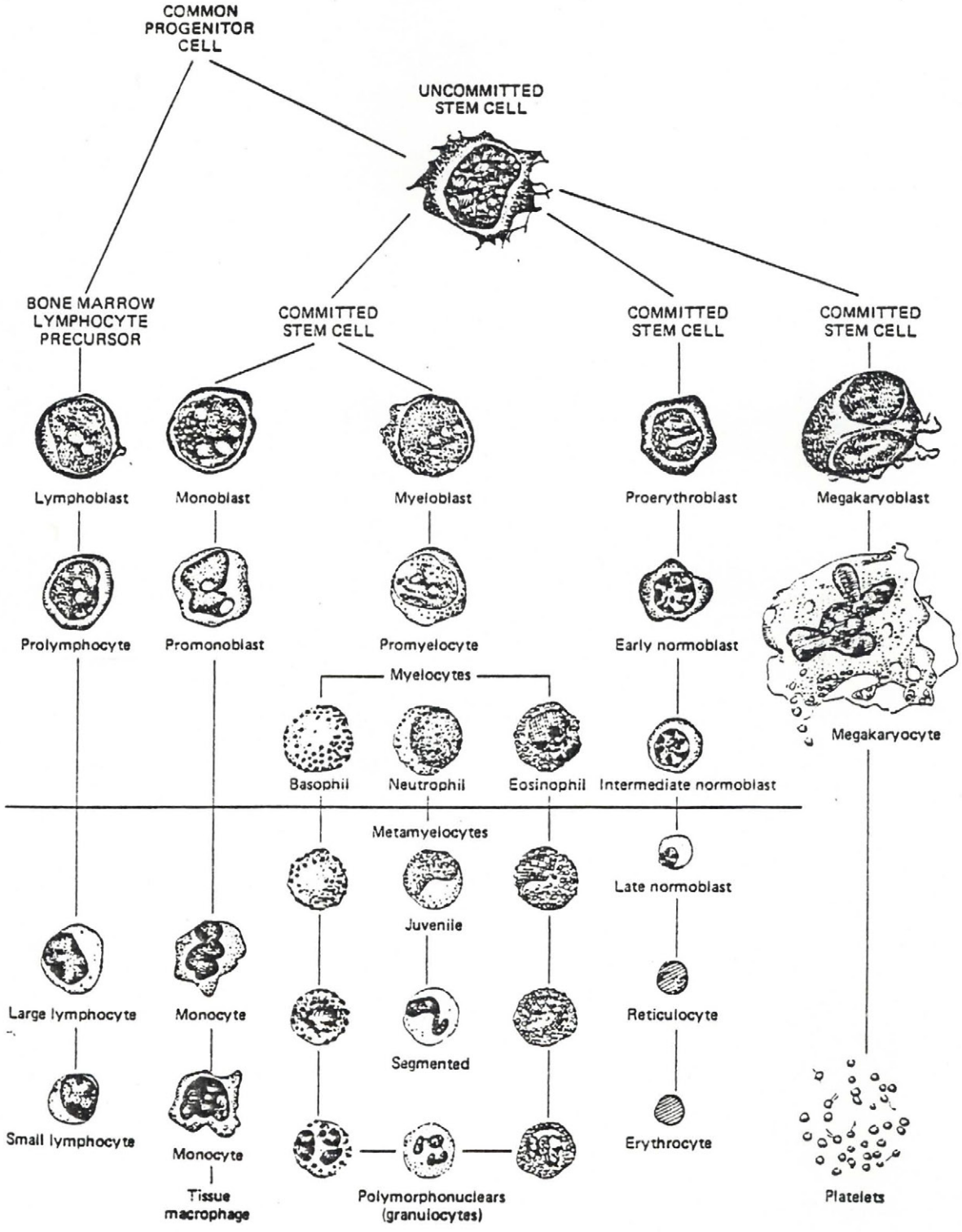


Figure 1. The development of the various blood elements from bone marrow cells.

derived from a common myeloid stem cell. In contrast, B and T lymphocytes are most probably derived from a common lymphocytic stem cell. Note that both pluripotent and unipotent or "committed" stem cells, with varying proliferative and self-renewal capacities are thought to exist.

Hematopoietic stem cells are present in the bone marrow in very low concentrations. The ratio of stem cell to nucleated marrow cell is about 1:2000 (Baboir and Stossel, 1984). The pluripotent stem cell has never been identified morphologically or isolated in pure form. Rather the stem cell is usually identified by its differentiated daughter cells. A number of different experimental approaches have been used to identify, characterize and classify the various stem cells. From these studies a general scheme of hematopoiesis has been derived.

1.3 Classification of stem cells

One of the experimental means of examining the nature of the stem cell is the colony forming assay described by Till and McCulloch in 1961. They found that when mice were subjected to doses of radiation sufficient to destroy all hematopoeisis and then injected with marrow cells (10^4-10^5) from syngeneic untreated mice, discrete colonies of hematopoietic cells could be observed in the spleen 8-10 days later. These nodules consisted of erythroid, granulocytic, megakaryocytic and undifferentiated cells in varying mixtures. By following chromosomal markers these colonies were shown to be clonal in origin indicating that one marrow cell could differentiate along different pathways within the myeloid system (Curry and Trentin, 1967; Abramson et al., 1977). The cells from these nodules were also shown to have self-renewal capability as demonstrated by the ability of cell suspensions from such colonies to form new colonies when injected into previously uninfected mice

(Siminovitch et al., 1963). These cells were termed CFU-S (colony forming unit-spleen) (see Figure 2).

There is extensive evidence that stem cells more primitive than CFU-S must exist. "The most stringent definition of a primitive stem cell is one which is able to reconstitute the hematopoietic system of lethally irradiated mice or mice bearing a mutation in some early step in stem cell development" (Dick et al., 1986). These are termed reconstitution assays. Such reconstitution assays suggest that there is an earlier stem cell (S_p) capable of differentiating into both myeloid and lymphoid lineages.

In vitro assays are also used to characterize stem cells. Cultivation of bone marrow in an appropriate environment can lead to the formation of colonies of differentiating cells of specific lineages. These assays select for the expression of committed stem cells. The pathway taken will depend on the local environment, specifically the growth and differentiation factors which are present. The development of these colonies requires the presence of the appropriate stimulatory molecules. Both multipotential and lineage restricted growth factors have been described. In the absence of these colony stimulating factors (CSFs) proliferation and maturation will not occur.

A model summarizing these observations is presented in Figure 2. All committed progenitor cells shown on the right in Figure 2 can produce in vitro colonies when provided with the appropriate growth and differentiation factors. CFU-Mix is also detected in vitro, and is a pluripotent progenitor. However, unlike CFU-S, it has a limited self-renewal capacity. Reconstitution assays have identified a pluripotent stem cell termed Sp in Figure 2. There is also indirect

Figure 2. Hematopoietic stem cell development. Reconstitution assays suggest that all blood elements can be derived from a common pluripotent progenitor cell (S_P). Evidence also exists for stem cells which have extensive self-renewal capabilities but are restricted to either the lymphoid (S_L) or myeloid (S_M) lineages. These early stem cells which have extensive self renewal capabilities are shown on the left of the figure. CFU-S, a partially committed stem cell retricted to the myeloid lineage, is identified by spleen colony assays. It has significant self renwal capacity and is therefore shown in the middle frame. Committed stem cells with very little self renewal capacity are identified by <u>in vitro</u> colony assays and are shown on the right of the figure. From J.E. Dick et al. (1986): Trends in Genetics, Vol.2 No.6, p165

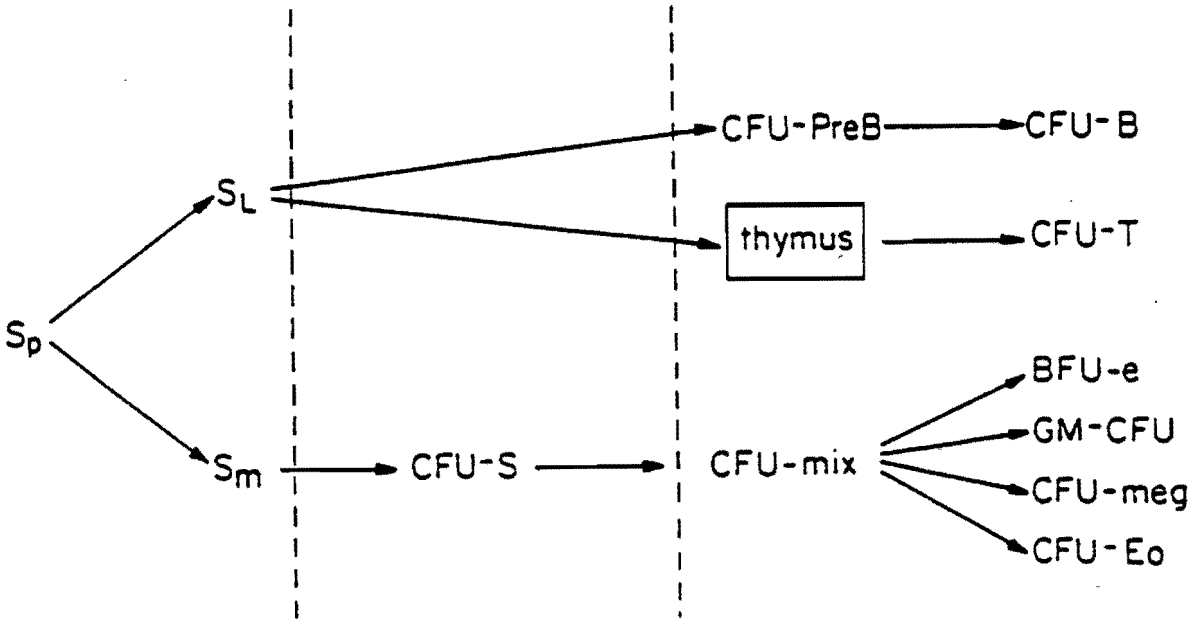


Figure 2. Hematopoietic stem cell development.

evidence that these are cells with extensive self-renewal capacity, but which are committed to either lymphoid (S_L) or myeloid (S_M) lineages (Abramson et al., 1977; Mintz et al., 1984). Recently, these observations have been substantiated by experiments using retroviral mediated gene transfer into primitive hematopoietic stem cells, as a means of tagging them. Briefly, bone marrow is removed and infected with retroviruses carrying marker genes. The retroviruses have been genetically altered so that they will integrate into the genome but will be unable to replicate. The infected bone marrow is then transplanted back into lethally irradiated mice or mice which are genetically deficient in hematopoiesis. By following the unique integration sites of these retroviral vectors one can unequivocally identify the progeny of a single stem cell. This experimental procedure, therefore, allows one to identify a variety of stem cells. In experiments such as described, evidence for S_P, S_M and S_L type stem cells has been presented (Dick et al., 1985).

1.4 Conclusion

In conclusion, the bone marrow comprises a great variety of hematopoeitic cell types with varying proliferative capacities and at various stages of commitment. To understand normal hematopoiesis it is important to analyse the regulatory mechanisms controlling the development of a stem cell capable of self-renewal into mature differentiated cells that cease dividing. Such mechanisms may be blocked or become unbalanced in leukemic transformation resulting in continued proliferation. Also it is of interest to investigate how a stem cell becomes selectively committed to a specific lineage, exactly what changes in gene expression occur and how they are regulated to result in the final cell phenotype. For example, developing erythroid cells are characterized by their ability

to synthesize large amounts of hemoglobin. Iron is a vital component of the hemoglobin molecule and must be delivered to the erythroid cell from the external milieu. Considering the physio-chemical properties of iron (see Introduction, section 4), one can predict that during erythroid differentiation there are dramatic changes in iron metabolism. Furthermore, these changes would likely be highly regulated and coordinated with the induction of hemoglobin synthesis. The specific aim of this study is to examine changes which occur in iron metabolism during erythropoiesis and their possible significance with respect to the programming of erythroid differentiation.

2. ERYTHROPOIESIS

2.1 Introduction

The process of erythropoiesis consists of the production of red blood cells as they develop from stem cell to mature erythrocyte. Commitment of the multipotential hematopoeitic stem cell to erythroid differentiation involves a series of developmental stages. Regulation of the rate of erythropoeisis may occur at a number of critical steps in this pathway. These include, 1. proliferation of the pluripotent hematopoeitic stem cells; 2. commitment of hematopoietic stem cells to erythroid differentiation ; 3. proliferation of the committed erythroid precusor cells, which includes several recognized sequential stages of development; and 4. induction of the erythroid precursors to the expression of biosynthetic and morphogenic changes characteristic of erythroid differentiation (Marks and Rifkind, 1978). In this section the features of erythroid development will be discussed.

2.2 Erythroid differentiation

Terminal erythroid differentiation is characterized by a) the accumulation of hemoglobin; b) the condensation of chromatin and prior to release into the blood stream, the extrusion of the nucleus (Babior and Stossel, 1984), and c) the eventual loss of protein synthesizing machinery including RNA, endoplasmic reticulum and mitochondria. An overview of the sequential development of the erythrocyte can be seen in Figure 3. The earliest identifiable stage is that of the pronormoblast. This is a large cell with an active nucleus and little, if any, hemoglobin. It takes 3-5 days for a pronormoblast to become a mature red cell. Within this period the cell gradually changes; it shrinks in size, hemoglobin accumulates and the nucleus condenses. Note that only the first three

precursor stages, pronormoblasts, basophilic normoblasts and polychromatophilic normoblasts are able to divide. These cells are said to be in the mitotic compartment (see Figure 3). The final step is the extrusion of the nucleus forming the "reticulocyte" which is then released into the blood stream. The reticulocyte still expresses transferrin receptors (required for iron uptake),contains some RNA and has a few mitochondria. These disappear within 24-48 hours to produce the mature erythrocyte.

2.3 Control of red cell production

Red cell production is largely controlled by erythropoietin which was the first humoral agent shown to control hematopoietic differentiation. It is a glycoprotein produced mainly in the kidney, and its production can be significantly increased in response to tissue hypoxia (Krantz and Jacobson, 1970). Erythropoietin acts at the level of the committed stem cell to induce both proliferation and differentiation (Goldwasser, 1975). There are at least two classes of erythroid precursors as characterized by in vitro clonogenic assays. An immature committed stem cell termed BFU-E (burst forming unit-erythroid), which responds to high levels of erythropoeitin, producing large colonies of differentiated erythroid cells after 8-10 days of culture, and a more mature stem cell, CFU-E (colony forming unit-erythroid) can both be detected (Queensberry and Levitt, 1979; Till and McCullough, 1980). The latter responds to lower levels of erythropoietin producing small differentiated colonies within two days. Of the two, BFU-E is thought to have the greatest proliferative capacity (Queensberry and Levitt, 1979). In conclusion, erythropoietin acts in two ways. First, it stimulates proliferation of the committed precursors and in so doing augments red cell production by increasing the number of stem

Figure 3. Erythropoiesis. The stages of red cell development, from the pluripotent stem cell to the mature red blood cell are outlined. It takes approximately 3-5 days for a pronormoblast to become a red cell and once in the blood stream the red cell will live approximately 120 days. Within the mitotic compartment division and maturation occur at the same time so that a single pronormoblast will produce several basophilic normoblasts and they in turn will produce several polychromatophilic normoblasts. Within the postmitotic compartment the cells continue to differentiate but no longer divide. From B.M. Babior and T.P. Stossel (1984): Hematology a Pathophysiological Approach p15.

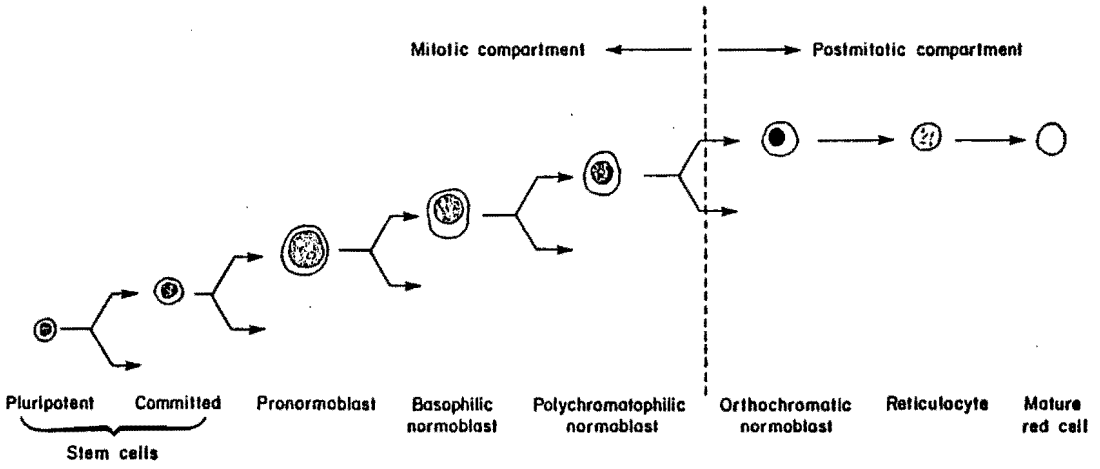


Figure 3. Erythropoiesis.

cells committed to the erythroid lineage. Secondly, it shortens the time required for these committed stem cells to mature to reticulocytes (Popovic and Adamson, 1979; Babior and Stossel, 1984). A summary of erythropoietin action is shown in Figure 4.

Figure 4. The action of erythropoietin. Red cell production is largely controlled by erythropoietin which both stimulates the proliferation of the committed precursor cells and shortens the time required for these committed stem cells to mature to reticulocytes. From B.M. Babior and T.P. Stossel (1984): Hematology a Pathophysiological Approach p 16.

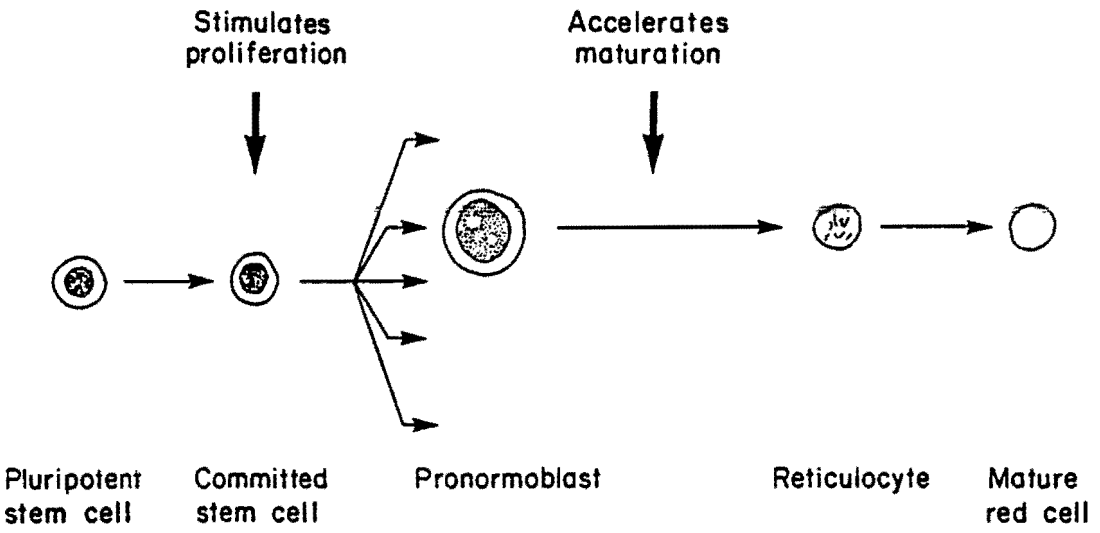


Figure 4. The action of erythropoietin.

3. HEMOGLOBIN

3.1 Introduction

The main function of the red blood cell is to transport oxygen to the tissues. This is accomplished by the hemoglobin molecule which is uniquely structured such that under the high partial pressure gradient for oxygen (PO_2) in the lung it picks up oxygen, while at the PO_2 in the tissues oxygen is released. It therefore plays a critical physiological function, transporting oxygen to the tissues.

Hemoglobin is a protein with a molecular mass of 64,500 daltons. It is composed of four polypeptide chains each conjugated to a heme moiety (Fig. 5). The polypeptide chains, known as globins, are a family of protein subunits. In normal adult hemoglobin there are two types of globin chains α and β which combine in dissimilar pairs forming the final tetrameric molecule $\alpha_2\beta_2$. This structure is held together primarily by attractive forces between the dissimilar subunits. In addition within each globin subunit is a region known as the heme crevice where the heme moiety is held by strong, but non-covalent forces. Hemoglobin structure and function have been the subject of intense study. For more extensive reviews refer to; Dickerson and Geis, 1983; Bunn and Forget, 1985; Stamatoyannopoulos et al., 1987.

3.2 Heme

Heme is an iron containing porphyrin derivative. It is ubiquitous and functions as the prosthetic group in a variety of different hemoproteins. In the proteins hemoglobin (described above) and myoglobin (a single polypeptide chain with one heme group, found in muscle and functions as an oxygen store), heme serves as a functional group for the binding of oxygen. Oxygen binds to the ferrous iron within the heme moiety. Ferrous

Figure 5. Hemoglobin structure. Hemoglobin is composed of four polypeptide chains each conjugated with a heme moiety. Normal adult hemoglobin contains two types of globin chains, α and β . Alpha/beta dimers combine to form the final tetrameric molecule. From B.M. Babior and T.P. Stossel (1984): Hematology a Pathophysiological Approach p22.

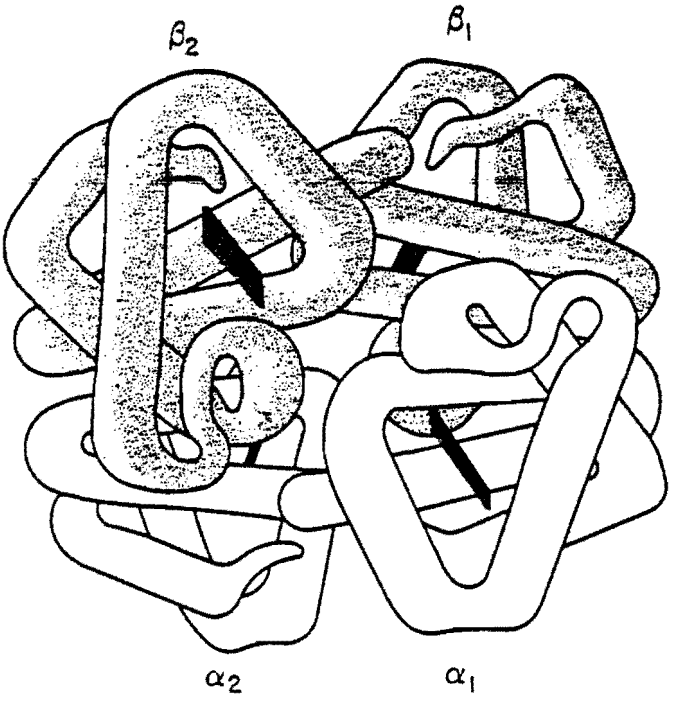


Figure 5. Hemoglobin structure.

iron is stabilized within the hemoglobin and myoglobin structures such that the binding and release of oxygen does not under normal conditions affect the oxidation state of the iron atom. This is a unique feature of these two proteins and is important since hemoglobin and myoglobin containing ferric heme (methemoglobin, metmyoglobin) are unable to reversibly bind oxygen.

In addition to hemoglobin and myoglobin, heme is also the prosthetic group for a number of vital enzymes including; cytochrome P450; the cytochromes of the electron transport chain; catalase, which decomposes H_2O_2; peroxidase, which activates H_2O_2; and tryptophan pyrrolase, which catalyses the oxidation of tryptophan. Within these enzymes the iron in the heme moiety undergoes reversible changes between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states (Lehninger, 1975). It is the ability of the iron within the heme moeity to pick up and donate electrons which makes it so useful in such a variety of reactions. In these forms therefore, the complexed heme moiety serves as an electron carrier whereas in hemoglobin and myoglobin it acts as an oxygen carrier.

3.3 Heme synthesis and regulation

Heme synthesis begins in the mitochondria with the condensation of glycine and succinyl-coenzymeA to form δ-aminolevulinic acid (ALA) (see Fig. 6). This reaction is catalysed by the mitochondrial enzyme δ-aminolevulinic acid synthase (ALA synthase). ALA then passes into the cytosol where, catalysed by the enzyme ALA dehydratase, two molecules of ALA combine to form the monopyrrol porphobilinogen (PBG). Four molecules of PBG then condense to form the tetrapyrrol structure uroporphobilinogen III. This reaction is catalysed by two enzymes, PBG deaminase, which leads to the formation of a linear tetrapyrrol structure (Battersby et

Figure 6. Heme biosynthesis. Presented here is an overview of the heme biosynthetic pathway, showing the intracellular localization of the heme biosynthetic enzymes and intermediates. For detailed description refer to the text. Modified from N.G. Ibraham et al., (1983): Progress in Hematology Vol. 13 p76.

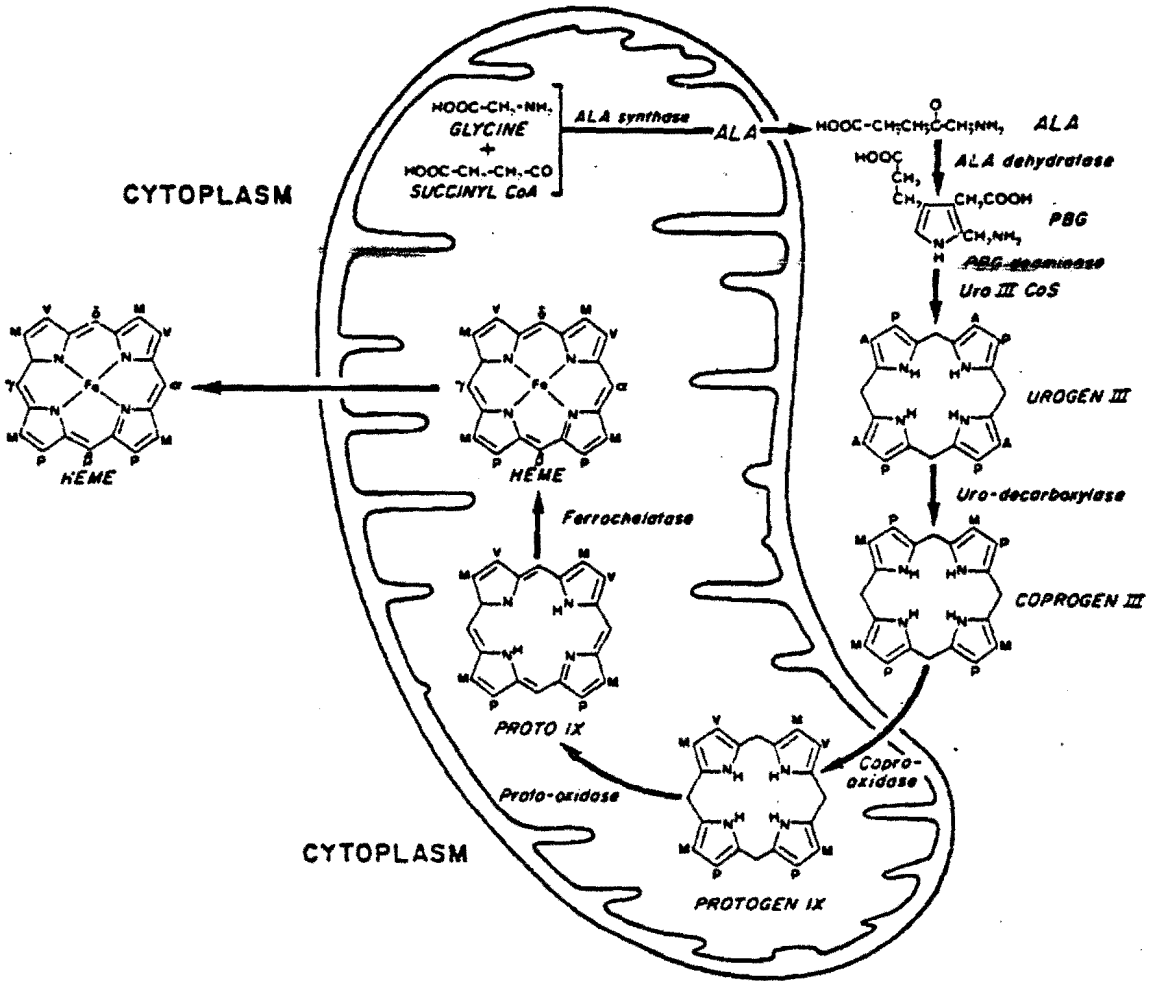


Figure 6. Heme biosynthesis.

al., 1979); and uroporphorinogen III cosynthase, which leads to the formation of the cyclic tetrapyrrol structure uroporphyrinogen III (Levine, 1968). The rapid enzymatic formation of uroporphyrinogen III from PBG, and the absence of metabolic intermediates suggests that these two enzymes (PBG deaminase and uroporphyrinogen III cosynthase) exist as an enzyme complex (Frydman and Feinstein, 1974; Higuchi and Bogorad, 1975). This cyclic tetrapyrrol structure then undergoes a series of side chain modifications. First the cytoplasmic enzyme uroporphyrinogen decarboxylase converts four acetic acid side chains to methyl groups forming the structure coproporphyrinogen III. This structure then passes into the mitochondria where the remaining steps in heme biosynthesis take place. Coproporphyrinogen oxidase modifies two proprionic acid side chains to vinyl groups forming protoporphyrinogen IX and subsequently, protoporphyrinogen oxidase removes six hydrogen atoms forming protoporphyrin IX. The final step in heme biosynthesis is the enzymatic insertion of ferrous iron into protoporphyrin IX to form heme. Conceptually, this biosynthetic pathway may be considered as occurring in two distinct steps, the first being porphyrin biosynthesis and the latter, heme biosynthesis which is the enzymatic insertion of ferrous iron into protoporphyrin (for reviews see; Kappas et al., 1983; Ibraham et al. 1983).

As one might expect in any multistep biosynthetic pathway, the end product, heme, regulates its own synthesis. Studies in bacteria and hepatocytes have shown that ALA synthase is the rate limiting enzyme of this pathway (Burnham and Lascelles, 1963; Granick and Urata, 1963), and that heme regulates its own synthesis by feedback inhibition of the activity (Paterniti and Beattie, 1979) and repression of the synthesis of

this enzyme (Burnham and Lascelles, 1963; Granick et al 1975). Recent evidence suggests however, that this is not true in erythroid cells (Ponka and Neuwirt, 1970; Ponka et al., 1973; Woods, 1974; Sassa, 1976; Malik et al., 1979a,b; Ponka and Schulman, 1985a,b). It should be mentioned here that while all cells contain heme, not all cells make heme at the same rate. Immature erythroid cells in the bone marrow make large amounts of heme which is used to form hemoglobin. The second largest site of heme synthesis in the body is the liver which contains 15% of total body heme. Hepatic heme is used for a number of hemoproteins of which cytochrome P450 is the most abundant. While heme synthesis in hepatic tissue has been extensively studied, studies of heme synthesis in erythroid cells have been hampered by the inability to isolate homogeneous populations of early erythroid cells which would be actively synthesizing hemoglobin. One of the best studied erythroid models is the reticulocyte which can be collected, in a partially purified form, from the peripheral blood of anemic animals. However, reticulocytes appear late in erythroid development and, lacking nuclei, cannot be used for studying transcriptional events. To study erythroid hemoglobin synthesis one may therefore use alternative erythroid models such as the murine erythroleukemia (MEL) cell system (see Introduction, section 4). The regulation of heme synthesis and the possibility of a distinct regulatory mechanism in erythroid cells is discussed in detail in Chapter 3.

3.4 The coordinate regulation of heme and globin synthesis.

Mature erythroid cells contain only complete hemoglobin molecules and are not capable of synthesizing heme or globin. Only minute amounts, if any, of free heme or unbound globin chains are detectable. This means that during erythropoiesis there must be a very tight co-ordination

between polypeptide chain synthesis and heme formation. This is achieved by virtue of the fact that "free" heme, if allowed to accumulate, stimulates both globin translation (Zucker and Schulman, 1968; Jagus et al., 1981) and transcription (Hoffman and Ross, 1980; Fuchs et al., 1981). At the same time "free" heme will, as described earlier, feedback inhibit its own synthesis. How it does so is still debatable. It may a) feedback inhibit the activity and/or synthesis of ALA synthase as is the case in bacteria and hepatocytes (Burnham and Lascelles, 1963; Granick et al., 1975; Paterniti and Beattie, 1979) or b) in erythroid cells it may inhibit iron uptake from transferrin (Ponka and Neuwirt, 1969; Ponka and Schulman 1985a,b) and thereby limit the iron available for insertion into protoporphyrin, or c) there may be some other as yet uncharacterized mechanism. Clearly however, one of these must operate in erythroid cells to ensure feedback inhibition of heme synthesis, and thus coordinate expression of heme and globin. In addition it should be noted that there is virtually no non-hemoglobin iron in mature erythrocytes. This implies that heme synthesis and iron uptake, are also tightly coordinated during erythroid development.

4. IRON METABOLISM

4.1 General Introduction.

Iron is an essential nutrient required by all cells for growth and survival. In addition to its function as an iron-porphyrin complex described earlier, there are several iron-proteins which require non-heme iron. Iron complexed in this form is usually found in the active center of the protein where it is covalently associated with either acid-labile sulphide or cysteinyl sulphur (Worwood, 1977). Some mammalian iron containing proteins are listed in Table 1. What makes iron so useful in such a variety of reactions is that it is a transition metal, and as such it can exist in two stable oxidation states Fe^{2+} and Fe^{3+}. It is the ability of this metal to pick up and donate electrons that makes it so useful in biological redox processes.

At physiological pH and oxygen tension however, iron is insoluble and will precipitate as ferric hydroxide ($Fe(OH)_3$) (Spiro and Saltman, 1969). To overcome this solubility problem, nature has developed sophisticated iron chelating and transporting systems to utilize this metal. Microorganisms, for example, have evolved small non-protein molecules specific for iron chelation which are able to sequester and transport iron (Neilands, 1981). Higher animals, on the other hand, tend to use proteins to transport and store iron. In mammals the serum glycoprotein transferrin transports iron from sites of absorption to sites of utilization and excess iron is stored within cells in the protein ferritin.

4.2 Body iron distribution

Total body iron content of an average 75 kg man is estimated at 4 g. Of this amount only 1-2 mg is absorbed and excreted each day. There is no

Table 1. Some Mammalian Iron-containing Proteins

Protein	Mol. wt.	No. of Fe Atoms Per Molecule	Distribution	Function	Ref.
Heme containing					
Hemoglobin	65,000	4 protoheme	Red blood cells	O_2 carrier	Text
Myoglobin	17,000	1 protoheme	Muscle	O_2 carrier	Text
Cytochrome aa_3	180,000*	2 heme a	Mitochondria	Terminal oxidase	89
b	18,000–30,000*	1 Protoheme	Mitochondria	Electron transport	89
c_1	37,000*	1 heme c	Mitochondria	Electron transport	89
c	12,000	1 heme c	Mitochondria	Electron transport	89
b_5	12,000	1 protoheme	Endoplasmic reticulum	Electron transport	89
P-450	—	protoheme	Endoplasmic reticulum	Steroid, drug, hydroxylation	89
Catalase	240,000	4 protoheme	Red blood cells, peroxisomes	Peroxide breakdown	21
Lactoperoxidase	93,000	1 protoheme	Milk	Peroxide breakdown	21
Tryptophan pyrrolase		heme dependent	Liver cytosol	L-tryptophan → formylkynurenine	3
Nonheme					
Aconitase	66,000	2 Fe 3 S	Pig Heart†	Citric acid cycle	51
(Phenylalanine hydroxylase)	100,000	2 Fe	Rat liver†	Phenylalanine → tyrosine	51
Adrenodoxin	12,500	2 Fe 2 S	Adrenal mitochondria	Steroid hydroxylation	51
(Complex III Fe-S protein)	30,000	2 Fe 2 S	Mitochondria	Electron transport	51
(Succinate dehydrogenase Fe-S protein)	27,000	2 Fe 2 S	Mitochondria	Electron transport	51
(Succinate dehydrogenase flavoprotein)	70,000	4 Fe 4 S 1 FAD	Mitochondria	Electron transport	51
NADH dehydrogenase		23–28 Fe + S FMN	Mitochondria	Electron transport	51
Xanthine oxidase	275,000	8 Fe 8 S 2 FAD 2 Mo	Milk,† tissue	Hypoxanthine → uric acid	51
Transferrin	77,000	2	Plasma	Iron transport	Text
Lactoferrin	77,000	2	Milk, secretions	Iron transport	Text
Ferritin	450,000–900,000	0–4000	All tissues	Iron storage	Text
Hemosiderin		Up to 37% Fe (dry wt.)	Liver, spleen, bone marrow	Iron storage	Text

A number of iron-dependent enzymes or processes have not been included.

*Soluble preparations of membrane-bound cytochromes.

†Enzyme isolated from this source. Found in other tissues.

From M Worwood (1977): Seminars in Hematology, Vol 14, p.5

physiological route for iron excretion, it is lost passively in the gastorintestinal tract in the form of blood and sloughed epithelium (Green et al., 1968). Therefore body iron load depends on the regulation of iron absorption (Bothwell et al., 1979). The amount of iron absorbed depends in part on the amount of iron in the diet, the content of the diet, and the form in which the iron is present. Regulation of iron absorption from the intestinal epithelium is poorly understood; however, it appears to reflect both the level of body iron stores and the rate of erythropoiesis (Stamatoyannopoulos et al., 1987). Women for example will loose iron each month in the menstrual flow and this will be replenished by absorption of iron from the diet. Under normal physiological conditions however, iron cycles within the body in an essentially closed loop. Figure 7 presents the relative distribution of iron within the body. The largest pool of body iron is the red blood cells, which accounts for 2300 mg. Iron stores, found primarily in the reticuloendothelial system accounts for 1000mg and the remainder is distributed troughout the various cells of the body, where it is involved in a variety of metabolic processes. Iron which is bound to plasma transferrin represents a small proportion of total body iron (3mg); however, as transferrin circulates within the body this iron load is exchanged resulting in a daily turnover of about 30mg (Worwood, 1979).

An overview of the iron cycle is presented in Figure 8. Mature red blood cells circulate for approximately 120 days after which they undergo phagocytosis by macrophages, primarily in the spleen. Hemoglobin from these cell remnants is broken down and the iron is either deposited in the storage protein ferritin and its breakdown product hemosiderin, or transferred back to serum transferrin. Under normal physiological

Figure 7. Body iron distribution. The total body iron content of an average 75 kg man is 4000 mg. The major portion of body iron is found in the red blood cells (2300 mg). Body stores, the second largest compartment (100 mg), consist of the iron stored in ferritin and hemosiderin, the majority of which is found in the cells of the reticuloendothelial system. The rest is distributed throught the body in the form of various iron containing proteins (500 mg). Transferrin iron represents only 3 mg of the total iron load however it serves an important function in cycling the iron. Comparatively little iron is either absorbed or excreted daily and therefore iron cycles within an essentially closed loop. From T.H. Bothwell et al., (1979): Iron Metabolism in Man p2.

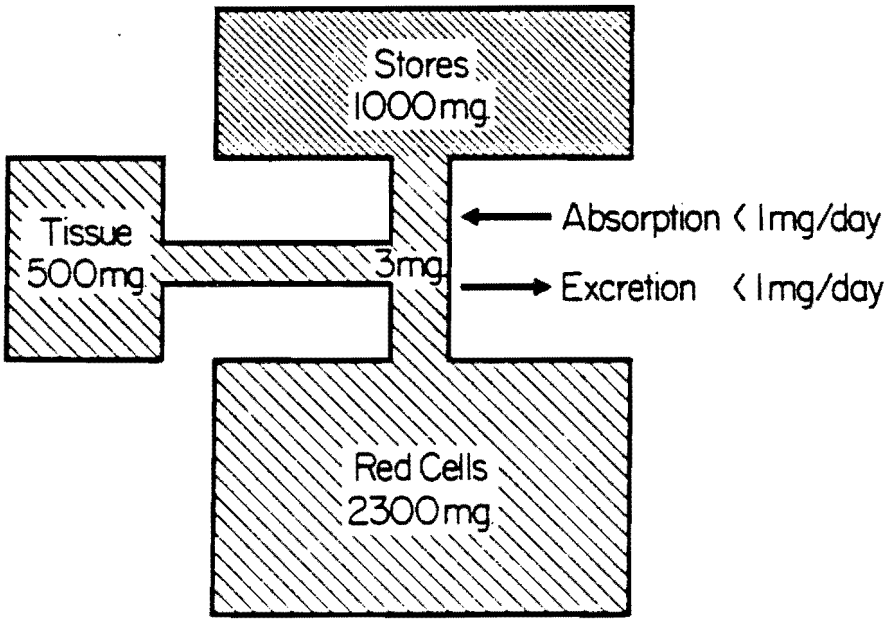


Figure 7. Body iron distribution.

Figure 8. The iron cycle. In the blood iron is circulated bound to the serum transport protein transferrin. Up to 80% of this iron will be directed towards immature erythroid cells where it is used for the synthesis of hemoglobin. These cells develop into mature red blood cells and will circulate in the blood stream for approximately 120 days after which they are removed by mononuclear phagocytes which digest them. Iron released in this process will subsequently be returned to serum transferrin to complete the cycle, or may be stored in ferritin for release at some later time. From B.M. Babior and T.P. Stossel (1984): Hematology a Pathophysiological Approach p41.

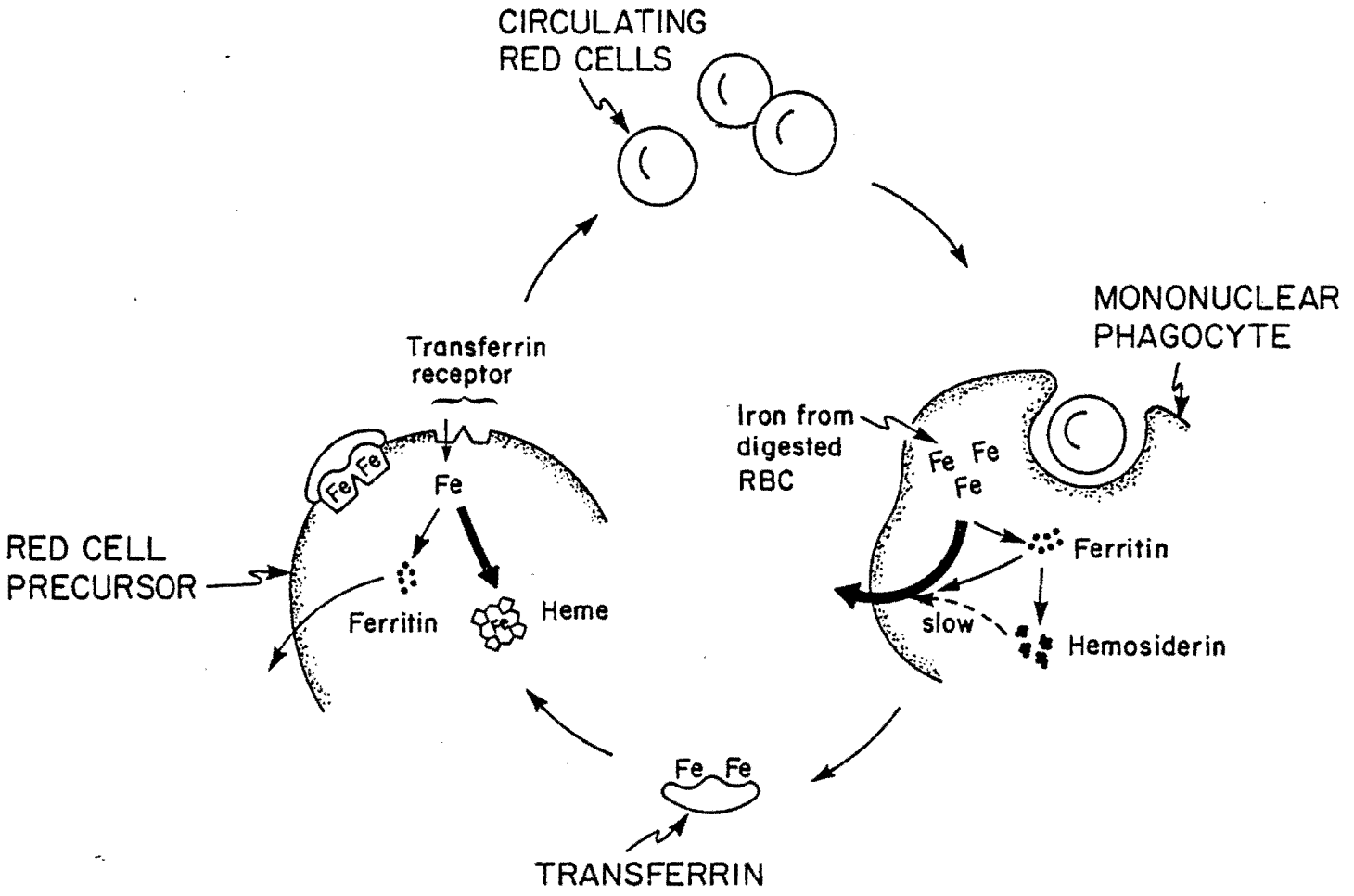


Figure 8. The iron cycle.

conditions a substantial amount of iron will accumulate in ferritin and so this macrophage pool represents a major iron storage site in the body. Finally the transfer of iron back to transferrin and the subsequent interaction of transferrin with the immature erythroid cells completes the iron cycle.

4.3 Transferrin

Transferrins are a class of closely related iron binding proteins which in addition to transferrin include lactoferrin, (found in milk, secretions and in neutrophils) and ovotransferrin (found in egg white) (Mazurier et al.,1983). Each is a monomeric glycoprotein with the capacity to bind reversibly two Fe^{3+} ions per molecule. While serum transferrin is required to transport iron in the plasma thereby supplying most tissues, the other two proteins are produced locally and transport iron in restricted areas. Ovotransferrin has been shown to supply iron to erythrocytes of developing chick embyros (Williams and Woodworth, 1973) and lactoferrin, because of its ability to bind and thereby sequester iron at low pH (Morgan, 1981), is thought to function, at least in part, as a bacteriostatic agent. In addition, at least two other proteins have been identified which show sequence homologies with normal transferrins: p97 - a membrane protein discovered on human melanoma cells (Brown et al., 1982); and B lym - a transforming protein found in chicken B cell lymphomas (Goubin et al., 1983). The significance of these homologies with respect to the evolution and function of normal transferrins remains to be determined.

Serum transferrin is a glycoprotein with an approximate molecular weight of 80,000. It is derived from a single polypeptide chain (Mann et al., 1970) which folds in such a way as to form two similar domains, each

of which contains an iron binding site (Gorinsky et al., 1979). Each transferrin molecule can therefore, bind two atoms of iron. Other metals can also be accommodated but iron is bound preferentially and is by far the most important physiologically. Analysis of transferrin's amino acid sequence (MacGillivray et al., 1977) and cDNA sequence (Park et al., 1985) has led to the suggestion that the two similar domains are a consequence of duplication of an ancestral gene during evolution.

Iron binds to transferrin in the ferric (Fe^{3+}) oxidation state and causes a conformational change in the molecule so that it becomes more compact (Charlwood, 1971; Jarritt, 1976). This iron-transferrin complex has a characteristic orange color with a maximum absorbance between 460 and 465 nm. The protein-metal binding function is dependent on the concomitant binding of a carboxylate anion, which functions to coordinate the iron within the binding site of each domain, and is thought to form a bridge between the iron and the protein (Schlabach and Bates, 1976). The nature of the iron binding sites in transferrin and the identity of the ligands within the transferrin protein which bind iron have been a subject of intensive study. Tertiary folding of transferrin creates, within each domain, an iron pocket which is highly anionic. In particular two tryosine residues (Pecoraro et al., 1981; Williams, 1982), one or possibly two histidines (Rogers et al., 1977) a water molecule and a carbonate ion (Pecoraro et al., 1981) are thought to be involved in the coordination of iron within these binding sites. There are several amino acids with cationic side chains in the vicinity of the tyrosines and the anion carbonate presumably neutralizes these charges allowing iron to bind (Chasteen, 1983). The protein-metal binding is also very sensitive to pH, iron being bound very tightly at physiological pH ($K_F=10^{28}\ M^{-1}$), but

as the pH is reduced to 6.5 iron begins to dissociate and completely dissociates by pH 4.5 (Lestas, 1976).

4.4 Transferrin Receptors.

In the early 60`s Jandl and Katz (1963) first provided evidence that, in reticulocytes, iron was obtained through the interaction of plasma transferrin with a component on the surface of the cell membrane. Numerous studies have since confirmed that cellular iron uptake is receptor mediated, showing that the binding of transferrin is saturable, reversible, and specific (for review see, Morgan, 1981; Huebers and Finch, 1987).

The transferrin receptor is a transmembrane glycoprotein with a molecular weight of 180,000 (Witt and Woodworth, 1978; Hamilton et al., 1979). Treatment with reducing agents shows that it consists of two disulfide bridged monomers of 90,000 each. It has a very high affinity for diferric transferrin (2-7 x 10^9 M) and may bind one or possibly two molecules of transferrin (reviewed in, Newman et al., 1982; Testa, 1985; Heubers and Finch, 1987). Recently cDNA and genomic clones (Schneider et al., 1983; McClelland et al., 1984; Kuhn et al., 1984) for the transferrin receptor have been isolated and sequenced. This should provide new and more detailed information on transferrin receptor structure, function and regulation.

4.5 Cellular Iron Uptake

The cellular uptake of iron from transferrin has been extensively studied and these results are summarized in the model described below (see Figure 9). Essentially, mammalian cells obtain iron through the interaction of transferrin with specific transferrin receptors expressed on the cell surface. Iron uptake involves the binding of iron-transferrin

Figure 9. The process of iron uptake from transferrin by mammalian cells. Iron uptake involves the binding of iron-transferrin to specific cell surface transferrin receptors; endocytosis of the transferrin/transferrin receptor complex in an acidic endosome; at acid pH the iron is released from transferrin and moves into the cytoplasm by an unknown mechanism; apotransferrin remains bound to its receptor and both are recycled to the surface where at physiological pH apotransferrin is released. From E. Morgan (1981): Mol. Aspects Med Vol 4 p46.

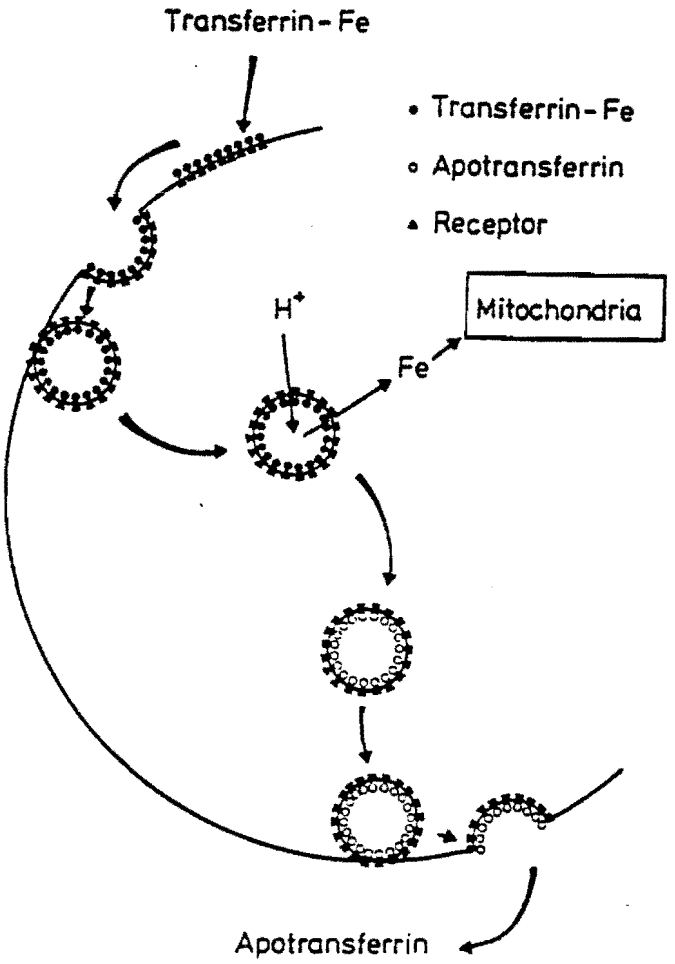


Figure 9. The process of iron uptake from transferrin by mammalian cells.

to transferrin receptors at the cell surface; endocytosis of the iron-transferrin/transferrin receptor complex in an acidic endosome; at acidic pH the iron is released from transferrin and moves into the cytoplasm by an unknown mechanism; apotransferrin remains bound to its receptor at the low pH and the endosome recyles to the surface where at physiological pH apotransferrin is released (for review see E. Morgan, 1981).

In mammals 80% of the iron carried by transferrin will be directed towards immature erythroid cells (Finch et al., 1970) which express large numbers of transferrin receptors because they require large amounts of iron for the synthesis of hemoglobin. Transferrin receptors are also highly expressed on cells which are actively proliferating such as mitogen stimulated lymphocytes and malignant cells. Furthermore, transferrin has been shown to be an essential growth factor for cells in culture (Barnes and Sato, 1980). The possible role of transferrin and transferrin iron in cell proliferation is discussed in detail in chapter 6.

Transferrin is the only physiological iron donor for the majority of cells in the body and until recently it was the only chelate which could supply iron for hemoglobin synthesizing cells. However, in the late 70`s and early 80's a new class of lipophilic synthetic chelating agents, derived from pyridoxal isonicotinoyl hydrazone, were developed. Some of these acyl hydrazones, complexed with iron, were shown to be very effective in donating iron to hemoglobin synthesizing and proliferating cells. In this laboratory's experience the most efficient chelate in supporting hemoglobin synthesis was ferric salicylaldehyde isonicotinoyl hydrazone (Fe-SIH). The chemical structure of salicylaldehyde isonicotinoyl hydrazone is shown in Figure 10. Recent experiments show

Figure 10. The biochemical structure of salicylaldehyde isonicotinoyl hydrazone.

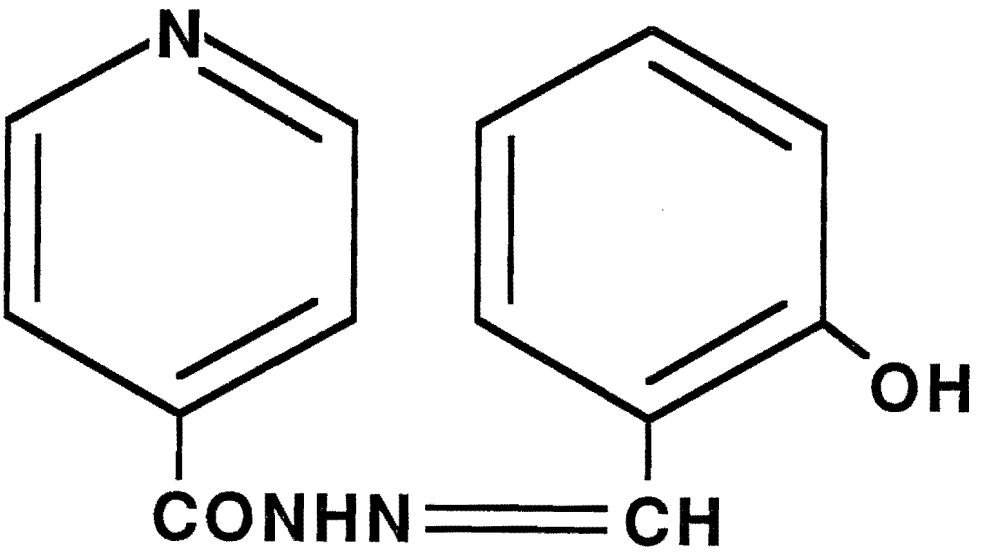


Figure 10. Salicylaldehyde isonicotinoyl hydrazone.

that ferric-SIH is able to deliver iron to cells independently of the transferrin/transferrin receptor pathway and in amounts greater than those seen with saturating levels of iron-transferrin (Laskey et al., 1986).

4.6 Regulation of iron uptake

Iron uptake may be regulated at a number of levels. These include iron availability, the number of transferrin receptors expressed, the rate at which they recycle, the rate at which iron is released from transferrin once the complex has been internalized, and the efficiency of iron extraction from internalized transferrin. It has been observed in a number of different cell systems that transferrin receptor expression is regulated, at least in part, by free intracellular iron levels (Ward et al., 1982). Removing intracellular iron using chelators results in an increase in the rate of denovo transferrin receptor synthesis (Mattia et al., 1984), while supplying iron in excess results in a decrease in receptor synthesis (Rouault et al., 1985; Rao et al., 1985). Recent evidence suggests that the regulation of transferrin receptor synthesis is controlled at the level of gene transcription (Louache et al., 1985; Rao et al., 1986).

Erythroid cells, which require comparatively large amounts of iron for the synthesis of hemoglobin may have an additional regulatory mechanism. It has been reported that in rabbit reticulocytes, addition of exogenous hemin inhibits iron uptake from transferrin (Ponka and Neuwirt, 1969, 1971), while the inhibition of endogenous heme synthesis leads to an increase in iron uptake. It would appear therefore, that free heme may regulate iron uptake in erythroid cells. Under these conditions heme, if allowed to accumulate, would feedback inhibit its own synthesis by limiting the availability of iron for insertion into protoporphyrin. This

in turn would function to provide a balanced supply of heme and globin during erythropoiesis.

5 FERRITIN

5.1 Introduction

Ferritin is an ubiquitous protein which functions to store iron in a soluble form and protect cells from the potentially toxic effects of free iron. It appears to have developed early during evolution as it is present in vertebrates, invertebrates, plants, fungi and bacteria (Munro and Linder, 1978). Such an iron storage protein is essential for living organisms since at physiological pH, in the presence of oxygen, iron is extremely insoluble and will precipitate as hydrous ferric oxides. Therefore, iron which is not bound to either protein or chelate will become unavailable. Furthermore, Fe^{2+}, in the presence of dioxygen will generate free radicals by the "Fenton" reaction (Halliwell and Gutteridge, 1984) (Figure 11). These reactive radicals will ultimately lead to cell death through the destruction of cell constituents including nucleic acids, lipids and proteins. Ferritin is therefore an essential housekeeping protein providing a reserve of iron in a soluble non-toxic form. It accounts for 15-30 percent of total body iron and is second only to hemoglobin as the most abundant iron protein in the body (Reeves et al., 1980). It was first observed by Granick in 1943 that ferritin synthesis is stimulated by iron. Since then it has been confirmed by several groups that the synthesis of apoferritin can be stimulated by loading cells with iron salts, both in vivo (Drysdale and Munro, 1966) and in vitro (Beck et al., 1974).

Evidence suggests that ferritin likely serves specialized functions in certain cell types. It may for example, play an important role in iron transport and absorption in the intestinal mucosa (Siimes and Dallman, 1974; Greenman and Jacobs, 1975); in the recycling of iron in macrophages

Figure 11. The Fenton reaction. If a single electron is accepted in the ground state by O_2 it produces the superoxide radical O_2^-. O_2^- is formed in almost all aerobic cells. In aqueous solution, O_2^- will produce H_2O_2 by the dismutation reaction. The subsequent mixture of H_2O_2 and an iron salt will generate •OH radicals. It can in fact provoke a whole series of radical reactions. These radicals are extremely reactive and have many damaging effects in living systems. From B. Halliwell and J.M.C. Gutteridge (1984): Biochem. J. Vol. 219 p2.

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \text{ (dismutation reaction)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^- \text{ (Fenton reaction)}$$

traces of Fe^{3+} can react further with H_2O_2:

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + O_2^- + H^+$$

and more reactions are possible:

$$\cdot OH + H_2O_2 \longrightarrow H_2O + H^+ + O_2^-$$
$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$
$$\cdot OH + Fe^{2+} \longrightarrow Fe^{3+} + OH^-$$

Figure 11. The Fenton Reaction.

(Hersko, 1977); and in long term iron storage in the adult liver (Siimes and Dallman, 1974; Unger and Hershko, 1974). In addition to its role in iron metabolism ferritin has also been described as a tumor marker (Hazard and Drysdale, 1975) and as a possible regulator of myelopoiesis (Broxmeyer et al., 1982).Thus while ferritin serves a common function of iron storage there is also evidence for cell specific variations in structure and function which may be genetically regulated features of specific cell types. The recent cloning of ferritin cDNAs from several species should provide new insight into ferritin gene organization and the regulation of ferritin gene expression and help to define structure-function relationships.

5.2 Structure

Ferritin is a large macromolecule, composed of 24 subunits assembled in a hollow sphere-like shape, with an aggregate molecular weight of 450,000 daltons. Within this apoferritin shell, anywhere from 0-4500 atoms of iron may be stored in the form of ferric oxyhydroxide crystals. Under normal physiological conditions however, it is rarely saturated. While the precise mechanism of iron entry is poorly understood it is known that iron enters ferritin as ferrous iron (Fe^{2+}) and is oxidized to be stored finally in the ferric (Fe^{3+}) form (Macara et al., 1972; Macara et al., 1973). There is evidence that this oxidation is a function of the protein itself (Crichton and Roman, 1978), while others speculate that Fe^{2+} is probably largely oxidized once inside the protein shell (Harrison et al., 1980). Iron can be removed from ferritin by using biological reducing agents such as reduced flavins. The particular efficiency of the reduced flavins to remove iron has led to the speculation that they may be the operative reductant in vivo (Sirivach et

al., 1974). X-ray diffraction analysis of horse spleen apoferritin (Baynard et al., 1978; Rice et al., 1983) has shown that the ferritin subunits share the same basic structure, which consists of 5 interlinked alpha helices (A-E, see Figure 12). High resolution X-ray crystallographic data reveal the presence of eight hydrophobic channels formed by the N terminal ends of three subunits, while interactions of four subunits form six channels of about 10 A^{o} in diameter, which penetrate into the central cavity (Figure 13). The presence of conserved residues within both types of channels suggests that they may be involved in iron transport. It remains to be seen which of these channels (the three-fold, the four-fold, both or perhaps neither) are involved in iron uptake and which are involved in iron release.

5.3 Isoferritins

Examination of ferritins from different tissues, by isoelectric focusing, reveals considerable heterogeneity, indicating that ferritin is in fact a family of closely related isoferritins. In the late 70's Drysdale and his colleagues suggested that these variations could be explained by their observation that there were in fact two distinct types of subunits (Drysdale et al., 1977a; Arosio et al., 1978; Kohgo et al., 1980). Identified by their respective mobility in SDS gels these two subunits were termed H or heavy subunit (21,000 daltons) and L or light subunit (19,000 daltons). These subunits combine in varying ratios in the different tissues. Thus, ferritins isolated from several tissues could be separated by isoelectric focusing into isoferritins which reflected both immunological differences and subunit composition (Drysdale et al., 1977a; Arosio et al., 1978)(see Figure 14). Isoferritins may also vary within the same tissue under different physiological conditions, such as iron

Figure 12. Ribbon diagram of ferritin subunit. This schematic representation of the carbon backbone of a horse spleen apoferritin subunit shows the folding of the four long alpha helixes A,B,C,D, as well as the smaller E helix. These helixes are composed of amino acid residues 10-39, 45-72, 92-120, 124-155, and 160-169 respectively. From E.C. Theil (1987): Ann. Rev. Biochem. Vol. 56 p292.

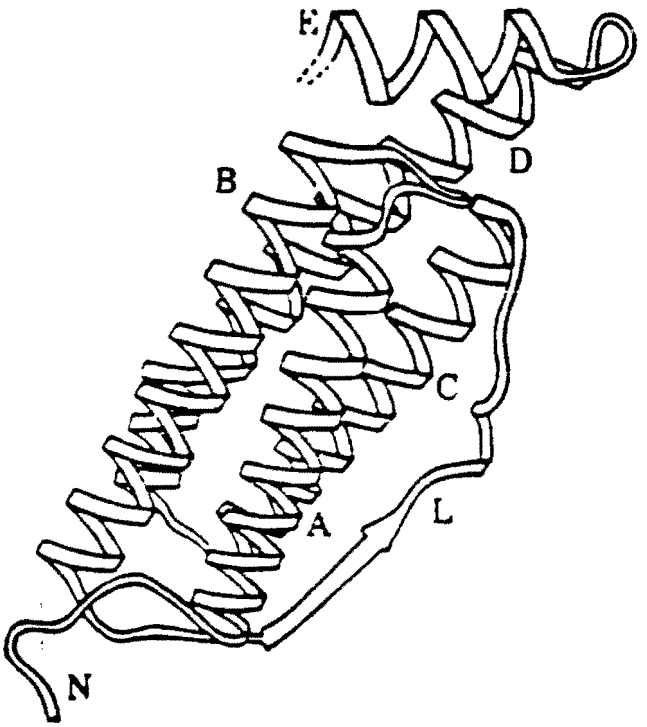


Figure 12. Ribbon diagram of ferritin subunit.

Figure 13. The quaternary structure of ferritin. Presented is a three dimensional model of horse spleen ferritin viewed down a molecular four-fold axis. Each subunit is represented by an oblong shaped building brick. Within each of these components, the area labeled N represents the N terminal region of each subunit while the area labeled E represents the E helix end of each subunit. From E.C. Theil

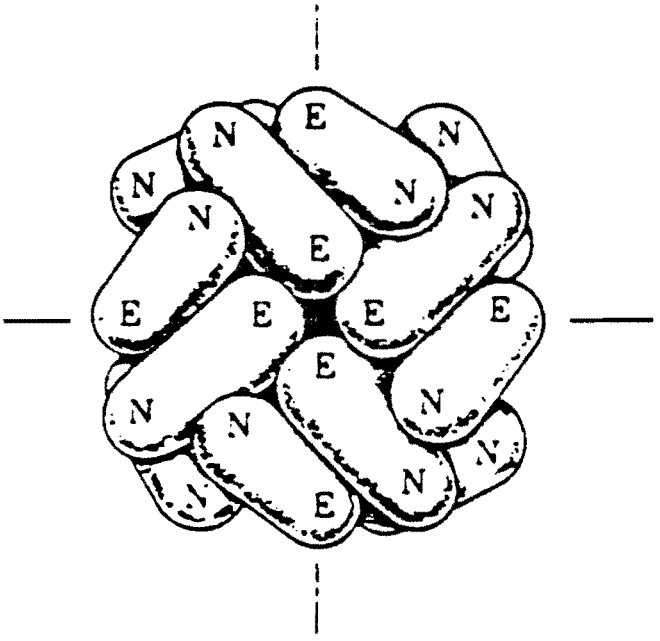


Figure 13. The quaternary structure of ferritin.

Figure 14. A model to explain isoferritins isolated from human tissues. The diagram shows the variations in pI values of ferritins found in different human tissues and diseased states. To explain these differences Drysdale (Drysdale, 1977a) proposed that isoferritins are composed of varying proportions of H and L subunits. From T.H. Bothwell et al. (1979): Iron Metabolism in Man, p318.

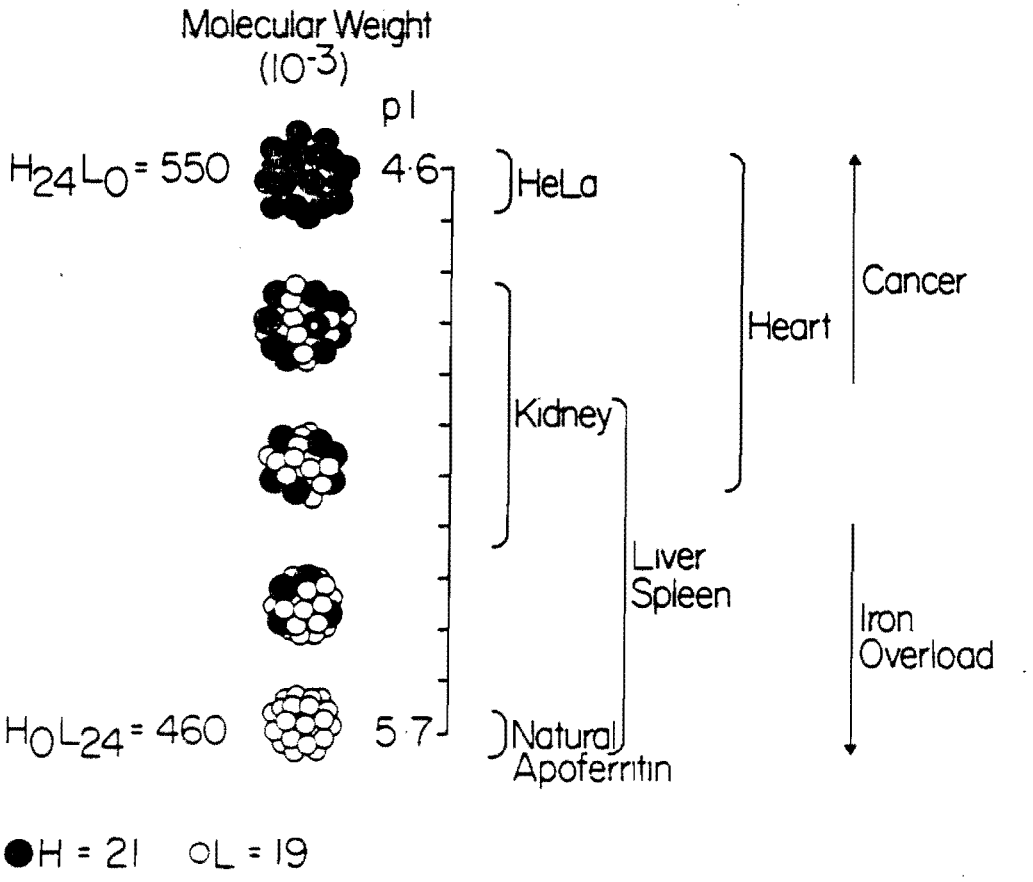


Figure 14. A model to explain isoferritins isolated from human tissues.

overload, inflammation, during development, or following neoplastic transformation (Drysdale et al., 1977). While the structural significance of these changes has not been established it is now widely believed that they reflect differences in the ability of the two subunits to pick up and store iron. This is suggested by the observation that in humans, H rich isoferritins tend to have a low iron content and are found in tissues with a high iron turnover such as the heart, whereas L rich isoferritins tend to have higher iron contents and predominate in iron overloaded tissue and in the liver, a site of long term iron storage (Arosio et al., 1978). Furthermore, iron challenge induces preferential synthesis of L subunits in both human (Dorner et al., 1983) and rat cells (Bomford et al., 1981). The possibility that H and L ferritin subunits differ in their ability to pick up and store iron is now being tested using recombinant isoferritins. Preliminary results suggest that H rich isoferritins take up iron more quickly. This results in a disordered crystalline core which is able to release iron faster (Harrison et al., 1987). In addition several groups are now using site directed mutagenesis to identify areas of the ferritin subunits which are involved in protein assembly, stability and iron uptake and deposition.

5.4 The ferritin gene family.

The recent cloning of ferritin cDNAs from several species (see Table 2) confirms that H and L ferritin subunits are derived from different mRNAs and provides new insight into the genetic organization of these genes and the regulation of their expression. It also provides new and neccesary tools to address the complex questions of ferritin structure-function relationships and ferritin heterogeneity.

Nucleotide sequence analyses of human H and L ferritin cDNAs indicate

Table 2. Ferritin cDNAs cloned from various species and cell types.

species	cell type	reference
human	liver	Boyd et al. (1985)
	lymphocyte	Boyd et al. (1984)
	HL60	Chou et al. (1986)
	U937	Dorner et al. (1985)
Rat	liver	Brown et al. (1983)
		Leibold et al. (1984)
Bullfrog	red cell	Didsbury et al. (1986)

that these two genes show extensive homology in their coding regions, but differ markedly in their non-coding regions (Drysdale et al., 1985). Amino acid sequences of the two subunits show about 60% homology of which 80% of those differences could have arisen from single base changes (Boyd et al., 1984). Comparison of the 5' untranslated region (UTR) of H and L ferritin cDNA from human and rat demonstrates extensive homologies between H and H or L and L of different species but no significant homologies between H and L within or between either species. This pattern of conservation suggests that the two subunits may be differentially regulated (Liebold et al., 1984). Within the coding region the residues involved in interactions of helices within and between subunits are strongly conserved (Munro et al., 1985). This allows them to form subunits of similar shapes which will be able to interact appropriately to form the apoferritin shell. The major difference in the functional groups in H and L subunits lies in the hydrophobic channel at the four-fold axis. These differences result in a loss of negative charge in the H subunit in this region. This may explain why the H rich subunits take up and release iron faster, while iron accumulates more in the L rich isoferritins. (Boyd et al., 1985).

Further sequence analysis suggests that these two genes are derived from a common precursor gene, which probably diverged about 200 million years ago (Boyd et al., 1985). Analysis of the ferritin genome reveals that in humans there are 5 copies of the L gene and 15 copies of the H ferritin gene, while in the rat there are approximately 20 copies of the L gene and 5 of the H gene (Brown et al., 1983). In both species however, only one functional gene of each type has so far been identified. In humans an expressed H gene is found on chromosome 11 (Worwood et al.,

1985) while an expressed L gene is found on chromosome 19 (Caskey et al., 1983). The other copies appear by various criteria (lack of introns, the presence of poly A tails and the presence of repeats at either end) to be non-expressed pseudogenes. However, it is conceivable that there are more than two expressed subunits and that these genes may in fact represent distinct subunit types that are expressed in a tissue specific or developmental fashion. This possibility is supported by a recent report there are three distinct subunit types in the bullfrog red cell (Dickey et al., 1987). These three ferritin subunits differ in their cell specificity and regulation by iron. Similar observations may easily have been overlooked in the past given that these three subunits are of very similar molecular weight (20.5, 20.6 and 19.9 as predicted from cDNA) and have similar mRNA length. This suggests that the ferritin genes may encode more than one species of H and L ferritin subunit and this may account for the complexity of tissue isoferritins. Alternatively, or possibly additionally, there is evidence for extensive post-translational modification of ferritin subunits in different tissues (Worwood et al., 1975). One or other of these observations may account for the observation that proteins with identical subunit proportions, i.e. H/L ratio, still may differ in their isoelectric point.

Genomic clones for human H and L ferritin subunits have recently been isolated (Santoro et al., 1986; Constanzo et al., 1986). Both H and L genes contain three introns and four exons and the exon lengths of the two subunits are similar, supporting the hypothesis that they are derived from a common ancestral gene. The intron/exon structure of both subunits corresponds well to the secondary structure of the protein obtained by X-ray diffraction (Baynard et al., 1978; Rice et al., 1983). The first

three exons correspond to A, B, and C alpha helices respectively whereas the fourth exon codes for D and E alpha helices (see Figure 12).

In the process of isolating these genomic clones several other independent clones were isolated, all of which were later determined to be processed pseudogenes. Using a probe prepared from the 5' flanking region of the genomic H ferritin clone in the case of the H ferritin study, and a 3' flanking probe from the L ferritin genomic clone in the L ferritin study it was observed that only one copy of each gene could be found. The authors therefore concluded that H and L apoferritin are each encoded by a single gene, giving rise to a common major transcript in all cells. It is possible however that multiple expressed genes may exist which differ in their 5' or 3' flanking sequences. Because of the complexity of the ferritin gene family, more information is required before it is possible to understand ferritin gene organization and the regulation of ferritin gene expression.

5.5 Regulation of ferritin synthesis.

Iron administration induces ferritin synthesis in many tissues of intact animals (Drysdale and Munro, 1966), in cultured cells (Beck et al., 1974) and in cell free systems (Drysdale and Shafritz, 1975). Furthermore, preincubation with actinomycin D (which inhibits transcription) or cordeycipin (which inhibits polyadenylation) does not inhibit the induction of ferritin synthesis by iron (Drysdale and Munro, 1966; Zahringer et al., 1976). The predominant site of ferritin subunit synthesis is on free polyribosomes (Puro and Richter, 1971), and following iron administration it was observed that there is a shift of ferritin mRNA from the cytoplasmic to the polysomal fraction. Together these results suggest that iron admistration leads to a mobilization of

preexisting ferritin mRNA, possibly through a derepression mechanism. These results are summarized in a translation control model first proposed by Munro and his collegues in 1976 (Zahringer et al., 1976), (Figure 15). In this model they speculate that the repressor which would bind to the 5' untranslated region of the ferritin mRNA might be a ferritin subunit. This suggestion was based on the observations that a) free ferritin subunits in the cytoplasm of hepatoma cells in culture will assemble into ferritin upon iron addition to the medium (Lee et al., 1975) and that b) in vitro , iron stimulates subunit assembly into ferritin (Drysdale and Shafritz, 1975). It was therefore suggested that iron entering the cytoplasm would mobilize these subunits and thus free the mRNA for translation (Figure 15).

The precise mechanism of translational control by iron is currently under investigation in a number of laboratories. Several groups are examining the 5' and 3' untranslated regions (UTR) of ferritin H and L cDNAs searching for regulatory sequences. In these studies the coding region of the mRNA is replaced by an indicator gene - bacterial CAT (chloramphenicaol acetyl transferase). This construct is placed into an expression vector and transfected into an appropriate eukaryotic cell line. The ability of iron to stimulate CAT expression is then measured. By selectively deleting regions of the 5' or 3' UTR one may identify the region which is responsible for the iron response. Munro and collegues recently reported evidence, derived from such an approach, that sequences in the 5' UTR of rat L ferritin mRNA contain the iron responsive element. Furthermore, sequences near the beginning of the 5' UTR are protected against RNase digestion by a protein present in the cell sap of untreated rats. This protein factor no longer protects this sequence if iron is

Figure 15. A model for the translational control of ferritin synthesis by iron. Following iron administration there is a shift of ferritin mRNA from the cytoplasmic to the polysomal fraction, which in turn leads to an increase in ferritin synthesis. This increase is not affected by treatment with actinomycin D or cordycepin, suggesting a cytoplasmic control mechanism. This model proposed by Zahringer et al. (1976) suggests that ferritin subunits adhere to ferritin mRNA preventing translation; however, iron administration promotes the assembly of subnints into ferritin and thereby removes this inhibition. From Zahinger et al. (1976): Proc. Nat. Acad. Sci. Vol. 73 No. 3 p860.

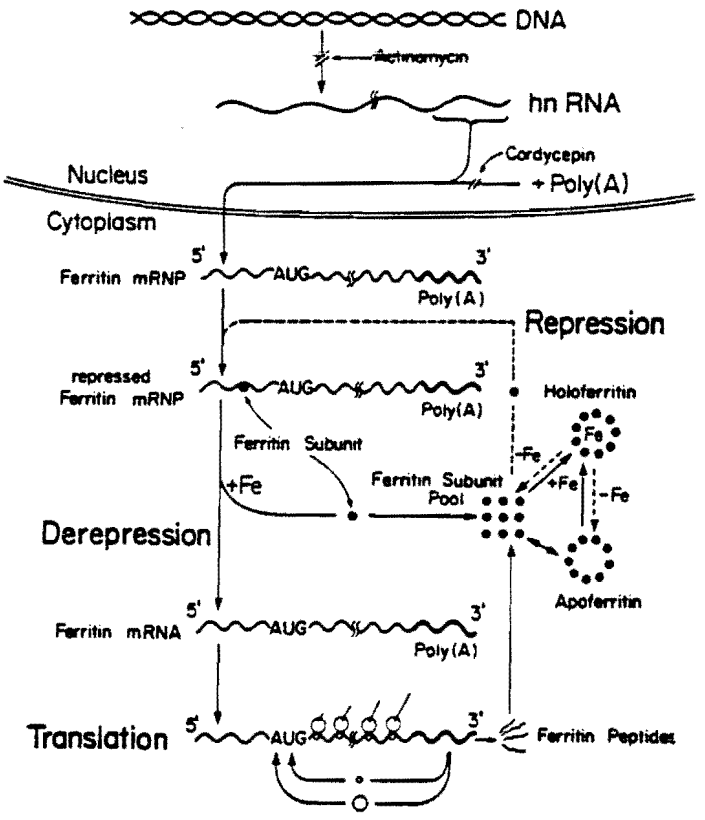


Figure 15. A model for the translational control of ferritin synthesis by iron.

administered (Munro et al., 1987). In a similar study using a CAT-fusion mRNA for human H ferritin, Klausner and his collegues (Klausner et al., 1987) reported that , as with rat L ferritin, the iron responsive element in the human H ferritin is contained in the 5' UTR.

While these results strongly suggest, as has been classically described, that iron regulates ferritin synthesis at the translational level, there is a recent report that in Hela cells iron may also stimulate ferritin transcription (Cairo et al., 1985). These results suggest that iron dependent regulation of ferritin synthesis may be more complex than previously hypothesized. Transcriptional control mechanisms may also be operative during development since it has been demonstrated that the relative proportion of H and L ferritin mRNA is altered during development in the bullfrog red cell (Dickey et al., 1987). In addition, ferritin mRNA levels have been shown to change during differentiation of HL-60 cells in culture (Chou et al., 1986). Evidence from studies which examined the regulation of tagged genomic ferritin clones (Teraoka et al., 1985) suggest that this is a transcriptional regulation requiring 5' upstream sequences to function correctly. The functional significance of such alterations in total ferritin mRNA levels and/or H/L ferritin ratios is not clear. It would however suggest that there is differential regulation of H and L ferritin. It also seems likely that the mechanism of regulation may vary depending on the cell type and physiological conditions. So, for example, ferritin synthesis may depend more on mRNA concentration (transcriptional control) in some cells and more on mRNA utilization (translational control) in others.

The regulation of ferritin gene expression and synthesis in differentiating murine erythroleukemia cells is the subject of chapters

five and six. These studies were undertaken in order to ascertain whether or not there is a cell specific regulation of ferritin synthesis in erythroid cells, i.e. cells which are actively synthesizing hemoglobin. In addition, these cells are capable of undergoing erythroid differentiation in vitro and so also provide a model for the study of ferritin regulation during differentiation.

6. MEL CELLS AS A MODEL FOR ERYTHROID DIFFERENTIATION

6.1 Introduction

The study of normal erythropoiesis is limited by a number of factors. These include the inability to establish long term cultures, to obtain a synchronized population of cells with respect to critical events of differentiation or to obtain a significant number of genetic variants useful for studying differentiation. To overcome these obstacles investigators have turned to the study of cell lines which are capable of differentiating *in vitro*. One such system is the Friend virus transformed murine erythroleukemia (MEL) cell line.

6.2 MEL cells

MEL cells are a virus-transformed erythroid cell line, first described by Charlotte Friend in 1967. They are derived from mice infected with the Friend virus complex (F-MuLV), which consists of two distinct viral activities. The first, Spleen Focus-Forming Virus (SFFV), is responsible for the onset of splenomegaly, hepatomegaly and erythroleukemia and is assayed by its ability to induce colonies in the spleens of suseptible mice. This virus, however, is defective and requires a second, helper virus which may be any one of a number of murine leukemia virus (MuLV) strains for replication. In 1957 Charlotte Friend first isolated this virus (F-MuLV) from the spleens of mice previously inoculated with filtrate from Ehrlich ascites tumor cells and who had developed erythroleukemia. Animals infected with Friend virus and who developed the disease showed enlarged spleens, liver and hyperplastic bone marrow all of which contained numerous cells with the biochemical markings of the erythroid lineage. The target of this virus induced leukemia, therefore, appeared to be immature erythroid cells. Interestingly, the Friend virus

complex (F-MuLV) does not contain a recognized viral oncogene, and the mechanism of cell transformation by this virus is still unknown.

In 1967, C. Friend was successful in establishing a number of cell lines derived from the spleen colonies of Friend virus infected mice. When cloned in semi-solid agar individual cells shed from these tumors gave rise to colonies containing both immature cells and differentiated erythroblasts, suggesting the potential for erythroid differentiation. These lines display a low level of spontaneuos differentiation ranging from .5 - 20 %. None of the cell lines required erythropoeitin to survive nor did they respond to erythropoietin by either dividing or differentiating. It seems likely, therefore, that the Friend virus complex infects an early erythroid precursor comparable to CFU-E, and viral transformation then makes the target cell independent of the normal physiological regulator erythropoietin.

Murine erythroleukemia cells (MEL) are, therefore, virus transformed erythroid cells blocked in a relatively early stage in the pathway of differentiation. When grown in culture these cells display a low level of spontaneous erythroid differentiation ($\leq$1%). However, erythroid differentiation can be induced (<u>in vitro</u>) by several known chemical inducers (Friend et al., 1971). There are several reported inducers of MEL cell differentiation (see Table 3). They vary in their ability to induce erythroid differentiation and in the time course of the differentiation program. However, this program of induced differentiation in many ways parallels that of normal erythroid development and, therefore, provides a model system in which to study the cellular and molecular events which occur during terminal erythroid differentiation.

MEL differentiation is characterized by a) an increase in the enzymes

Table 3. Inducers of murine erythroleukemic differentiation.

Generally strong inducers	Generally weak inducers
Polar-planar compounds[b]	**Polar-planar compounds**
Dimethylsulfoxide	2-Pyrrolidinone
1-Methyl-2-piperidone	Propionamide
N,N-Dimethylacetamide	Pyridine-N-oxide
N-Methylpyrrolidinone	Piperidone
N-Methylacetamide	Pyridazine
N,N-Dimethylformamide	Dimethylurea
N-Methylformamide	**Antibiotics and antitumor agents**
Acetamide	Vincristine
Triethylene glycol	5-Fluorouracil
Polymethylene bisacetamides (n = 2-8)	Methramycin
Hexamethylene bispropionamide	Cycloheximide
Acetamide	X irradiation
Tetramethyl urea	UV irradiation
Antibiotics and antitumor agents	Adriamycin
Bleomycin	Cytosine arabinoside
N-Dimethylrifampicin	Mitomycin C
Actinomycin D	Hydroxyurea
Actinomycin C	**Diamines**
Purine and purine derivatives	Cadaverine
Hypoxanthine	**Fatty acids**
1-Methylhypoxanthine	Acetate
2,6-Diaminopurine	Propionate
6-Mercaptopurine	Butyrate
6-Thioguanine	Isobutyrate
6-Amino-2-mercaptopurine	**Other**
2-Acetylamino-6-mercaptopurine	Hemin
Fatty acids	Methylisobutylxanthine
Butyrylcholine	
Other	
Ouabain	

Modified from P. Marks and R. Rifkind (1978). Ann. Rev. Biochem. Vol. 47, p.428.

of the heme biosynthetic pathway (Sassa, 1976) b) an increase in transferrin receptor expression (Hu et al., 1977; Yeoh and Morgan, 1979; Wilczynska et al., 1984; Laskey et al., 1986) and iron uptake (Glass et al., 1978; Laskey et al., 1986) and c) an increase in globin mRNA (Ross et al., 1976) all of which lead to the accumulation of large amounts of hemoglobin. This differentiation process is accompanied by the onset of terminal cell division, i.e., the loss of capacity for proliferation. Morphologically, the cells become smaller, show a decreased nuclear to cytoplasmic ratio and are comparable to orthochromatophilic normoblasts (Friend et al., 1971).

6.3 MEL cell differentiation

MEL cell differentiation can broadly speaking be divided into early and late events. Detectable commitment begins within the first 9-18 hours following exposure to inducer. "Committed" cells will complete terminal differentiation in the absence of inducer (Gusella et al. 1976). After 48-72 hours 80-90% of MEL cells will have undergone irreversible commitment (Fibach et al., 1977). It is within the initial lag phase of 9-18 hours that the signals which drive the cells to terminal differentiation are initiated. The late stage of MEL cell induction includes the accumulation of hemoglobin and the drop in proliferative capacity. The early events require the presence of inducer while late events do not.

An early event in the induction process is an increase in the rate of heme synthesis (Sassa, 1976). High intracellular heme levels are a later event and may play a role in modulating the late events (Friend et al., 1971; Mager and Bernstien, 1979). Heme treatment can potentiate DMSO induction of MEL cells, but will not by itself initiate terminal erythroid

differentiation (Ross and Sautner, 1976; Guesella et al., 1980). On the other hand, inhibition of endogenous heme synthesis will inhibit the onset of terminal cell division and hemoglobin accumulation, and this block is relieved by treatment with hemin (Mager and Bernstien, 1979). Therefore, heme may play a necessary role in eliciting the complete differentiation program, but only after cells reach a particular stage (presumably this would be the commitment stage).

Altered expression of a number of genes occurs during terminal MEL differentiation. These alterations can be considered in two distinct catergories, a) alterations which lead to the accumulation of the characteristic features of erythroid differentiation, e.g., hemoglobin and b) alterations which result in the cessation of proliferation and the loss of tumorogenic capacity. MEL cells can therefore be used to study alterations in, and the regulation of, gene expression during erythroid development as well as mechanisms which may be involved in controlling cell division. The regulation of globin gene expression for example, probably the most striking event, has been extensively studied and has provided valuable insight into transcriptional control mechanisms (refer to; Curtis, 1980; Marks et al., 1987).

A number of groups are currently investigating the possible involvement of proto-oncogenes in induced MEL differentiation. A number of cellular proto-oncogenes are expressed in MEL cells and their expression is altered following induction. Changes in the level of c-fos (increases), c-myb (decreases and remains low) and c-myc (decreases in the initial 1-4 hours and then returns to normal) expression occur during the initial latent period suggesting that their products may function as nuclear mediators of membrane transduced signals (Ramsay et al., 1986;

Marks et al., 1987). Furthermore, transfection of MEL cells with an expression vector containing c-myc leads to a block in DMSO induced differentiation (Coppola and Cole, 1986). While it is not yet clear how protooncogenes eilicit their appropriate cellular response or how the inducing agents give rise to the changes in protooncogene expression, they do appear to have some role in the differentiation program.

CHAPTER 2

CHARACTERISTICS OF DMSO INDUCED MURINE ERYTHROLEUKEIMIC DIFFERENTIATION

PREFACE

While considerable information has been gained from studies of erythroid differentiation using in vivo and in vitro culture systems, there are several problems which limit experimental design. In this study Friend virus transformed murine erythroleukemia (MEL) cells were selected as a model for erythroid differentiation. These cells provide a system in which to study the changes in cellular physiology and gene expression which lead to the development of the mature erythroid phenotype. The aim of this chapter therefore, is to discuss MEL cells as a model and to characterize the basic features of MEL differentiation in relation to iron metabolism.

ABSTRACT

As an initial step in the investigation of iron metabolism in differentiating murine erythroleukemia (MEL) cells, the ability of MEL cells to undergo erythroid differentiation in vitro was measured and the features of MEL cell differentiation were characterized. In this chapter benzidine staining is used to determine the percentage of cells which are actively synthesizing hemoglobin. To determine the time course and effectiveness of DMSO induction, cells were cultured in the presence and absence of DMSO (1.5%), and cell growth and the percentage of benzidine positive cells were measured over a 96 h period. Iron uptake and transferrin receptor expression were measured before and after DMSO induction. Finally the induction of α and β globin mRNA was measured to ascertain if the pattern of globin gene expression concurred with results previously reported for this cell line. Since the expected pattern of globin induction has already been verified in several laboratories, this system could be used to asses the northern and dot-blot techniques to be used in further experiments.

INTRODUCTION

The objective of this work is to characterize the changes which occur in iron metabolism and their significance during erythroid differentiation. While erythropoiesis constitutes one of the best characterized developmental systems in higher eukaryotes, the study of normal erythropoiesis is limited by several factors. These include the inability to establish normal erythroid precursors in long-term culture, limited availability of genetic variants of significance to differentiation and difficulty in creating a differentiating population synchronized with respect to critical events of differentiation (Marks and Rifkind, 1978). To circumvent these problems cell lines capable of undergoing cellular differentiation in vitro have been established. One such system is the in vitro erythroid differentiation of Friend virus-transformed murine erythroleukemia cells (termed MEL or Friend cells) (Friend et al.,1971).

The MEL model provides an excellent system for the study of erythroid differentiation. The cells can grow indefinitely in culture, it is relatively easy to generate mutant clones and they can be induced to differentiate in a synchronized fashion. However, the model also suffers from limitations. It is a transformed cell line, it is not responsive to the normal physiological regulator erythropoietin and regardless of the inducing agent used it rarely goes to complete erythroid maturation, e.g., it will not enucleate. So, while MEL cells provide an excellent model, results should be interpreted cautiously. In addition, the extent and kinetics of induced differentiation will depend on the MEL clone used, as well as on the inducing agent. Therefore, it is important first to characterize the features of differentiation of the system to be used in

these studies. The clone used was 745 (Friend et al., 1971) and the inducer was dimethlysulfoxide (DMSO).

MATERIALS AND METHODS

Chemicals. Iron free human transferrin was obtained from Behringwerke (Marburg, West Germany); mouse transferrin was kindly provided by Dr. Evan Morgan, the University of Western Australia; lactoperoxidase was from Calbiochem (Los Angeles, CA.). CM Sephadex and CNBr activated Sepharose 4B were obtained from Pharmacia (Dorval, Quebec). All other chemicals were of the highest purity available.

$^{59}FeCl_3$ (10-25 mCi/mg), ^{125}I (17 Ci/mg), and ^{32}P-dCTP (800 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA).

Media and reagents were from Flow Laboratories (McLean, VA) and tissue culture plastic ware from Falcon (Oxnard, CA).

Plasmids containing the cDNAs for mouse α-globin and β-major globin were kindly supplied by Dr. W.French Anderson (N.I.H.), nitrocellulose paper was from Schleicher & Schuell (Keene, NH.) pore size 0.45 μm, the nick translation kit was from Bethesda Research Laboratories (Gaithersburg, MD), and NIF-RX films were from Fugi (Japan).

Cells and Cell Culture. Friend murine erythroleukemia cells used were derived from clone 745A originally isolated by Dr. Charlotte Friend. Cell cultures were grown at 37 ^{o}C in an atmosphere of 95% air and 5% CO_2 in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum. Cells were subcultured every 96 h at an initial cell concentration of approximately 5 X 10^4 cells/ml. For induction, cells were grown in a medium containing 1.5% dimethlysulfoxide (DMSO) at the time of subculturing. After 96 h, both induced and uninduced cells were collected by centrifugation at room temperature and washed three times with serum-free medium. The percentage of benzidine positive cells was determined by the method of Orkin et al. (1975).

Studies with ^{59}Fe and ^{125}I transferrin. $^{59}FeCl_3$ in 0.5 mol/L HCl was converted to ferric citrate by the addition of a 20-fold molar excess of sodium citrate. ^{59}Fe-transferrin was prepared by mixing the ^{59}Fe-ferric citrate with transferrin in a molar ratio of 2 mol Fe:1 mol transferrin. After the addition of solid $NaHCO_3$ to a final concentration of 0.1 mol/l the pH was adjusted to 7.4 and the solution kept at room temperature for three hours. The preparation was then extensively dialyzed (Martinez-Medeillin and Schulman, 1972).

To prepare ^{125}I-transferrin, apotransferrin was saturated with unlabelled iron as its citrate complex (1:20) and labelled with ^{125}I using lactoperoxidase coupled to CNBr activated Sepharose 4B according to the procedure described by David (1972).

To measure transferrin and iron uptake, induced and uninduced Friend cells, at concentrations of 1 X 10^7 cells/ml, were incubated in serum-free medium containing human transferrin labelled either with ^{59}Fe or ^{125}I. At the indicated time periods samples were collected by centrifugation and washed three times with cold phosphate-buffered saline (PBS) and ^{125}I and ^{59}Fe associated with the cells were measured with an LKB Compugamma counter.

Measurement of cellular heme and non-heme ^{59}Fe were performed either by an acid precipitation method (Borova et al., 1973) or by acid methylethylketone extraction (Teale, 1959). There was an estimated 8% loss of total cellular heme during extraction with methylethylketone. When final heme estimates were corrected for this loss, the two methods yielded values which were virtually identical. In view of the similar results with the two methods, it is unlikely that tightly bound, poorly extractable ^{59}Fe is trapped by acid precipitation and as this method is

more rapid it was the method of preference.

Northern and Dot-Blot Analysis. To extract RNA, MEL cells were washed and resuspended in lysis buffer (0.14M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.0, .5% NP-40). The cell lysate was collected after centrifugation for 10 min in an eppendorf microcentrifuge. The lysate was then extracted with phenol/TE and precipitated in ethanol. The pellet was resuspended in DNAse buffer and treated with RNAse free DNAse for 30 min at room temperature. The preparation was then re-extracted with phenol/TE and precipitated in ethanol. Electrophoresis and transfer were performed as described by Davis et al. (1986), except that 2.2 M formaldehyde was used in the running buffer.

For dot blot analysis, a known concentration of total cellular RNA was resuspended in 0.1X SSC, 7.5% formaldehyde and heated to 65°C for 10 minutes. The sample was then cooled on ice for 5 minutes and serially diluted in the same buffer. Four microliters of each dilution was then spotted onto nitrocellulose paper (Schleicher & Schuell), which had been previously soaked for 15 minutes in 1X SSC. The filter was subsequently air dried and baked in a vacuum oven at 80°C for 2 h.

Hybridizations were carried out as described by Meinkoth and Wahl (1984) at 42°C in 50% formamide, 5X Denhardt's solution, 0.1% SDS, 5X SSPE, 200 ug/ml denatured herring sperm DNA and 10% dextran sulfate. The probes used were cDNAs for mouse α and β-globin respectively.

Nick translations were performed with ^{32}P-dCTP according to the manufacturers instructions. Specific activity of the probe was approximately 10^8 cpm/ug. The filters were washed according to the method described by Meinkoth and Wahl (1984).

RESULTS

Effect of DMSO on growth and differentiation. The effect of dimethlysulfoxide on growth and differentiation of MEL cells can be seen in Figure 1. Cells grown in the presence of 1.5% DMSO show a somewhat slower growth rate, reaching about 80% that of control cells after 4 days (panel A). The ability of DMSO to induce differentiation can be seen in panel B. In cells grown in the absence of DMSO the percentage of benzidine positive cells is always less than 1%. Cells grown in DMSO (1.5%) show a dramatic increase in benzidine staining after the third day reaching a maximum of 85% on the fourth day.

As can be seen in Figure 2, after four days of growth in the presence of DMSO there is a marked change in the color of the cell pellet. While the control pellet remains colorless throughout the induction period the DMSO treated pellet turns red, indicating the presence of hemoglobin. Figure 3 shows the benzidine stained cells as they appear under the light microscope. As is clearly evident, there is a marked increase in benzidine staining following DMSO induction.

Transferrin binding and iron uptake. Figure 4 shows that the rate of iron incorporation from saturating concentrations of ^{59}Fe-transferrin into both whole cells and heme are significantly increased following DMSO induction.

Estimation of transferrin receptor numbers (Figure 5) shows that following 4 days of exposure to DMSO the number of transferrin binding sites increases roughly three-fold. These results, together with previous studies (Hu et al., 1977; Yeoh and Morgan, 1979; Wilczynska and Schulman, 1980) suggest that following induction, in addition to an increase the enzymes of the heme biosynthetic pathway (Sassa, 1976), there is also an

Figure 1. The effect of dimethylsulfoxide (DMSO) on growth and differentiation of MEL cells in culture. Cells were grown in the presence or absence of 1.5% DMSO. At the indicated times samples were counted, and stained with benzidine, according to the method of Orkin et al. (1975). The effect of DMSO on growth (panel A) and differentiation (panel B) were recorded.

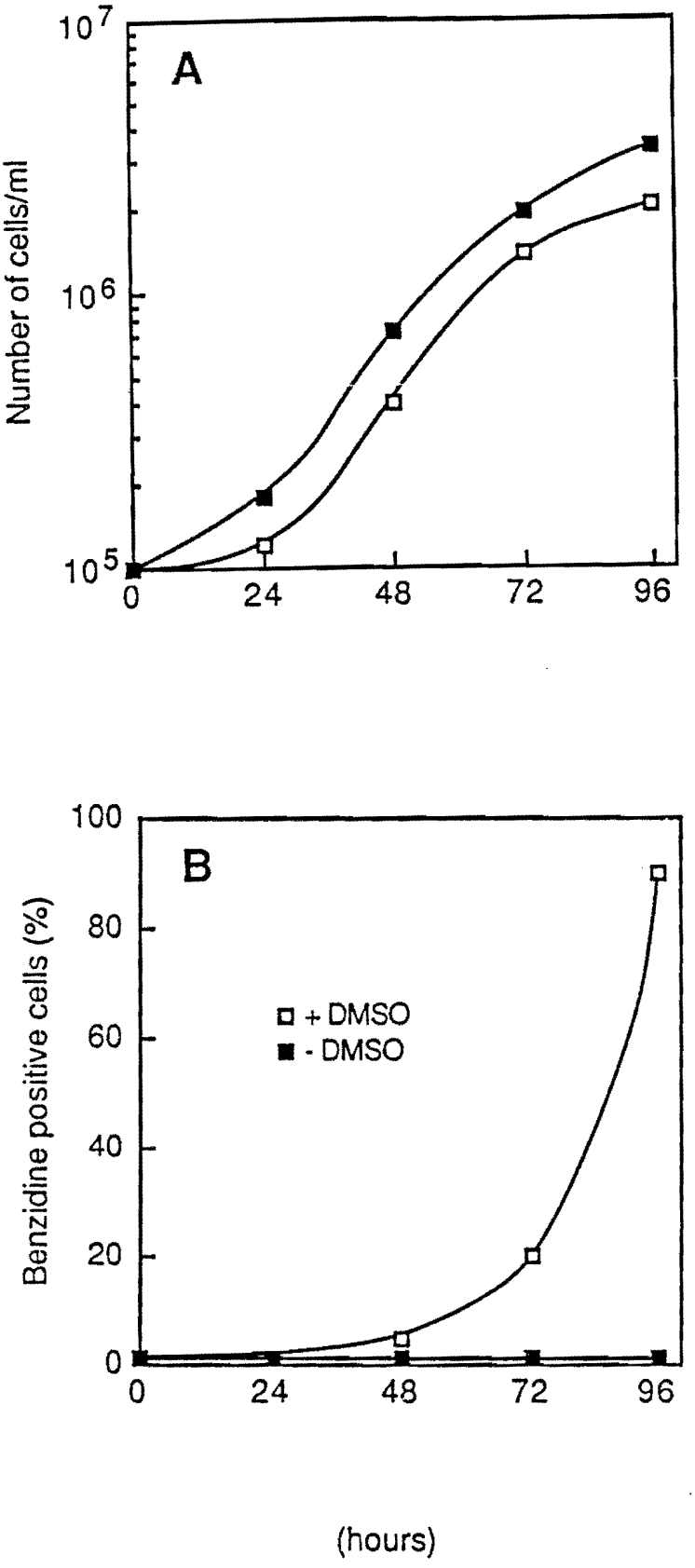

A
10^7
10^6
10^5
Number of cells/ml
0
24
48
72
96
B
100
80
60
40
20
0
Benzidine positive cells (%)
+ DMSO
- DMSO
(hours)

Figure 2. The appearance of MEL cell pellets following growth in the presence and absence of dimethylsulfoxide (DMSO). Cells were grown in the presence of 1.5% DMSO for four days, after which they were collected by centrifugation and washed three times in PBS. Control cell pellets, shown on the right of the photograph, are off-white. Cells grown in the presence of DMSO, shown on the left of the photograph, are distinctly red, indicating the presence of hemoglobin.

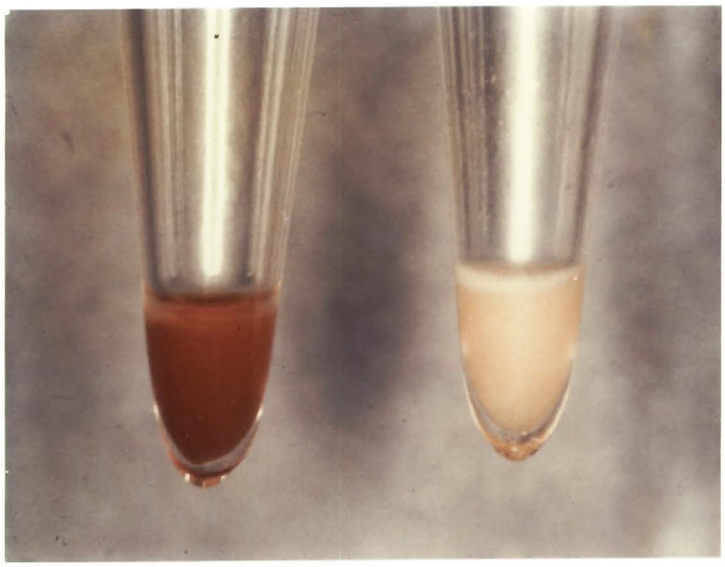


Figure 3. Benzidine stained MEL cells as they appear under the light microscope. MEL cells were grown in the presence and absence of DMSO for four days. Samples were then taken and stained with benzidine, according to the method of Orkin et al. (1975). Control cells, as they appear under the light microscope, are shown in the top frame of the photograph, all cells in this view are benzidine negative. The bottom frame of the photograph shows induced cells, most of which are seen to be positively stained.

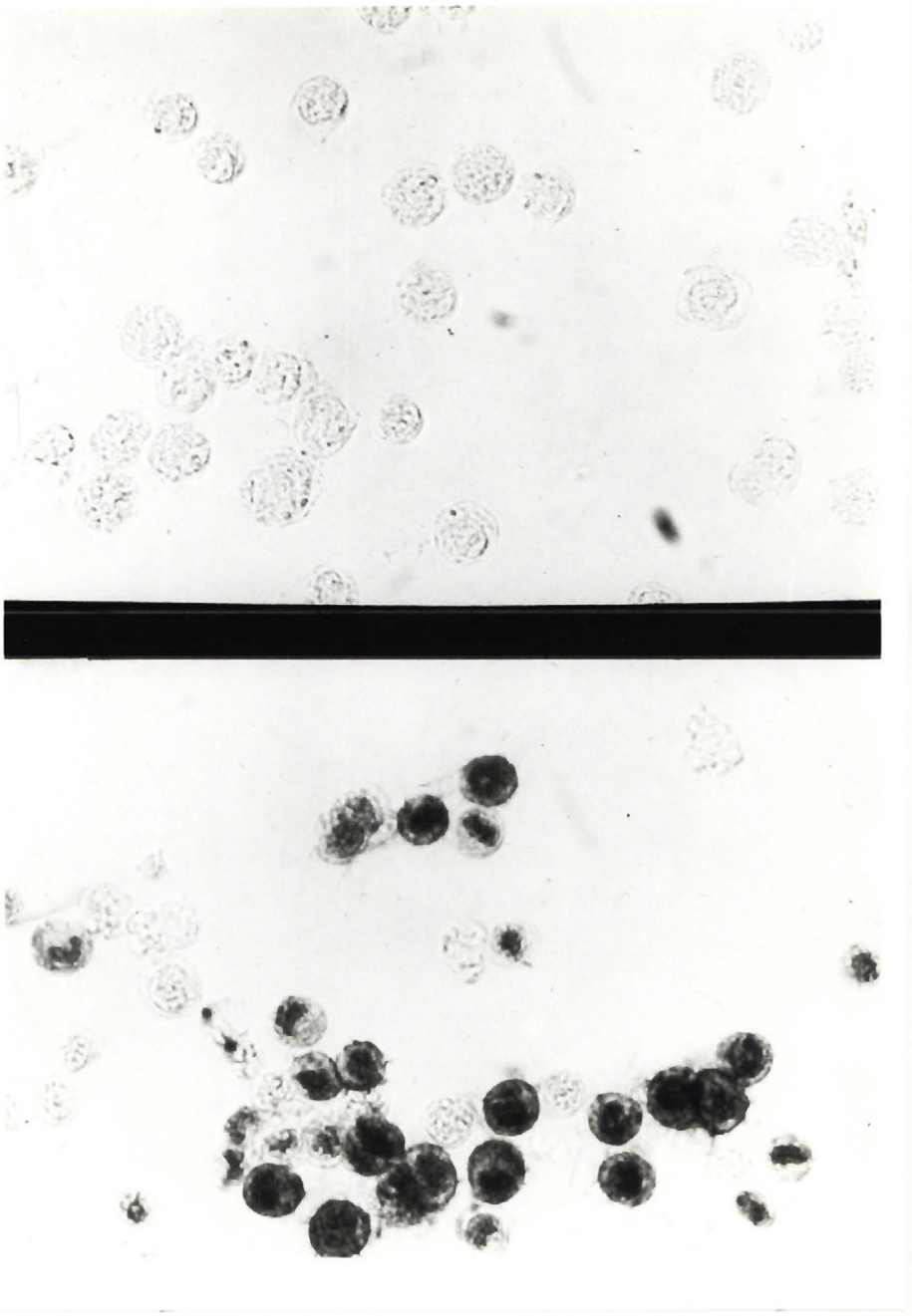


Figure 4. The incorporation of ^{59}Fe from saturating concentrations of ^{59}Fe-transferrin by induced (●) and uninduced (o) MEL cells. MEL cells were incubated with or without 1.5% DMSO for four days, washed, and resuspended in fresh medium without serum. Samples of 1 X 10^7 cells were incubated at 37°C in 0.5 ml (MEM medium with 15mM Hepes, pH 7.4) containing ^{59}Fe-transferrin at a final concentration of 20 μM. At the indicated time intervals cells were chilled, washed three times in phosphate buffered saline, and their incorporated radioactivity measured in both total cells and heme fractions. Since previous studies showed that mouse reticulocytes utilize iron equally well from human and from mouse transferrin (A. Wilczynska, and H.M. Schulman 1978, unpublished), commercially available human transferrin was used in these studies.

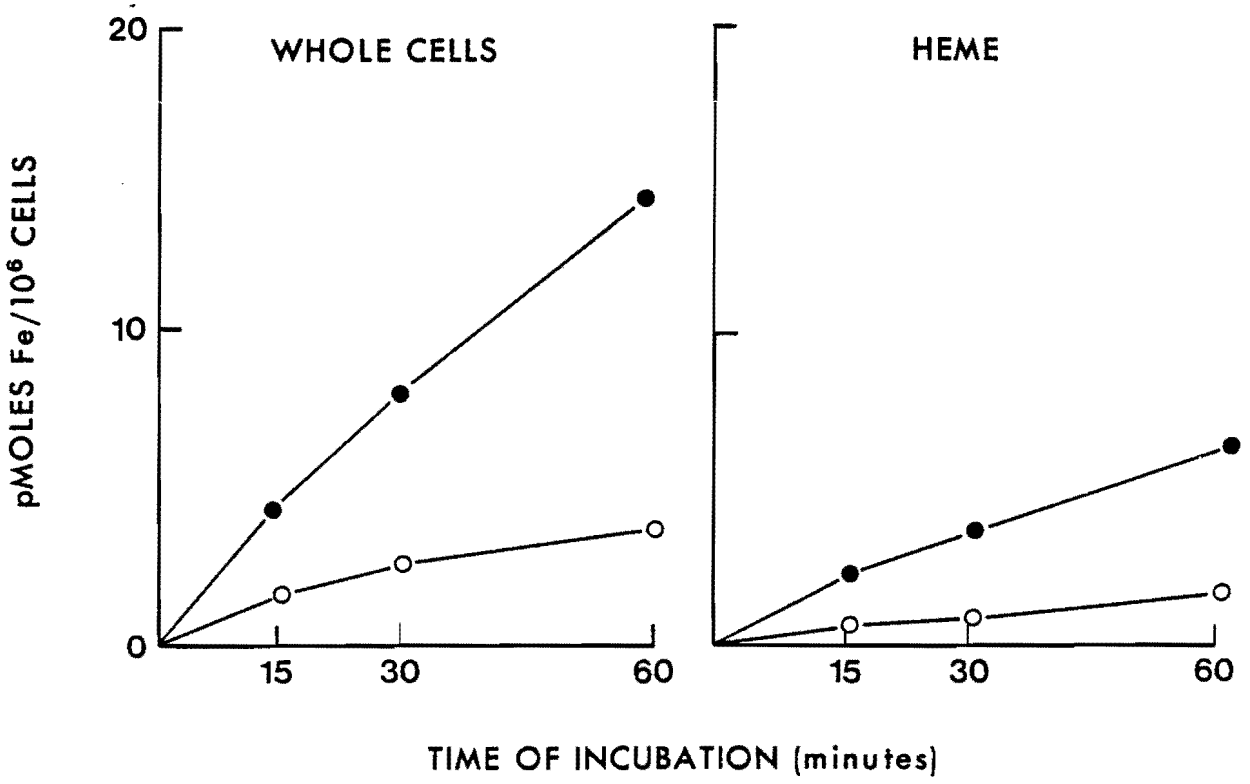
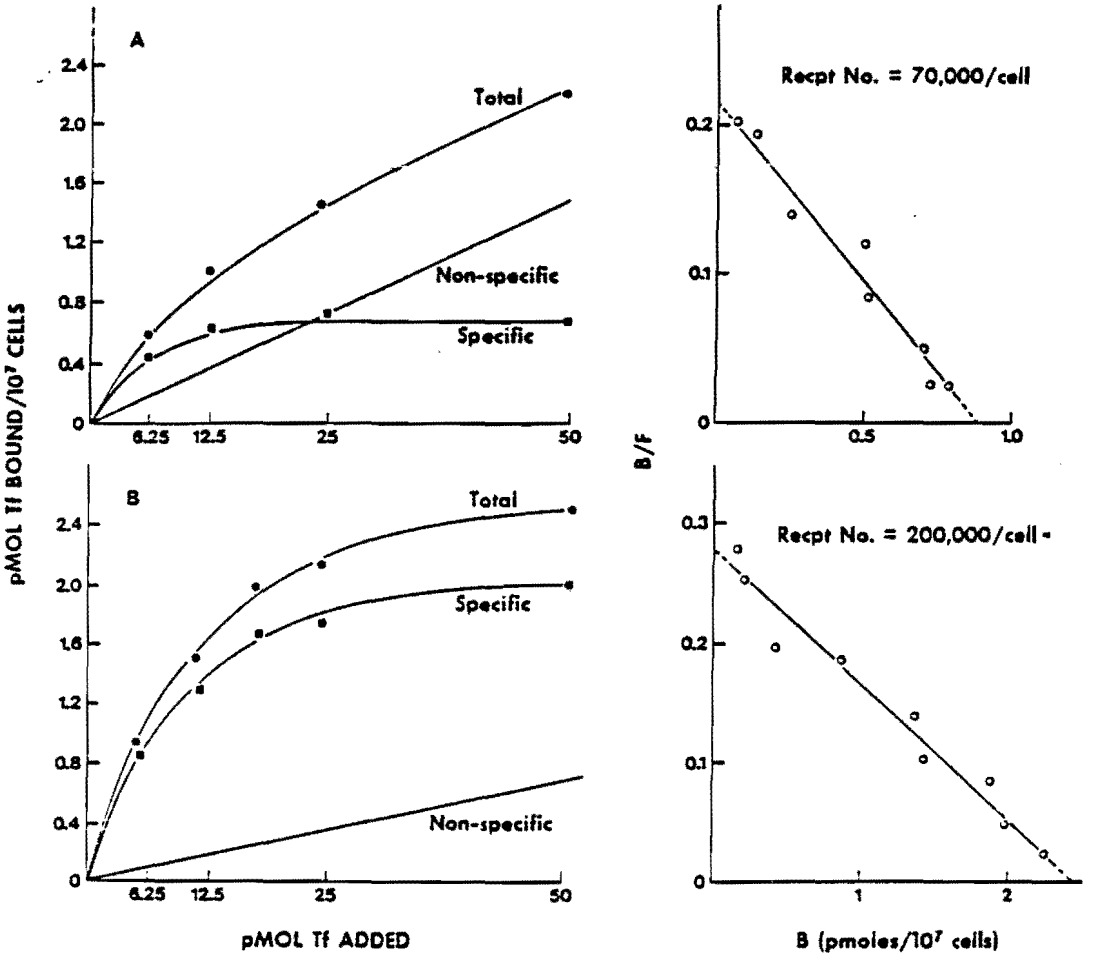

20
10
0
WHOLE CELLS
HEME
pMOLES Fe/10⁶ CELLS
15
30
60
15
30
60
TIME OF INCUBATION (minutes)

Figure 5. Left: Effect of transferrin concentrations on transferrin uptake by uninduced (A) and induced (B) MEL cells. To measure transferrin binding, the cells were incubated with a constant amount of ^{125}I-labelled transferrin but increasing amounts of unlabelled transferrin. Each sample was incubated for 30 min at 37^{o}C. The cells were then washed three times with cold PBS and the cell-bound ^{125}I-radioactivity measured. Specific transferrin binding was calculated by taking the difference between total and non-specific binding curves. Non-specific binding was calculated from the slope of the curve between 25 and 50 pmol of transferrin (0.5 ml, final volume).

Right: The Scatchard analysis of the data from the transferrin saturation curves representing specific binding. This analysis plots the bound over free transferrin versus the amount of transferrin bound. The linearity of the graph indicates the presence of one class of receptors with the x axis intercept representing the B_{max}, and the negative inverse of the slope representing the K_a of the receptor.


A
Total
Non-specific
Specific
B
Total
Specific
Non-specific
pMOL Tf BOUND/10⁷ CELLS
pMOL Tf ADDED
Recpt No. = 70,000/cell
Recpt No. = 200,000/cell
B/F
B (pmoles/10⁷ cells)

increase in transferrin mediated iron uptake probably due to an increase in the number of transferrin receptors.

Cellular RNA levels. It has been previously reported (Grandchamp et al., 1985; Parker and Houseman, 1985; Sherton and Kabat, 1976), that following DMSO induction there is a decrease in cellular RNA content. Our experiments confirm this finding as shown in Table 1. After four days of culture in the presence of DMSO there is an approximate decrease of 40% in total cellular RNA as compared to control cells. Similar decrease in poly A^+ RNA has also been reported (Grandchamp et al.,1985),(see Table 1B). It is important to note however, that the proportion of mRNA/total RNA remains constant throughout the induction period.

Induction of globin mRNA by DMSO. In order to determine the time course of α and β globin mRNA accumulation total RNA was isolated from MEL cells after various periods of time in culture, in the presence of DMSO (0,24,48,72 and 96 h), and was subjected to northern analysis (Figures 6 and 7). As can be seen, treatment of MEL cells with DMSO results in a dramatic increase in both α and β globin mRNA between 48 and 96 h. Similar results are also observed when globin mRNA levels are measured by dot-blot analysis (Figures 8 and 9). This pattern of induction is in accord with previous data (Grandchamp et al., 1985; Lowenhaupt and Lingrel, 1979). Previous studies have shown that the increase in globin mRNA is due to an increase in the rate of transcription, and that the rate of globin transcription can be further stimulated by treatment with exogenous heme (Ross and Sautner, 1976; Dabney and Beaudet, 1977).

Table 1A. The effect of DMSO on RNA/cell. MEL cells were counted and harvested after different times of culture in the presence of 1.5% DMSO. Total RNA was prepared and the RNA/cell estimated. Table 1A shows these results, expressed as the mean ± S.D. from seven independent experiments.

Table 1B shows similar results obtained by Grandchamp et al. (1985). They also measured poly A^+ and showed that it decreased proportionately.

Table 1A. The effect of dimethylsulfoxide (DMSO) on total RNA.

time after DMSO addition (hours)	total RNA pg/cell
0	7.15 ± 1.84
96	4.51 ± 1.20

Table 1B. The effect of dimethylsulfoxide (DMSO) on total and poly A^+ RNA.

time after DMSO addition (hours)	total RNA pg/cell	poly A^+ RNA pg/cell
0	8.2 ± 0.3	0.53 ± 0.02
96	5.5 ± 0.2	0.31 ± 0.01

Figure 6. Induction of α-globin mRNA during DMSO induced differentiation of MEL cells. Samples of total RNA (10 µg) from MEL cells grown for various times (0,24,48,72 and 96 h) in the presence of 1.5% DMSO were electorphoresed in a 1% agarose gel as described in Materials and Methods. The top panel shows the ethidium bromide staining of total RNA prepared from each sample, indicating that the same amount of RNA was applied to each lane and that this RNA was not degraded. Following electrophoresis and transfer to nitrocellulose paper, the filter was hybridized with ^{32}P-labelled cDNA probe for mouse α-globin, specific activity 5 x 10^7 cpm/µg, 2 x 10^6 cpm/ml, and autoradiographed (bottom panel).

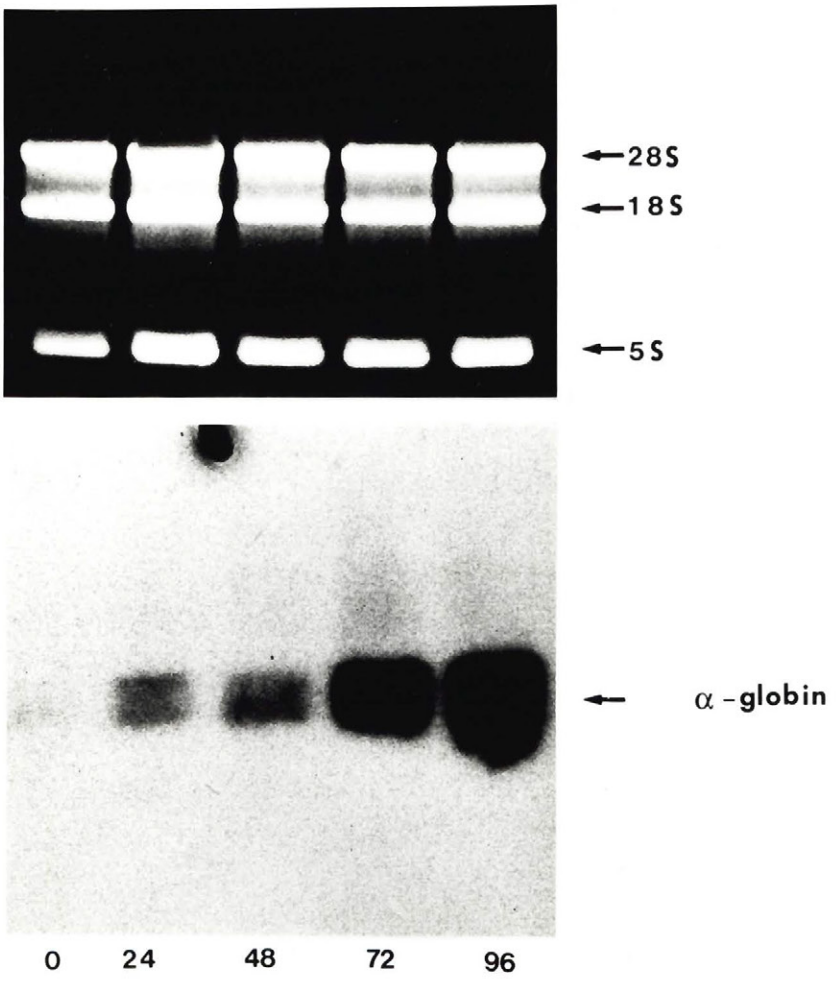

28S
18S
5S
α-globin
0
24
48
72
96
TIME (h)

Figure 7. Induction of β-globin mRNA during DMSO induced differentiation of MEL cells. Samples of total RNA (10 µg), from MEL cells grown for various times (0,24,48,72, and 96 h) in the presence of 1.5% DMSO, were electrophoresed in a 1% agarose gel as described by Davis et al. (1986) except that the concentration of formaldehyde was increased to 2.2 M. The top panel shows the ethidium bromide staining of total RNA prepared from each sample, indicating that the same amount of RNA was applied to each lane and that this RNA was not degraded. After transfer to nitrocellulose, the filter was hybridized with ^{32}P-labelled cDNA probe for mouse β-globin (specific activity 5×10^7 cpm/µg, 2×10^6 cpm/ml) and autoradiographed.

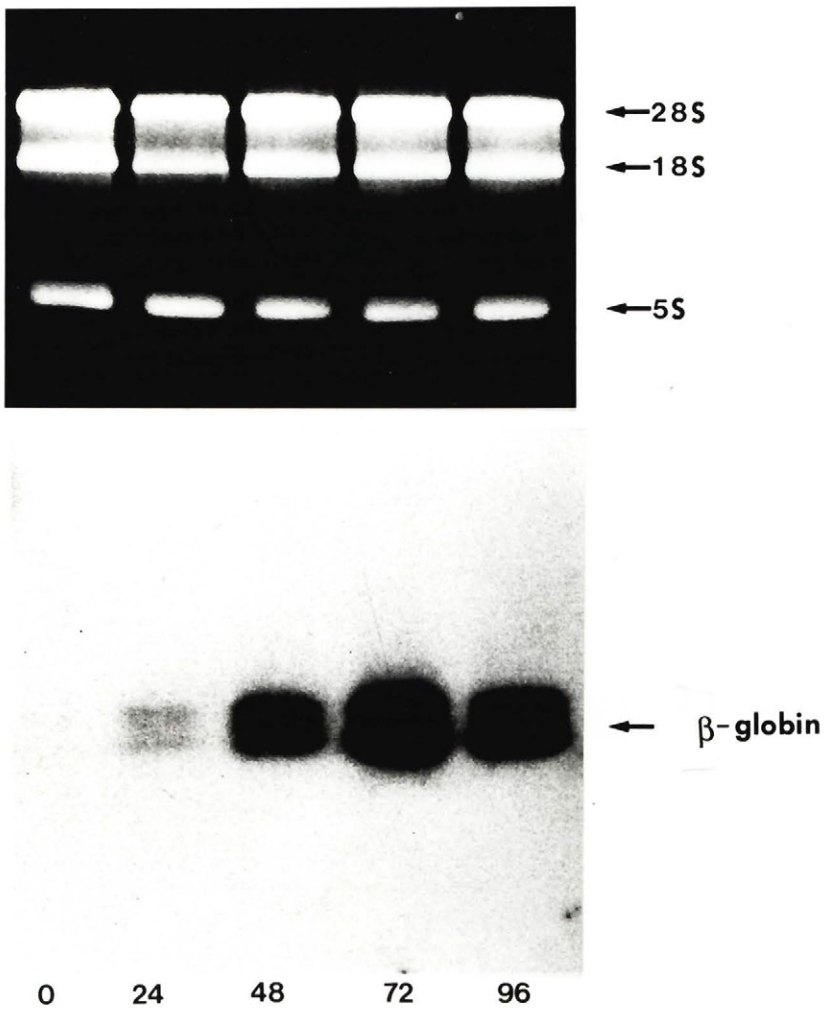

28S
18S
5S
β-globin
0
24
48
72
96
TIME (h)

Figure 8. Dot blot anaylsis of α-globin mRNA induction during DMSO induced differentiation of MEL cells. The samples of total RNA isolated from MEL cells after various periods of exposure to DMSO (described in Figures 6 and 7) were also analyzed by dot blotting. For each time interval (0,24,48,72, and 96 h), 10 μg of total RNA and serial dilutions were spotted onto nitrocellulose paper, as described in Materials and Methods. Filters were prepared in duplicate and hybridized with ^{32}P-labelled cDNA probes for α and β-globin respectively. Specific activity of both probes was 5 x 10^7 cpm/μg and hybridization mixtures contained 2 x 10^6 cpm/ml. Autoradiograms of these filters are presented here (Figures 8 and 9). To analyse this type of data, such autoradiograms can be subsequently scanned with a laser densitometer and arbitrary scanning units calculated for each spot. For any one experimental condition, concentration limits can be selected where the intensity of the RNA spots is proportional to the amount of RNA spotted. In this way a linear range for each experimental condition can be defined. To compare between groups, a single concentration which falls within the linear range of all groups, is selected and at this concentration, arbitrary scanning units compared. This type of analysis is used in Chapter 5.

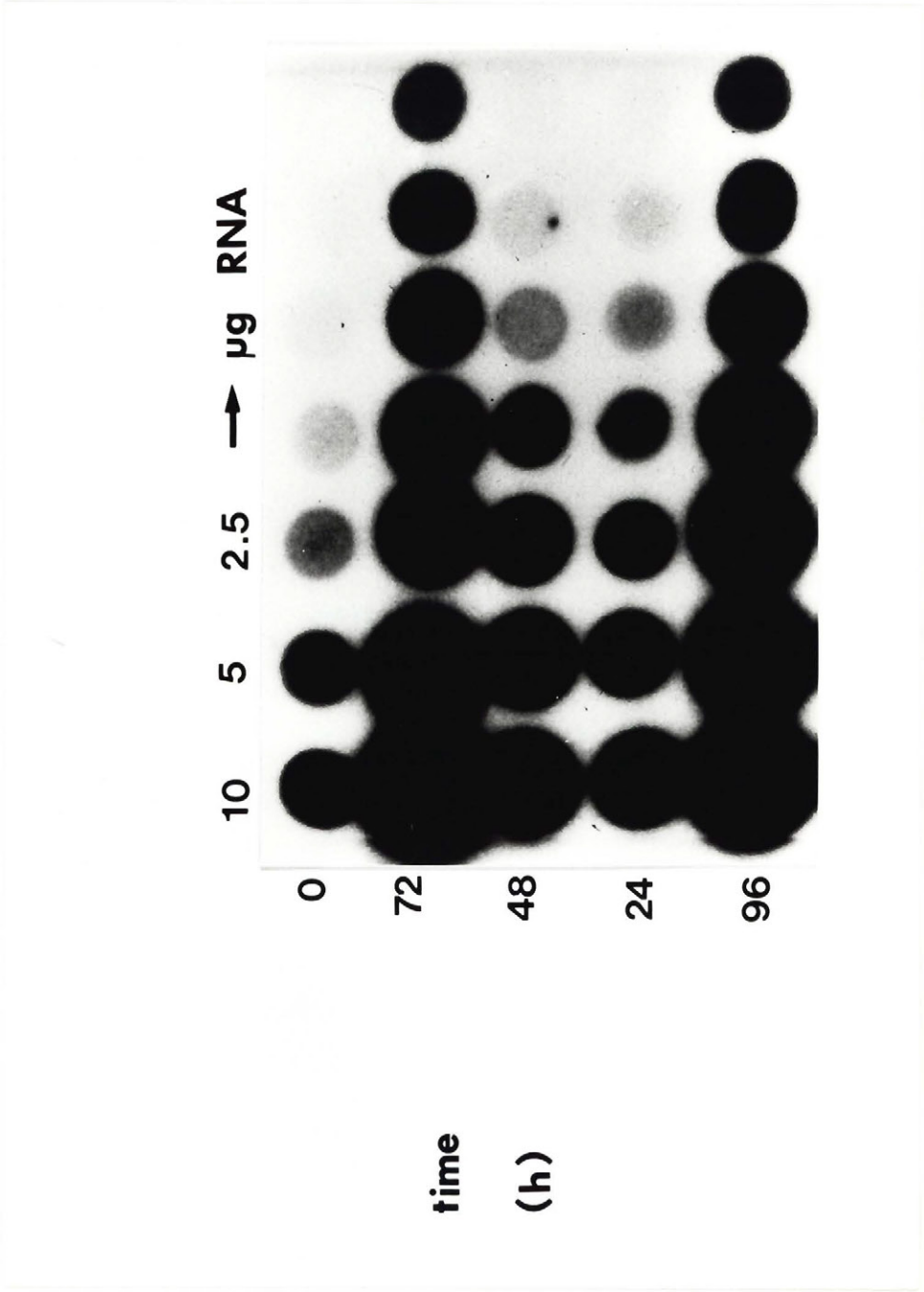
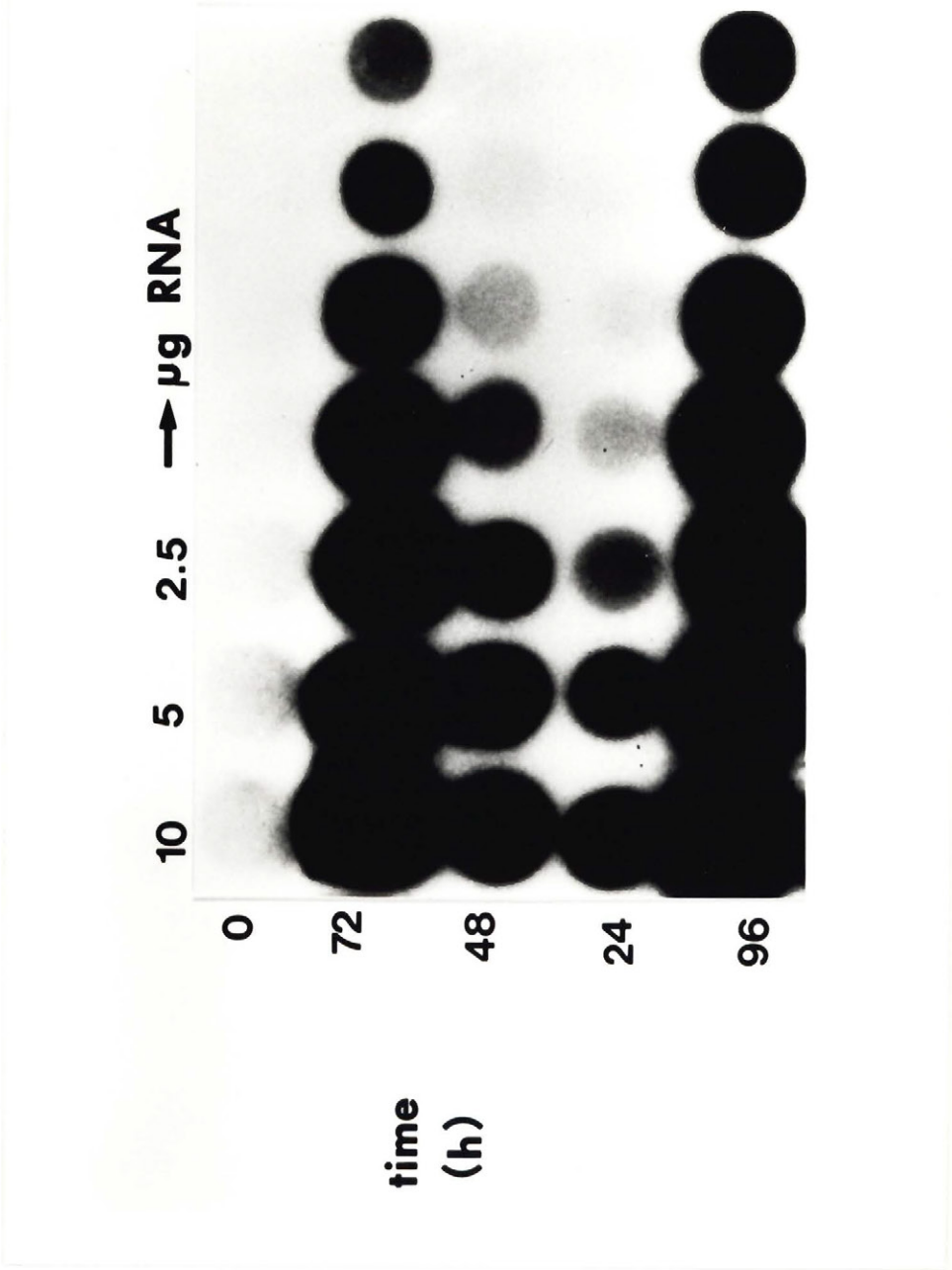

10
5
2.5
→ µg RNA
0
72
48
24
96
time (h)

Figure 9. Dot blot analysis of β-globin mRNA induction during dimethylsulfoxide (DMSO) induced differentiation of MEL cells. Samples of total RNA isolated from MEL cells after various times of exposure to DMSO 1.5%, were analysed by dot blots and the relative amounts of β-globin mRNA visualized by autoradiogram. RNA preparation and blot analysis is described in detail in Figure 8.


10
5
2.5
→ µg RNA
0
72
48
24
96
time
(h)

DISCUSSION

The terminal differentiation of MEL cells has been described in terms of morphological, biochemical and molecular changes. However, a number of different MEL cell strains have been cloned and these may vary in their ability to differentiate, in their responsiveness to specific inducers, and in the time course and exact pattern of induction. Perhaps one of the most commonly used clones, and the clone used in this study, is clone 745A (Friend et al., 1971). The aim of this study was to characterize the pattern of MEL differentiation in response to the inducing agent dimethlysulfoxide (DMSO), under the conditions and methods used in this laboratory.

The results confirmed that the clone used (745) followed the expected pattern of development, and provide a basis for comparison for other experiments in which perturbations will be introduced. The results indicate that, in our hands, DMSO was an effective inducer of MEL cell clone 745. After four days of culture in the presence of DMSO (1.5%), benzidine staining reached a maximum of 85% which is in accordance with previous studies (Friend et al., 1971). Also confirmed were the following: an increase in iron uptake (Glass et al., 1978; Wilczynska and Schulman, 1980; Laskey et al., 1986); an increase in the number of transferrin receptors (Hu et al., 1977; Yeoh and Morgan, 1979; Wilczynska and Schulman, 1980; Laskey et al., 1986); an increase in heme synthesis, as measured by ^{59}Fe incorporation into heme (Granick and Sassa, 1978; Glass et al., 1978; Laskey et al., 1986); and an increase in α and β globin mRNA accumulation (Ross and Sautner, 1976: Grandchamp et al., 1985)

It can be concluded therefore, that MEL cells, when treated with DMSO, initiate a coordinated program of erythroid development which resembles

normal erythroid differentiation and leads to the accumulation of large amounts of hemoglobin in these cells.

CHAPTER 3

CONTROL OF HEME SYNTHESIS DURING FRIEND CELL DIFFERENTIATION: ROLE OF IRON AND TRANSFERRRIN

PREFACE

In Chapter 3 the hypothesis that the regulation of heme synthesis is distinctly different in erythroid and non-erythroid cells is tested. As reviewed in Chapter 1, previous work has shown that in rabbit reticulocytes heme controls cellular acquisition of iron from transferrin rather than controlling the synthesis of ALA, which is known to regulate heme synthesis in hepatocytes and bacteria. Moreover, recent work has provided evidence that in rabbit reticulocytes the rate of iron uptake from transferrin, rather than ALA synthase, limits the rate of heme formation.

In Chapter 2, it was demonstrated that MEL cell differentiation results in an increase in the number of cell transferrrin receptors, increased iron uptake from transferrin and an increase in the rate of heme synthesis. In this respect these changes parallel those thought to occur during normal erythroid development. In this chapter MEL cells are used to study the control of heme synthesis before, and after DMSO induction. The question is asked whether erythroid specific control of heme synthesis develops as the cells acquire the ability to synthesize large amounts of heme.

ABSTRACT

In many types of cells the synthesis of δ-aminolevulinic acid (ALA) limits the rate of heme formation. However, results from our laboratory with reticulocytes suggest that the rate of iron uptake from transferrin (Tf), rather than ALA synthase activity, limits the rate of heme synthesis in erythroid cells. To determine whether changes occur in iron metabolism and the control of heme synthesis during erythroid cell development Friend erythroleukemia cells induced to erythroid differentiation by dimethlysulfoxide (DMSO) were studied. While added ALA stimulated heme synthesis in uninduced Friend cells (suggesting that ALA synthase is limiting) it did not do so in induced cells. Therefore the possibility was investigated that, in induced cells, iron uptake from Tf limits and controls heme synthesis. Several aspects of iron metabolism were investigated using the synthetic iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH). Both induced and uninduced Friend cells take up and utilize Fe for heme synthesis directly from Fe-SIH without the involvement of transferrin and transferrin receptors and to a much greater extent than from saturating levels of Fe-Tf (20 μM). Furthermore, in induced Friend cells 100 μM Fe-SIH stimulated 2-^{14}C-glycine incorporation into heme up to 3.6-fold as compared to the incorporation observed with saturating concentrations of Fe-Tf. In contrast, Fe-SIH, even when added in high concentrations, did not stimulate heme synthesis in uninduced Friend cells but was able to do so as early as 24 to 48 h following induction. In addition, contrary to previous results with rabbit reticulocytes, Fe-SIH also stimulated globin synthesis in induced Friend cells above the levels seen with saturating concentrations of

transferrin. These results indicate that some step(s) in the pathway of iron from extracellular Tf to protoporphyrin, rather than the activity of ALA synthase, limits and controls the overall rate of heme synthesis and possibly hemoglobin synthesis in differentiating Friend erythroleukemia cells.

INTRODUCTION

The first step in the formation of heme, which is synthesized in all aerobic cells, is the condensation of glycine and succinyl coenzyme A to form δ-aminolevulinic acid (ALA); a reaction catalyzed by the mitochondrial enzyme ALA synthase. In some bacteria (Burnham and Lascelles, 1963) and in hepatocytes (Granick and Urata, 1963) ALA synthase is the rate-limiting enzyme of heme biosynthesis and both the synthesis(Burnham and Lascelles, 1963; Granick et al., 1975) and activity of the enzyme (Paterniti and Beattie, 1979) are controlled by the level of intracellular heme. Although it has been suggested that heme synthesis in erythroid cells is regulated by a similar mechanism (London et al., 1964; London, 1980; Ibraham et al., 1983), this view has been challenged (Neuwirt et al., 1969) and a growing body of evidence suggests that ALA synthase activity does not control heme synthesis in these cells (Ponka and Neuwirt, 1970; Ponka et al., 1973; Woods, 1974; Sassa, 1976; Malik et al.,1979a,b; Sassa, 1980; Ponka and Schulman, 1985a,b). The existence of distinct regulatory features of heme biosynthesis in erythroid cells seems plausible because of the large quantities of heme that are synthesized by erythroid as compared to other cells.

In erythroid cells the control of iron supply to mitochondrial ferrochelatase, the last enzyme in the heme pathway, may regulate heme synthesis (Ponka and Neuwirt, 1974; Ponka and Schulman, 1985a,b). The uptake of iron for heme synthesis in erythroid cells involves the binding of Fe-transferrin to specific receptors at the cell surface, internalization of the Fe-transferrin:receptor complexes in endocytic vesicles, release of iron and its transport to mitochondrial ferrochelatase by an unknown mechanism, and return of transferrin:receptor

complexes to the surface where apotransferrin is released (Morgan. 1981). In reticulocytes iron uptake is coordinated with its utilization for heme synthesis by a mechanism in which heme feedback inhibits the uptake of iron from Fe-transferrin (Ponka and Neuwirt, 1969; Ponka et al., 1974; Schulman et al., 1974) and this control is probably specific for erythroid cells (Schulman et al., 1981). Since heme does not inhibit ALA synthase activity in hemoglobin-synthesizing cells (Ponka and Schulman, 1985a,b), one of the steps in the Fe-transferrin cycle may limit the overall rate of heme synthesis in such cells. Evidence for this was obtained (Ponka and Schulman, 1985a,b) by using specific iron chelators (such as Fe-SIH which bypasses the Fe-transferrin cycle) and showing that by increasing the iron taken up by reticulocytes heme synthesis is increased above the maximum observed with saturating amounts of Fe-transferrin. However, reticulocytes appear late in erythroid differentiation and, lacking nuclei, cannot be used for studying transcriptional events.

The aim of this present work was to extend our experiments to less mature erythroid cells by using the Friend murine erythroleukemia cell system (Friend et al., 1971). These virus-transformed cells resemble erythroblasts and can be induced to erythroid differentiation with dimethlysulfoxide (DMSO) or a variety of other agents (Reuben et al., 1980; Marks and Rifkind, 1978). Following induction the cells synthesize globin mRNA (Ross et al., 1972), increase enzymes of the heme pathway (Sassa, 1976), increase transferrin receptors (Wilczynska et al., 1984) and iron uptake (Glass et al., 1978) and synthesize and accumulate large quantities of hemoglobin. Therefore, these cells are well suited for the study of iron metabolism and heme synthesis during erythroid differentiation.

In this study Fe-SIH was used as a source of iron for Friend cells in order to examine the effect of increased iron uptake on heme synthesis during erythroid cell development. The results obtained indicate that iron utilization from transferrin limits the rate of heme production in Friend cells which are actively synthesizing hemoglobin but not in uninduced cells.

MATERIALS AND METHODS

Chemicals. Iron-free human transferrin was obtained from Behringwerke (Marburg, West Germany); mouse transferrin was kindly provided by Dr. Evan H. Morgan, the University of Western Australia; 4,5-dioxoheptanoic acid (succinylacetone, SA) was from U.S. Biochemical Corp. (Cleveland, Ohio) and lactoperoxidase was from Calbiochem (Los Angeles, CA). CM Sephadex and CNBr activated Sepharose 4B were obtained from Pharmacia (Dorval, Quebec). All other chemicals were of the highest purity available. $^{59}FeCl_3$ (10-25 mCi/mg), 2-^{14}C-glycine (40-60 mCi/mg) and ^{125}I (17 Ci/mg) were purchased from New England Nuclear Corp. (Boston, MA).

Media and reagents were obtained from Flow Laboratories (McLean, VA) and tissue culture plasticware from Falcon (Oxnard, CA).

Cells and cell culture. Friend erythroleukemia cells used were derived from clone 745A originally isolated by Dr. Charlotte Friend. Cell cultures were grown at 37°C in an atmosphere of 95% air and 5% CO_2 in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum. Cells were subcultured every 96 h at an initial cell concentration of approximately 5 X 10^4 cells/ml. For induction, cells were grown in a medium containing 1.5% dimethlysulfoxide (DMSO) at the time of subculturing. After 96 h, both induced and uninduced cells were collected by centrifugation at room temperature and washed three times with serum-free medium. The percentage of benzidine positive cells was determined by the method of Orkin et al. (1975).

Studies with ^{59}Fe-ligands and ^{125}I-transferrin. $^{59}FeCl_3$ in .5 mol/L HCL was converted to ferric citrate by the addition of a 20-fold molar excess of sodium citrate. ^{59}Fe-transferrin was prepared by mixing the ^{59}Fe-ferric citrate with transferrin in a molar ratio of 2 mol Fe:1

mol transferrin. After addition of solid $NaHCO_3$ to a final concentration of 0.1 mol/L the pH was adjusted to 7.4 and the solution was kept at room temperature for 3 h. The preparation was then extensively dialysed (Martinez-Medellin and Schulman, 1972).

SIH (2-hydroxybenzal isonicotinoyl hydrazone) was prepared as described by Ponka et al. (1979). SIH was dissolved with a few drops of 1N NaOH and diluted with incubation medium. ^{59}Fe-ferric citrate was added to a final molar concentration of Fe:SIH of 1:1. The preparation was adjusted to pH 7.4 and allowed to sit at room temperature for 1 h.

To prepare ^{125}I-transferrin, apotransferrin was saturated with unlabelled iron as its citrate complex (1:20) and labelled with ^{125}I using lactoperoxidase coupled to CNBr activated Sepharose 4B according to the procedure described by David (1972).

To measure transferrin and iron uptake, induced and uninduced Friend cells, at a concentration of 1×10^7 cells/ml, were incubated in serum-free medium containing human transferrin labelled either with ^{59}Fe, ^{125}I, or with ^{59}Fe-SIH. At the indicated time periods samples were collected by centrifugation and washed three times with cold phosphate-buffered saline (PBS) and ^{125}I and ^{59}Fe associated with the cells were measured with an LKB Compugamma counter.

Measurement of cellular heme and nonheme ^{59}Fe were performed either by an acid precipitation method (Borova et al., 1973) or by acid methlyethlyketone extraction (Teale, 1959). There was an estimated 8% loss of total cellular heme during extraction with methlyethylketone. When final heme estimates were corrected for this loss, the two methods yielded values which were virtually identical. In view of the similar results with the two methods, it is unlikely that tightly bound, poorly

extractable ^{59}Fe is trapped by acid precipitation and as this method is more rapid it was the method of preference.

2-^{14}C-glycine incorporation into heme and globin. Induced and uninduced Friend cells (10 X 10^6) were incubated for 30 min with nonradioactive iron-transferrin, added at saturating concentrations (20 µM) or iron-SIH (100 µM), then 1.5 to 2 µCi of 2-^{14}C-glycine (pH 7.4) was added per 500-µl-sample and the incubation continued for indicated time intervals. The cells were washed three times with PBS, lysed in 200 µl of double distilled water, and kept frozen overnight.

Following thawing, each sample was centifuged at 2,800 rpm at 4°C for 5 min and the supernatant was applied to a CM-Sephadex column equilibrated in 0.1 M phosphate buffer, pH 6.6. The CM-Sephadex column was then washed with three volumes of ice cold 0.1M phosphate buffer, pH 6.6. Relatively pure hemoglobin, as judged by SDS-PAGE, was eluted using 0.2 M Na_2HPO_4/0.2 M NaCl, pH 8.9.

Purified hemoglobin obtained in this manner was then separated into heme and globin fractions by "acid acetone" extraction and precipitation (Ponka and Schulman, 1985a,b).

For determination of specific activity, the hemoglobin recovered from the CM-Sephadex column was divided into two equal fractions. One fraction was processed by the acid acetone procedure as described above, and the counts in heme and globin were determined. The other fraction was assayed by the micro Lowry technique for the determination of protein (Lowry et al., 1951). The specific activity was expressed as cpm/pmoles of hemoglobin.

RESULTS

Iron uptake from ^{59}Fe-SIH. To test the possibility that iron delivery from transferrin may limit the rate of heme formation, induced Friend cells, which are actively synthesizing hemoglobin, were incubated with ^{59}Fe-SIH. Whereas the uptake of iron from transferrin by Friend cells is saturated within the physiological range of plasma iron (20 μM), iron uptake from Fe-SIH is not saturable in the range of 20-100 μM (not shown). As shown in Figure 1, following 60 min of incubation, the total cellular iron uptake from 100 μM ^{59}Fe-SIH is up to six times greater than that from 20 μM transferrin and ^{59}Fe incorporation into heme is increased three-fold. Succinylacetone (1 mM), a specific inhibitor of heme synthesis (Ebert et al., 1979; Ponka et al., 1982) inhibits the incorporation of ^{59}Fe from ^{59}Fe--SIH into heme by 70%, indicating that ^{59}Fe from ^{59}Fe-SIH is incorporated into de novo synthesized protoporphyrin (data not shown).

To exclude the possibility that iron incorporation from Fe-SIH is mediated by transferrin or transferrin receptors, induced cells were preincubated with pronase (200 μg/ml) for 10 min at 4°C (Schulman et al., 1983), washed, and then incubated with ^{59}Fe-transferrin or ^{59}Fe-SIH. As seen in Figure 2, pronase treatment inhibited iron uptake from transferrin, probably by digesting surface transferrin receptors, but had virtually no effect on either the uptake or utilization of ^{59}Fe from ^{59}Fe-SIH.

It can be argued that increased ^{59}Fe incorporation into heme from ^{59}Fe-SIH may reflect an increase in the specific activity of the preheme ^{59}Fe pool, since the total cellular radioiron uptake is so increased with Fe-SIH as compared to Fe-transferrin. If this were the case,

FIGURE 1. ^{59}Fe-incorporation into induced Friend cells from diferric transferrin and ferric salicylaldehyde isonicotinoyl hydrazone (SIH-Fe). Samples of 1 X 10^7 cells were incubated at 37°C in 0.5 ml of serum-free medium containing either ^{59}Fe-transferrin at a final concentration of 20 μM or ^{59}Fe-SIH at a final concentration of 100 μM. At the indicated time intervals the cells were chilled, washed three times with cold phosphate-buffered saline, and the radioactivity incorporated into both whole cells and heme fractions was measured.

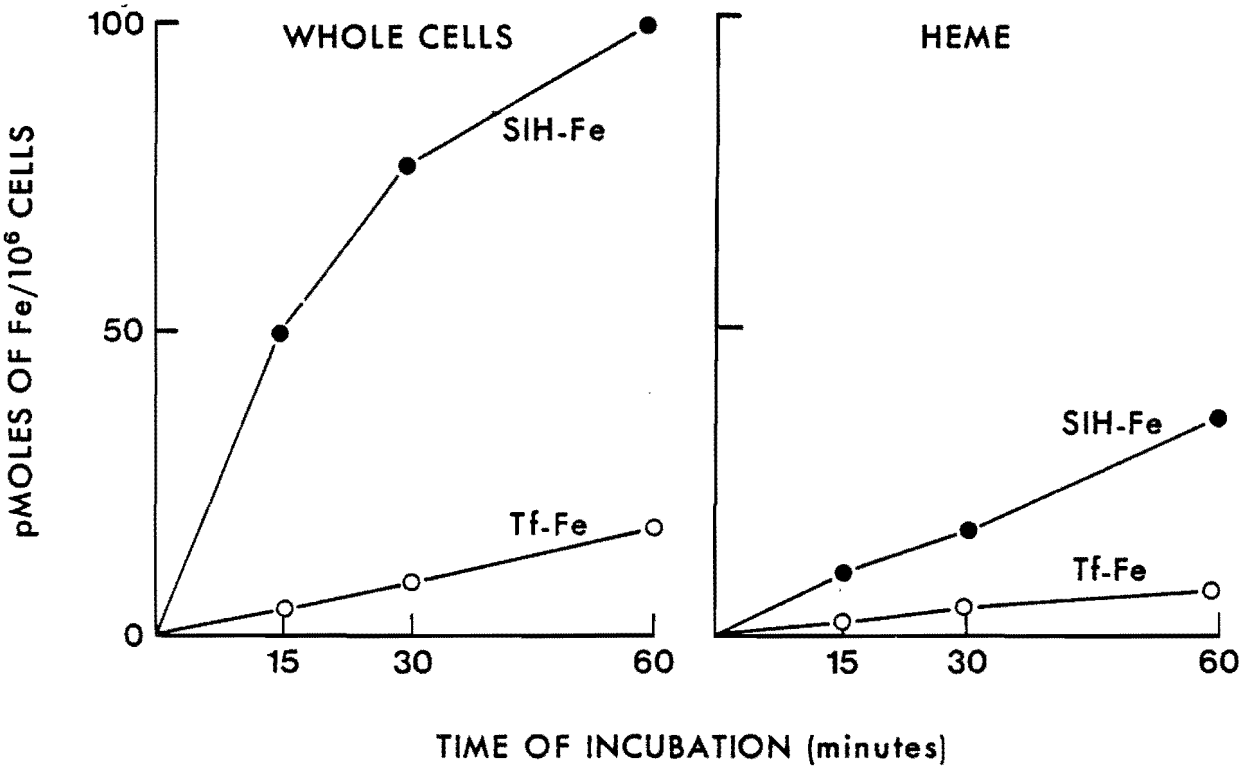
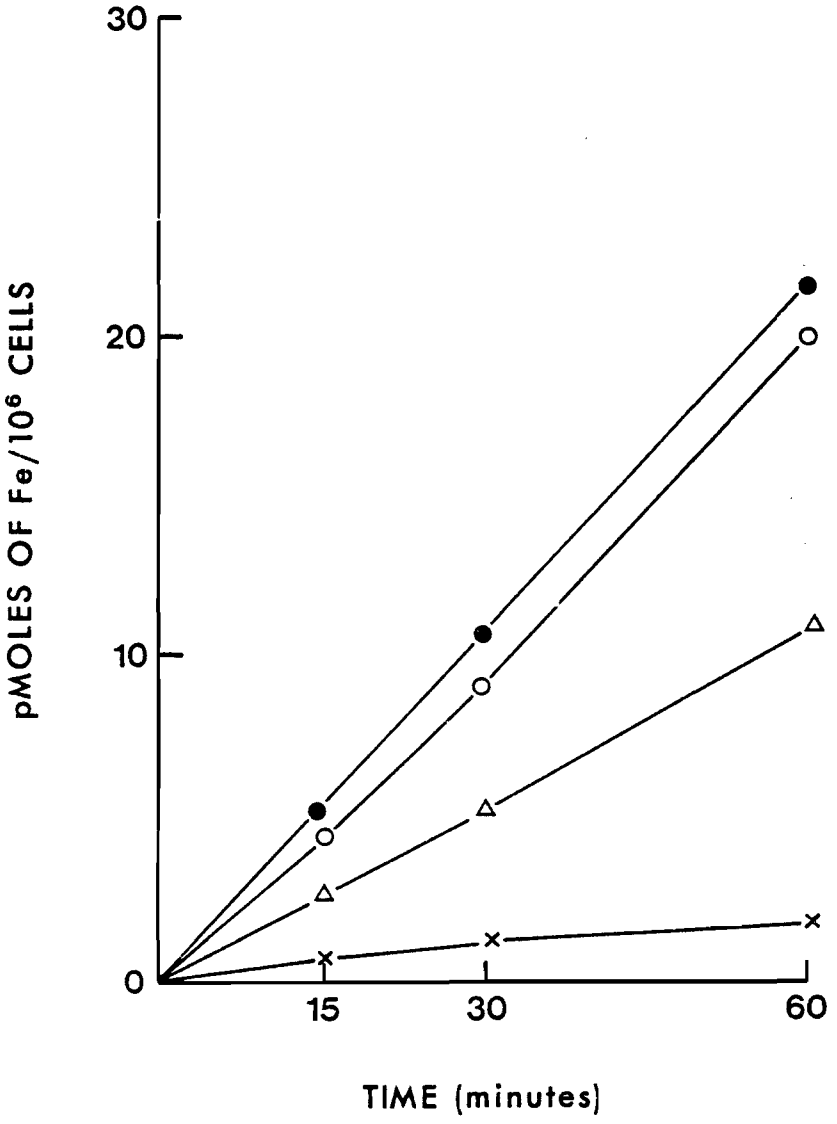

WHOLE CELLS
HEME
100
50
0
SIH-Fe
Tf-Fe
SIH-Fe
Tf-Fe
15
30
60
15
30
60
pMOLES OF Fe/10⁶ CELLS
TIME OF INCUBATION (minutes)

FIGURE 2. The effect of preincubation with pronase (● , ×) on the incorporation of ^{59}Fe, from transferrin (△ , ×) and SIH-Fe (○ , ●), respectively, into heme in induced Friend cells. Induced Friend cells were preincubated with or without pronase (200 μg/ml) for 10 min at $4^{\circ}C$. The cells were then washed, resuspended in incubation medium containing either ^{59}Fe-transferrin (20 μM) or ^{59}Fe-SIH (100 μM), and incubated for the indicated time periods.


30
20
10
0
pMOLES OF Fe/10⁶ CELLS
15
30
60
TIME (minutes)

increased radioactivity in heme need not reflect an actual increase in heme production. The aim of further experiments was to exclude the possibility and investigate the effects of higher than physiological concentrations of iron, supplied as Fe-SIH, on the rate of heme synthesis measured by 2-^{14}C-glycine incorporation.

Studies with 2-^{14}C-glycine. In initial experiments we examined the rate of heme synthesis, as measured by 2-^{14}C-glycine incorporation, in induced Friend cells incubated with saturating concentrations of either mouse or human Fe-transferrin. Since the results with both transferrins were identical, we used human Fe-transferrin for convenience.

Figure 3 shows the results of a typical experiment and Table 1 summarizes the results of several experiments in which induced Friend cells were preincubated with either Fe-transferrin or Fe-SIH for 30 min and then incubated with 2-^{14}C-glycine for 1 h. The incorporation of 2-^{14}C-glycine into heme and globin was then measured. As seen in Figure 3, in induced Friend cells, Fe-SIH (100 μM) stimulates the rate of heme production up to 3.6-fold as compared to Fe-transferrin (20 μM). Interestingly, in uninduced cells this stimulation is nearly absent.

The stimulation of heme formation by Fe-SIH was also examined at various times after induction. Friend cells were grown with DMSO for 24, 48, 72 and 96 h, after which time the cells were harvested and the effects of either Fe-SIH or transferrin on 2-^{14}C-glycine incorporation into heme were measured and compared. Figure 4 shows that when transferrin is the iron donor, 2-^{14}C-glycine incorporation into heme gradually increases as the cells become induced, reaching a plateau after 4 days. However, between 24-48 h following DMSO induction, the rate of heme production can be significantly increased by providing more iron to the cells with

FIGURE 3. 2-^{14}C-glycine utilization for heme synthesis by induced and uninduced Friend cells incubated without an iron ligand (empty columns), with 20 μM Fe-transferrin (dotted columns), or with 100 μM Fe-SIH (dashed columns). Samples were preincubated under the above conditions for 30 min at 37°C, 2-^{14}C-glycine was then added, and the cells were incubated for an additional 60 min at 37°C. The cells were then washed and 2-^{14}C-glycine incorporation into heme measured. The results shown are values for a typical experiment and are averages of quadruplicate samples. Table 1 summarizes the results of several such experiments.

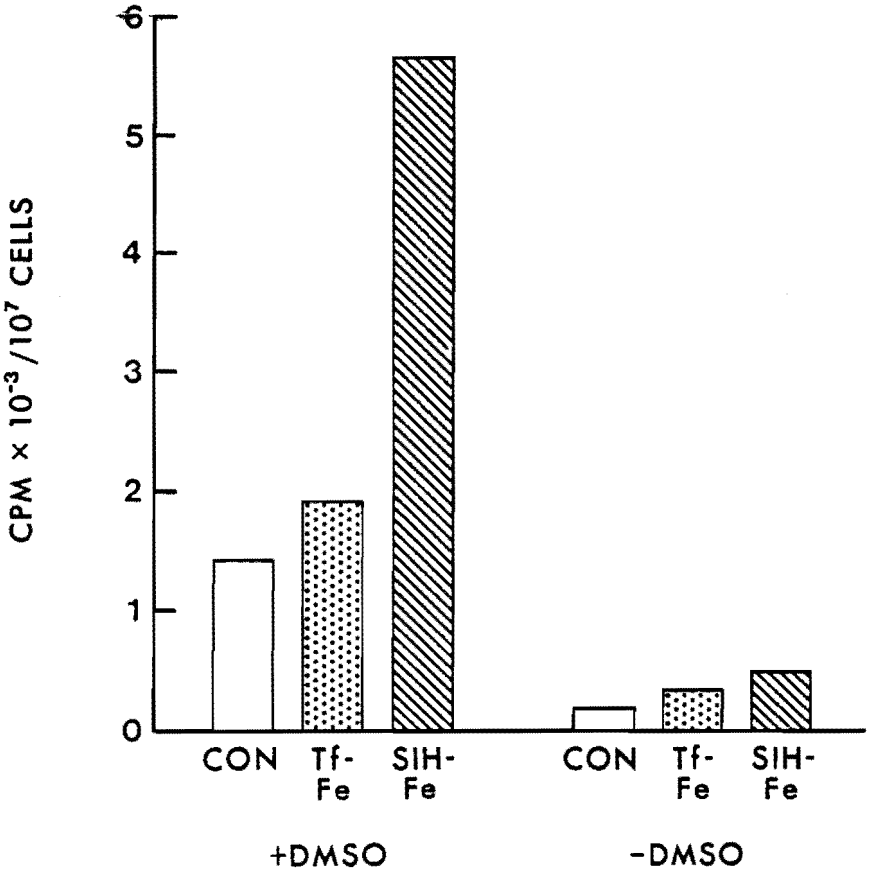
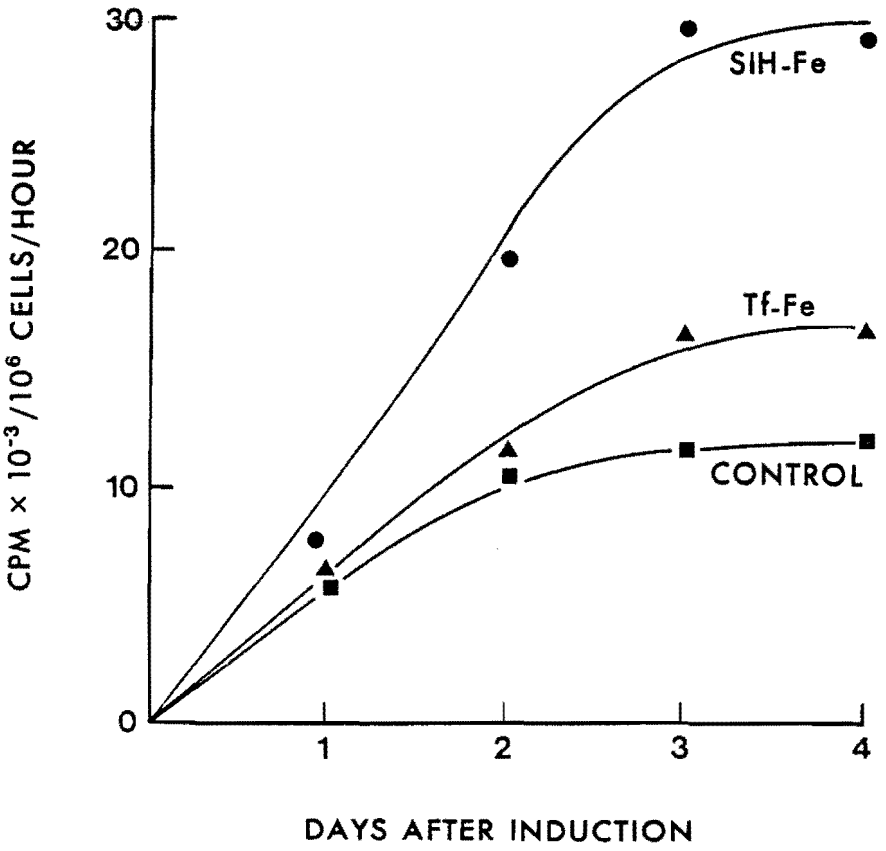

CPM x 10^{-3} /10^{7} CELLS
0
1
2
3
4
5
6
CON
Tf-Fe
SIH-Fe
CON
Tf-Fe
SIH-Fe
+DMSO
-DMSO

TABLE 1. The effect of 100 μM Fe-SIH on 2-^{14}C-glycine incorporation into heme of induced Friend erythroleukemia cells.

Conditions	Average ± SEM (n = 6)
-Fe	82 ± 13.4
Fe-Tf	100
Fe-SIH	221 ± 72.4

Results are expressed as percentages of values obtained with cells incubated with saturating concentrations of Fe-transferrin (Fe-Tf, 20 μM). The time of incubation with 2-^{14}C-glycine was 60 min.
n, Number of experiments.

FIGURE 4. The effect of Fe-transferrin or Fe-SIH on 2-^{14}C-glycine utilization for heme synthesis in differentiating Friend erythroleukemia cells. Friend cells of line 745 were incubated with 1.5% DMSO for various time periods and at the indicated times (24, 48, 72, 96 h), the cells were harvested and resuspended in serum-free medium. Samples (from each time period) were preincubated for 30 min at 37^{o}C either a) without an iron ligand (■), b) with 20 μM Fe-transferrin (▲), or c) with 100 μM Fe-SIH (●). 2-^{14}C-glycine was then added to each sample and the cells incubated for an additional 60 min after which time cells were washed and 2-^{14}C-glycine incorporation into heme measured. Results are averages of quadruplicates.


30
20
10
0
1
2
3
4
SIH-Fe
Tf-Fe
CONTROL
CPM × 10⁻³/10⁶ CELLS/HOUR
DAYS AFTER INDUCTION

Fe-SIH, as compared to the maximun amounts of iron the cells can incorporate from transferrin.

Contrary to previous results with rabbit reticulocytes (Ponka and Schulman, 1985b) Fe-SIH stimulates the incorporation of 2-^{14}C-glycine into globin to a greater extent than Fe-transferrin, probably by increasing the intracellular levels of heme. When heme synthesis is inhibited by succinylacetone, this stimulation of globin synthesis is not seen (not shown). Furthermore, exogenous hemin (25 μM) increases the rate of globin production in induced Friend cells to the level seen with 100 μM Fe-SIH. In contrast, Fe-SIH does not increase globin production in uninduced cells, which is not surprising since Fe-SIH does not significantly increase the formation of heme in uninduced cells. The slight stimulation of heme synthesis that is seen is probably insufficient to produce any detectable changes in the rate of globin formation.

The effect of ALA on ^{59}Fe use for heme synthesis. To examine whether or not ALA synthase is the rate limiting enzyme of heme synthesis in Friend cells, the effect of ALA (0.2 mM) on the incorporation of ^{59}Fe into heme in uninduced and induced cells was determined. If ALA synthase were limiting added ALA should stimulate the production of heme as measured by ^{59}Fe insertion into protoporphyrin. Uninduced Friend cells synthesize trace amounts of heme and when ALA is added to the cells heme synthesis, as measured by ^{59}Fe incorporation from transferrin, is increased. These results together with the results from other laboratories (Malik and Djaldetti, 1979) suggest that prior to induction ALA synthase limits the rate of heme formation. Conversely in induced Friend cells, when ^{59}Fe-transferrin is the iron donor, ALA synthase is not limiting since the addition of ALA does not change the rate of ^{59}Fe

TABLE 2. The effect of ALA on ^{59}Fe incorporation from either ^{59}Fe-Tf or ^{59}Fe-SIH into heme of uninduced and induced Friend cells.

Conditions	Iron donor	Incubation conditions	Iron incorporation into heme (pmol/10^6 cells/h)[a]	% Control (n = 4)
-DMSO	Tf	Control	2.1	100
		+ ALA	3.0	142 ± 21
+DMSO	Tf	Control	6.3	100
		+ ALA	6.2	98 ± 6
+DMSO	SIH-Fe	Control	15.8	100
		+ ALA	21.0	133 ± 15

[a]Sample values from one experiment.

Uninduced (-DMSO) or induced (+DMSO, 4 days) Friend cells were preincubated for 30 min at 37°C in the presence and absence of ALA (0.2 mM), ^{59}Fe-Tf (20 μM) or ^{59}Fe-SIH (100 μM) was then added to each of the control and "+ ALA" samples and incubated for an additional 60 min at 37°C. The samples were then chilled, washed three times in PBS, and the ^{59}Fe incorporation into heme measured. Higher concentrations of ALA had an apparent toxic effect on Friend cells.

incorporation into heme. However, when iron delivery to Friend cells is increased by using ^{59}Fe-SIH, the addition of ALA stimulates ^{59}Fe incorporation into heme, suggesting that under these conditions ALA synthase has become limiting.

DISCUSSION

Although all aerobic cells synthesize heme, the largest quantities of heme are produced in developing erythroid cells following the onset of hemoglobin synthesis. Therefore, the regulation of heme synthesis may be different in erythroid and nonerythroid cells, in which heme synthesis is controlled by the rate of ALA formation (Granick and Urata, 1963; Granick et al., 1975; Paterniti and Beatie, 1979). The results of this study provide evidence for this suggestion. Although addition of ALA stimulates heme synthesis in uninduced Friend cells (indicating that ALA synthase is limiting), it does not do so in induced cells. This finding and the previous report of Malik and Djaldetti (1979) indicate that the mode of control of heme synthesis changes during erythroid differentiation.

In erythroid cells, the regulation of heme synthesis involves both the regulatory mechanisms which induce hemoglobin synthesis and the control of heme synthesis after the pathway's enzymes are fully induced. With respect to induction, earlier reports (Sassa, 1976; Rutherford et al., 1979) suggested that ferrochelatase is the controlling enzyme of heme synthesis during differentiation since this enzyme activity was the last to be increased during the induction of Friend cells. However, a recent report indicates this may not be so (Beaumont et al., 1984), since ferrochelatase was induced as early as ALA synthase during differentiation of Friend cells. Nevertheless, in spite of this, the accumulation of heme occurs relatively late during Friend cell differentiation (Beaumont et al.,1984).

Once the hemoglobin synthesizing machinery is fully induced in erythroid cells, it appears that the heme pathway enzymes are available in amounts allowing maximum possible production of heme for hemoglobin

synthesis. Previous studies (Ponka and Neuwirt, 1970; Ponka and Schulman, 1985a,b) showed that in rabbit reticulocytes heme formation is not limited by either the rate of protoporphyrin synthesis or the activity of ferrochelatase but by the utilization of iron from transferrin. The results of this present study confirm and extend this conclusion to Friend cells induced to erythroid differentiation. Uninduced and induced Friend cells incorporate iron from the synthetic chelator SIH in amounts several-fold higher than from transferrin. In induced cells this results in substantially increased heme production over the maximun obtained with transferrin, while in uninduced cells only a very limited stimulation of heme synthesis by Fe-SIH is observed. It is possible that increased iron uptake does not stimulate heme synthesis prior to induction because in these cells the enzymes of the heme pathway are present in limiting amounts.

Since the utilization of iron from transferrin limits heme biosynthesis only in induced cells, it was of interest to examine the stimulation of heme production by Fe-SIH at various times after induction. During the first 24 h following the addition of DMSO there was no significant difference between the stimulation of heme production by Fe-transferrin and Fe-SIH. However, as early as 48 h after DMSO addition, Fe-SIH stimulated heme production up to two-fold as compared to Fe-transferrin. It is interesting that Fe-SIH stimulates heme production only after 24 h, reaching a maximum after 72 h, which follows the time course of ALA synthase induction (Sassa, 1976; Beaumont et al., 1984). Since ferrochelatase also increases as soon as 24 h following DMSO (Beaumont et al., 1984), it appears that it is the iron incorporation from transferrin that limits heme production during the induction period. It

is interesting that in induced Friend cells ALA stimulates ^{59}Fe incorporation into heme from ^{59}Fe-SIH but not from ^{59}Fe-transferrin, suggesting that unlike rabbit reticulocytes (Ponka and Schulman, 1985b) ALA synthase becomes rate-limiting for heme synthesis when induced Friend cells are supplied with nonlimiting amounts of iron from Fe-SIH.

There is also some evidence that iron utilization from transferrin may limit not only heme but also hemoglobin synthesis in Friend cells, since contrary to previous results with rabbit reticulocytes (Ponka and Schulman, 1985a,b), Fe-SIH also stimulated globin synthesis in the induced cells. It seems reasonable that the stimulation of globin synthesis by Fe-SIH is mediated by an increase in the level of intracellular heme, which could increase globin synthesis at either the translational (Zucker and Schulman, 1968; Jagus et al., 1981) or transcriptional (Hoffman and Ross, 1980; Fuchs et al., 1981) level or both.

These results therefore provide further evidence that some step(s) in the pathway of iron from extracellular transferrin to protoporphyrin, rather than ALA synthase, limits and controls the overall rate of heme and possibly hemoglobin synthesis in erythroid cells. However, some of our results are paradoxical and require special comment. When iron is provided to induced Friend cells by transferrin, only a relatively small proportion (30-40%) of the iron taken up by the cells is used for heme synthesis. This finding appears to be in conflict with our main conclusion but there are two possible explanations. First, following release of iron from transferrin there may be two pathways of iron utilization in Friend cells, one leading to heme and another to ferritin, which cannot provide iron for heme synthesis (Ponka et al., 1982). It is tempting to speculate that in uninduced cells almost all the iron enters

the ferritin pathway and that erythroid differentiation is accompanied by the induction of a new iron pathway which shunts increasing amounts of iron to mitochondria for heme synthesis during cell maturation. Thus, in reticulocytes, virtually only the mitochondrial heme pathway exists (Martinez-Medellin and Schulman, 1972; Ponka et al., 1982). Second, following iron release from transferrin there may be an intermediate involved in the iron transport to or within mitochondria that is bypassed when iron is supplied from Fe-SIH. If this intermediate is limiting, then its level, rather than the cellular uptake of iron, would appear to limit iron incorporation into heme.

CHAPTER 4

FERRITIN AND FERRITIN IRON METABOLISM IN MURINE ERYTHROLEUKEMIA CELLS

PREFACE

Iron which is not utilized for heme synthesis, or for the synthesis of iron requiring proteins is stored in ferritin. The exact role of ferritin in intracellular iron metabolism however, is not clear. This is of particular interest in erythroid cells which take up large amounts of iron for the synthesis of hemoglobin.

As discussed in Chapter 1 ferritin is an extremely complex protein and may serve different functions in different cell types. In this chapter intracellular iron distribution and the effect of iron and heme on ferritin protein levels are examined. The question of whether ferritin iron can be utilized for heme synthesis is also addressed. These studies were performed in order to better understand the interrelationships between iron uptake, the regulation of heme and ferritin synthesis, and intracellular iron flux in MEL cells, and how these may alter during erythroid differentiation.

ABSTRACT

Intracellular ^{59}Fe distribution and the regulation of ferritin protein levels were studied in murine erythroleukemia (MEL) cells following dimethylsulfoxide induced erythroid differentiation. MEL cell differentiation is accompanied by an increase in transferrin receptor expression, an increase in total cellular iron uptake, and an increase in heme synthesis. Following incubation of induced and uninduced cells with ^{59}Fe-labelled transferrin the ^{59}Fe incorporation into heme, ferritin, and stromal fractions was measured. The results show that the majority of newly incorporated iron, which accompanies differentiation, is shunted to the mitochondria for use in heme synthesis. Furthermore, chase experiments prove that in the presence of saturating concentration of transferrin, cells which are actively synthesizing hemoglobin do not use ferritin iron to support heme synthesis. The synthetic iron chelate, ferric salicylaldehyde isonicotinoyl hrydrazone (Fe-SIH), is able to supply cells with iron, independently of transferrin and transferrin receptors, and in amounts greater than those seen with saturating concentration of transferrin. Using this source to supply iron in great excess, it was demonstrated that in induced MEL cells it is only when iron supply exceeds that required for heme synthesis (these levels are above those seen with saturating concentrations of transferrin) that the proportion of iron in ferritin increases. Furthermore, iron supplied in this form stimulates ferritin protein accumulation in both induced and uninduced cells, while removal of iron with desferrioxamine results in a drop in ferritin content. Addition of exogenous hemin also stimulated ferritin accumulation in both induced and uninduced cells, and while inhibition of heme synthesis, with succinyl acetone, had no effect in

uninduced cells it stimulated ferritin accumulation in induced cells, probably by increasing intracellular iron levels. These results suggest that in addition to iron, endogenous heme levels may play a role in regulating ferritin synthesis in differentiating MEL cells.

INTRODUCTION

Ferritin is an ubiquitous iron storage protein found in animals, plants fungi and bacteria (Munro and Linder, 1978). Its role is both to store iron and to protect cells from the potentially toxic effects of free iron (Halliwell and Gutteridge, 1984). It is well documented that ferritin synthesis is stimulated by iron (Munro and Linder 1978; Theil, 1987). This control has long been thought to occur at the translational level (Munro and Linder 1978) although recently evidence for transcriptional control in Hela cells has also been described (Cairo et al., 1985). Ferritin is a large macromolecule (450,000 daltons) composed of 24 subunits which assemble to form a hollow sphere like shape within which iron gets deposited as ferric hydroxide crystals. There are two unique subunits, the H or heavy subunit (21,000 daltons) and the L or light subunit (19,000 daltons). These combine in varying proportions in different cell types to form tissue specific isoferritins (Arosio et al., 1978). Isoferritins may also vary within the same tissue under different physiological conditions. In both rat and human cell types, for example, iron loading stimulates the accumulation of L rich isoferritins. These observations suggest that in addition to its ubiquitous intracellular housekeeping role, there may be cell specific variations in ferritin function and regulation. One such variation may be the role of ferritin in intracellular iron metabolism.

The role of ferritin in iron flux is of particular interest in erythroid cells which are actively synthesizing hemoglobin. These cells take up large amounts of iron, most of which is diverted for heme synthesis. The significance of intracellular ferritin in these cells and its relationship to hemoglobin synthesis is controversial. Some have

suggested that ferritin merely serves as a sink for surplus iron (Zail et al., 1964; Primosigh and Thomas, 1968; Borova et al., 1973) while others argue that ferritin is an intermediate, possibly an obligatory intermediate, in the pathway of iron utilization for heme synthesis (Mazur and Carleton, 1963; Speyer and Feilding, 1979).

To address this question we examined iron distribution and ferritin synthesis in murine erythroleukemia cells (MEL). These cells were selected for study as they are a useful model for the investigation of iron metabolism in developing erythroid cells. They are virus transformed immature erythroid cells which upon treatment with a chemical inducing agent, such as dimethylsulfoxide (DMSO), initiate a program of erythroid differentiation which in many ways parallels normal erythropoeisis. Differentiation in these cells is characterized by increases in transferrin receptor number (Hu et al., 1977; Wilczynska et al 1984; Laskey et al., 1986), iron uptake (Glass et al., 1978; Laskey et al., 1986), enzymes of the heme biosynthetic pathway (Sassa, 1976), and an induction of globin mRNA (Ross et al., 1972), all of which leads to the accumulation of large amounts of hemoglobin.

In this study we have used ^{59}Fe to trace iron incorporation into various cell fractions. Two distinct iron sources were used, one transferrin (Tf), the obligatory physiological iron donor and the other a synthetic iron chelate ferric-salicylaldehyde isonicotinoyl hydrazone (Fe-SIH) which we have previously shown can deliver iron to cells independently of the transferrin:transferrin receptor pathway, in amounts far greater than those seen with saturating levels of iron transferrin (Laskey et al., 1986). In this way, iron distribution and ferritin synthesis can be examined, under normal physiological conditions and under

conditions of iron loading, in both induced and uninduced cells. The results suggest that there is a tight coordination between iron uptake from transferrin and heme synthesis under normal physiological conditions and that ferritin serves primarily as a storage site for surplus iron rather than as an intermediate in iron delivery for heme.

MATERIALS AND METHODS

Cells and cell culture. Murine erythroleukemia cells, clone 745A originally isolated by Dr. Charlotte Friend (Friend et al., 1971), were grown in suspension cultures in MEM supplemented with 10% fetal calf serum at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were subcultured every 96 h at an initial cell concentration of approximately 5 x 10^4 cells/ml. For induction, 1.5% dimethlysulfoxide (DMSO) was added to the medium at the time of subculturing. After 96 h induced and uninduced cells were collected by centrifugation and washed three times with phosphate buffered saline.

Studies with ^{59}Fe-transferrin and ^{59}Fe-SIH. To prepare ^{59}Fe-transferrin (^{59}Fe-Tf), ^{59}Fe-ferric citrate (20-fold molar excess of citrate over iron) was mixed with transferrin in a molar ratio of 2 mol Fe:1 mol Tf. Solid $NaHCO_3$, at a final concentration of 0.1 mol/L , was then added and the pH was adjusted to 7.4. The prepartion was kept at room temperature for 3 h and then dialysed extensively against phosphate buffered saline.

SIH (salicylaldehyde isonicotinoyl hydrazone or 2-hydroxybenzal isonicotinoyl hydrazone) was prepared as described by Ponka et al (1979). SIH was dissolved in a few drops of 1 N NaOH and diluted with medium. To prepare ^{59} Fe-SIH, ^{59}Fe ferric-citrate was added to the SIH solution at a final molar concentration of Fe-SIH of 1:1. The preparation was then adjusted to pH 7.4 and allowed to sit at room temperature for 1 h.

To label cells, induced and uninduced cells (10 x 10^6) were incubated at 37°C for the indicated time preiods with either saturating concentrations of ^{59}Fe-Tf (20 μM) or ^{59}Fe-SIH (100 μM). The cells were then washed three times with phosphate buffered saline and the

^{59}Fe associated with the cells was measured with an LKB Compugamma counter. Quadruplicate samples were then divided into two groups, one for the determination of ^{59}Fe-heme, and the other for stromal and ferritin ^{59}Fe measurements.

^{59}Fe-heme measurements were performed by acid precipitation (Borova et al., 1973) as described previously (Laskey et al., 1986).

For fractionation, lysing buffer (10mM Tris, 25 mM NaCL, 10 mM $MgCl_2$, 1 mM PMSF, 1% Triton-X-100, pH 7.5) was added to the cell pellets and the prepartion was vortexed vigorously and left on ice for ten minutes. The solution was then spun at 10,000 rpm for 20 min and separated into stromal and supernatant fractions. The ^{59}Fe in the stromal fraction was then measured. To measure ^{59}Fe in ferritin, an aliquot of supernatant was removed and incubated at 37°C for 1 h with polyclonal rabbit anti-mouse ferritin antiserum. The ferritin-antibody complex was then precipitated with goat-anti-rabbit-immunobead matrix as described by the manufacturer (BioRad), and the ^{59}Fe associated with the pellet was measured. Knowing the volume of the aliqout and of the supernatant, and the specific activity of the ^{59}Fe added, the radioiron content in the ferritin fraction was then calculated.

Ferritin radioimmunoassay. The ferritin assay developed for use in these studies is essentially a coventional radioimmune assay where an antigen competes with labelled antigen for a limited number of available antibody binding sites. The antibody-antigen complexes are then precipitated using a second antibody coupled to a solid matrix. Quantification of the label in the pellet shows an inverse relationship between the level of radioactivity and the concentration of antigen. Using known concentrations of unlabelled ferritin a standard curve is

prepared. The concentration of the unknown ferritin sample is then determined from the standard curve.

Purified mouse ferritin was prepared and labelled with I^{125} using lactoperoxidase coupled to CNBr activated Sepharose 4B according to the procedure described by David (1972). Polyclonal rabbit anti-mouse ferritin antiserum was collected and shown by SDS-PAGE to precipitate selectively mouse ferritin.

To measure cellular ferritin content, cells (1.0×10^7) were incubated under the specified conditions for the indicated times, collected by centrifugation and washed three times in phosphate buffered saline. Ice cold lysis buffer was then added and the solution was vortexed and left on ice for 10 minutes. This solution was then centrifuged in an Eppendorf Microfuge for 10 minutes and the clarified lysate collected. Known ferritin standards, and aliquots of clarified cell lysates were incubated at $37^{o}C$ for 1 h with polyclonal rabbit anti-mouse ferritin antiserum in the presence of constant amounts of labelled ferritin. After one hour goat anti-rabbit immunomatrix (Becton Dickinson) was added to precipitate the ferritin-antibody complexes. This reaction was allowed to continue for 30 minutes at room temperature, after which the solution was centrifuged and the immune complex precipitated. The radioactivity associated with the pellet was measured and a standard curve was constructed. From this curve the concentration of ferritin in the lysates was determined.

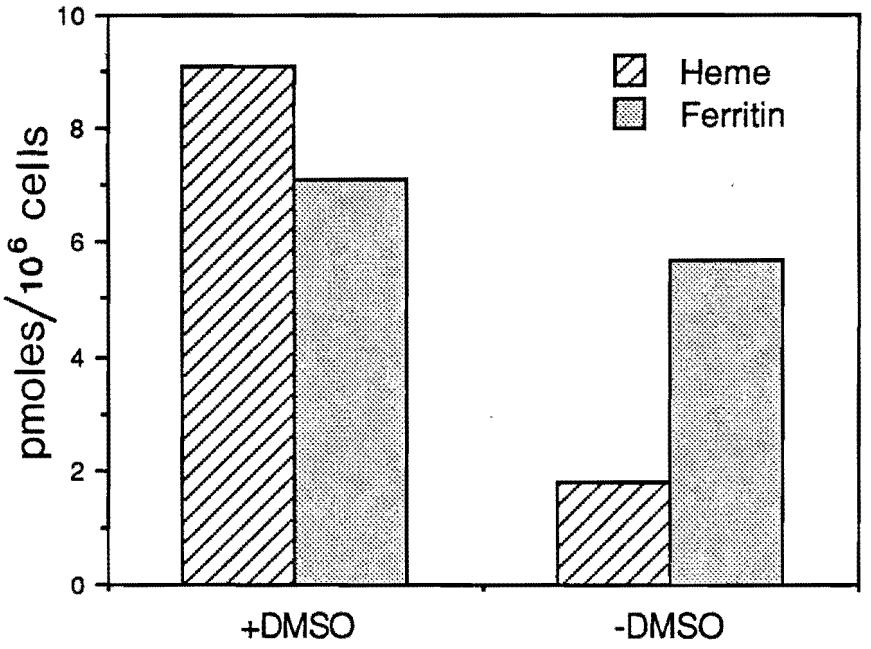
RESULTS

Iron distribution in induced and uninduced MEL cells. We have previously shown that following DMSO induced erythroid differentiation in MEL cells there is an approximate 3-fold increase in the number of transferrin receptors which in turn results in a several fold increase in total iron uptake (Laskey et al., 1986). In order to determine how this increase in total cellular iron uptake affects intracellular iron distribution we examined iron distribution in MEL cells, before and after induction of hemoglobin synthesis.

Induced and uninduced MEL cells were incubated in the presence of saturating levels of ^{59}Fe-Tf for 60 minutes after which the cells were washed, lysed and fractionated. Previously we showed that following DMSO induced differentiation there is a large increase in the amount of ^{59}Fe incorporated into the heme fraction (Laskey et al., 1986). Here we further show that while there is an increase in ^{59}Fe recovered in the ferritin fraction in induced cells it is not comparable to the increase in heme ^{59}Fe incorporation (Figure 1). Furthermore, the concentration of iron recovered in the stromal fraction (which is the Triton X insoluble pellet) did not change following induction. This suggests that under normal physiological conditions most, but not all, of the additional iron which is taken up following induction is utilized for heme synthesis.

Is ferritin iron utilized for heme synthesis? To test if iron in ferritin could be used for heme synthesis, pulse-chase experiments were performed. Induced and uninduced cells were incubated with saturating concentrations of ^{59}Fe-transferrin for 1 h, after which they were washed and resuspended in serum free medium containing saturating amounts of cold Fe-transferrin. The chase was continued for 1 h and the ^{59}Fe in

Figure 1. The incorporation of ^{59}Fe from saturating concentrations of ^{59}Fe-transferrin into the heme and ferritin fractions of induced (+DMSO) and uninduced (-DMSO) MEL cells. MEL cells were cultured in the presence or absence of 1.5% DMSO for four days. The cells were then collected by centrifugation, washed and resuspended in serum free medium. Samples of 1 x 10^7 cells/ml were incubated at 37^{o}C in the presence of saturating concentrations of ^{59}Fe-transferrin (20 μM) for 60 min, cells were chilled, washed three times in phosphate buffered saline and the ^{59}Fe in the heme and ferritin fractions measured. The results shown here are values for a typical experiment and are averages of triplicate samples. This pattern of expression held true in three repeated experiments.


10
8
6
4
2
0
pmoles/10^6 cells
Heme
Ferritin
+DMSO
-DMSO

ferritin before and after the chase was measured. Over this period of time there was no significant loss of ^{59}Fe from the ferritin fraction in either the induced or uninduced cells (data not shown). Furthermore, in parallel experiments using similar conditions, it can be demonstrated that induced cells do use iron from exogenously added transferrin for their heme synthesis. These results show that in short term incubations in the presence of Fe-transferrin, ^{59}Fe from ferritin is not utilized for heme synthesis and suggests that there is a preferential use of transferrin iron.

^{59}Fe uptake from ^{59}Fe-transferrin and ^{59}Fe-SIH. It was also of interest to examine intracellular iron distribution under conditions of normal and excess iron supply. To do this we took advantage of a chelator developed in our laboratory salicyladehyde isonicotinoyl hydrazone (SIH). Previously, we showed that Fe-SIH could deliver iron to MEL cells in a manner which was transferrin and transferrin receptor independent, and in excess as compared to saturating concentrations of Fe-Tf (Laskey et al., 1986). Moreover, Fe-SIH was able to stimulate heme synthesis in induced but not uninduced cells suggesting that once committed to erythroid differentiation iron delivery from transferrin becomes limiting for heme synthesis.

We therefore compared the distribution of iron from the two different iron donors, Fe-transferrin and Fe-SIH. Induced MEL cells, which are already taking up large amounts of iron and are actively synthesizing hemoglobin were incubated for 1 h with either saturating amounts of ^{59}Fe-Tf which would be the maximal physiological iron loading condition or 100 μM Fe-SIH which should supply iron in great excess compared to saturating levels of Fe-Tf. Table 1 shows that when transferrin is the

Table 1. Distribution of iron in induced MEL cells pulsed with either ^{59}Fe-transferrin or ^{59}Fe-SIH

intracellular fraction	Iron donor	
	^{59}Fe-Tf	^{59}Fe-SIH
stroma	8.19 ± 0.61	81.17 ± 7.03
heme	7.17 ± 0.31	28.53 ± 2.29
ferritin	9.94 ± 0.24	50.36 ± 2.86

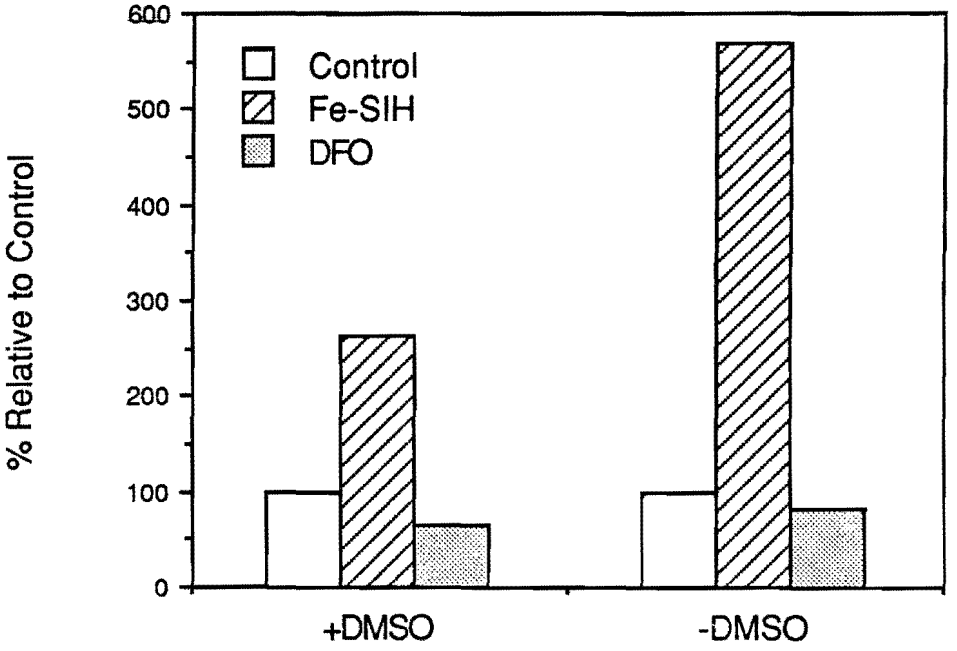
MEL cell were grown in the presence of 1.5% DMSO for four days, after which they were collected by centrifugation and washed three times in phosphate buffered saline (PBS). Samples of 1 x 10^7 cells were then resuspended in serum free medium in the presence of either, saturating concentration of ^{59}Fe-Tf (20 μM), or ^{59}Fe-SIH (100 μM) and incubated for 60 minutes at 37°C . The samples were then chilled, washed three times in PBS, and the ^{59}Fe incorporation into the various cell fractions measured. The results are expressed in pmol Fe/ 10^6 cells.
Data are mean ±S.D., n=4.

iron donor, the total cellular iron uptake is 25.3 pmol/10^6 cells/h. This amount is distributed more or less equally among the three measured fractions stroma, heme and ferritin. The iron found in the stromal fraction is thought to consist mainly of hemosiderin iron - hemosiderin being a breakdown product of ferritin (Munro and Linder, 1978). Fe-SIH, on the other hand, is able to supply iron to cells in great excess as compared to Fe-Tf and as previously reported this excess iron is able to stimulate heme synthesis (Laskey et al., 1986). When Fe-SIH is the iron donor the cells took up 160 pmol/10^6cells/h, and incorporated four times more iron into heme as compared with Fe-Tf incubated cells. However, the major portion of this excess iron is recovered in the stromal and ferritin fractions (Table 1).

The effect of iron on cellular ferritin content. The effect of iron loading and iron depletion on ferritin content was also measured (Figure 2). Induced and uninduced cells were preincubated under the specified conditions for 18 h after which total cellular ferritin content was measured by radioimmune assay (RIA). Fe-SIH (100 μM) was used to iron load the cells while desferrioxamine, an iron chelator, was used to deplete intracellular iron. In both induced and uninduced cells preincubation with Fe-SIH resulted in ferritin accumulation while cells treated with desferrioxamine had decreased ferritin contents. Interestingly, in uninduced cells the response to Fe-SIH is greater than in induced cells.

The effect of heme on cellular ferritin content. Since heme is well known to control various biochemical processes in erythroid cells (Hoffman and Ross, 1980; Jagus et al., 1981; Ponka et al., 1969) it is possible that intracellular heme levels may affect ferritin synthesis. To test this possibility induced and uninduced MEL cells were preincubated for 18

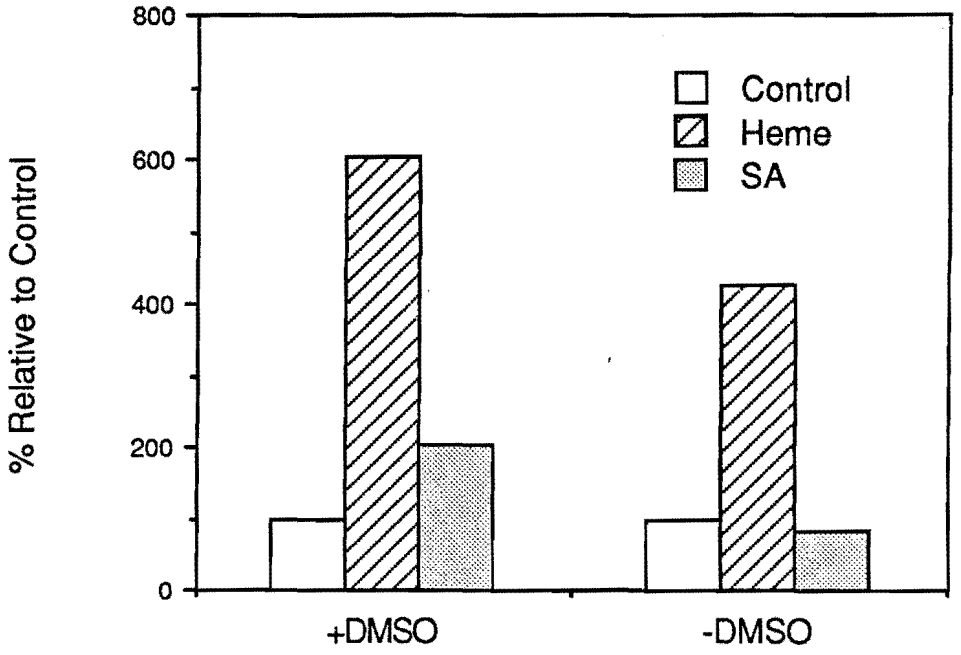
Figure 2. The effect of iron on ferritin content of induced (+DMSO) and uninduced (-DMSO) MEL cells. MEL cells were cultured in the presence or absence of 1.5% DMSO for four days. Prior to collecting the cells on the fourth day, MEL cells were incubated for 18 h without an iron ligand (empty columns) with 50 μM Fe-SIH (dashed columns) or with 50 μM desferrioxamine (dotted columns). The cells were then collected by centrifugation, washed three times in phosphate buffered saline, and assayed for ferritin content. Using an estimated molecular weight of 490,000 for mouse ferritin, ferritin concentrations were calculated in pmol/10^6 cells. The results are expresed here as percent of induced (+DMSO) and uninduced (-DMSO) controls, respectively. The results are mean of four experiments. Total estimated ferritin content for +DMSO and -DMSO controls are, 0.15 ± 0.045 and 0.14 ± 0.018 pmol/10^6 cells, respectively (data are mean ± S.D., n=4).


% Relative to Control
600
500
400
300
200
100
0
Control
Fe-SIH
DFO
+DMSO
-DMSO

hours in the presence of added exogenous hemin (50 μM) or succinylacetone (SA, 1mM) an inhibitor of heme synthesis (Ebert et al., 1979). Figure 3 shows that in both induced and uninduced MEL cells, added hemin leads to an increase in intracellular ferritin content. This may be a direct effect of heme itself or could alternatively result from iron released from heme. When cells were treated with both hemin and SIH (which will chelate and remove iron (Ponka et al., 1979)) ferritin levels remained at control values (data not shown). This could mean either that the response of ferritin to heme is due solely to its ability to deliver iron, or alternatively that both heme and iron can affect ferritin content and in this case they effectively compete.

Inhibition of heme synthesis, using SA, results in a slight drop in ferritin levels in uninduced MEL cells (Figure 3). This is not entirely unexpected as uninduced cells are synthesizing only minute amounts of heme. In contrast, induced MEL cells respond to SA treatment by increasing their ferritin content. It has previously been reported that in rabbit reticulocytes inhibition of heme synthesis leads to an accumulation of intracellular iron (Ponka et al., 1982). It seemly likely therefore that inhibition of heme synthesis in induced cells which are taking up large amounts of iron leads to an increase in intracellular iron levels which in turn stimulates ferritin accumulation.

Figure 3. The effect of heme on ferritin content in induced (+DMSO) and uninduced (-DMSO) MEL cells. MEL cells were cultured in the presence (+DMSO) and absence (-DMSO) of 1.5% DMSO for four days. Prior to collecting the cells on the fourth day, MEL cells were incubated for 18 h with nothing added (empty columns), with 50 μM hemin (dashed columns), or with 1mM succinylacetone (dotted columns). The cells were then collected by centrifugation, washed three times with phosphate buffered saline, and assayed for ferritin content. Using an estimated molecular weight of 490,000 for mouse ferritin, the results were calculated in pmol/10^6 cells, and are expressed here as percent control. The mean of four different experiments is shown. The ferritin content of +DMSO and -DMSO controls are, 0.014 ± 0.018 and 0.15 ± 0.045, respectively (data are mean ± S.D., n=4).


800
600
400
200
0
% Relative to Control
Control
Heme
SA
+DMSO
-DMSO

DISCUSSION

Differentiating MEL cells provide a model system for the study of iron metabolism in developing erythroid cells. Of particular interest is the relationship between ferritin iron and heme iron, as the cells initiate and maximize hemoglobin synthesis. We reported previously that following dimethlysulfoxide (DMSO) induced erythroid differentiation in MEL cells, there is an approximate three-fold increase in transferrin receptor expression which in turn results in a several fold increase in total iron uptake (Laskey et al., 1986). In the present study cellular iron distribution was compared in induced MEL cells, which are synthesizing large amounts of hemoglobin, and uninduced MEL cells which synthesize little if any hemoglobin. Three major cell fractions were identified, the heme iron fraction, the ferritin iron fraction and stromal iron, which most probably represent hemosiderin, a breakdown product of ferritin.

Our data show that following DMSO induction, the level of ferritin and stromal iron increases marginally, if at all, while the majority of the increased iron taken up is recovered in the heme fraction. In similar studies Ofer et al. (1981) have used Mossbauer spectroscopy to study iron distribution. They identify only two fractions namely, heme and ferritin though they also show, using electron microscopic analysis, that much of the ferritin is present in lysosomes, which in our study would be measured as the stromal fraction. They reported that when transferrin was the iron donor, the level of ferritin iron remained constant following differentiation and the increase in total iron was recovered in the heme fraction. Together, the results suggest that there is a preferential use of Tf-Fe for heme synthesis. As is suggested by the recent results of Offer et al. (1981), ferritin iron is, most probably, only used when

extracellular iron sources are insufficient. Furthermore, they suggest, that following erythroid differentiation there is a shunting of transferrin iron to the mitochondria for utilization in heme synthesis. This is also reflected by the observation that Tf receptor expression and heme synthesis both increase in a coordinated manner during erythroid differentiation in these cells.

Peto et al., (1983) have previously reported that in MEL cells iron in ferritin can be chased into the heme fraction. In these experiments cells were labelled for 16 h with $^{59}FeCl_3$, washed and then resuspended in medium with or without DMSO and chased for periods of 4-5 days, during which time the cells remain in logarithmic growth phase. At the end of this period however up to 30% of the ^{59}Fe was lost, presumably due to cell death (Peto et al., 1983). They concluded that there is a movement of iron from the ferritin fraction into the heme fraction, however, it is equally possible that in the process of cell death iron is released and subsequently reincorporated and therefore, long term chase studies such as theirs may be inconclusive. In our study we used a short term labelling and chase period (1 h respectively) to test if iron in ferritin can be utilized for heme synthesis. The results indicate that, in the presence of unlabelled Fe-Tf, the iron in ferritin is not utilized for heme synthesis in either induced or uninduced cells. This suggests that there is a preferential use of Fe from Fe-TF for heme synthesis.

Taking advantage of the synthetic iron chelate, Fe-SIH, the effect of iron overload on iron distribution and ferritin synthesis was also examined. As described previously, in differentiated MEL cells increasing iron supply above the amounts obtained with saturating levels of Fe-Tf stimulated heme formation (Laskey et al.,1986), however, the majority of

this excess iron was recovered in the ferritin and stroma fractions. These data suggest that in cells which are actively synthesizing hemoglobin, the increase in iron uptake which accompanies erythroid differentiation is used exclusively to synthesize heme, and only if maximum levels of physiological iron uptake are surpassed, is iron then deposited in ferritin. Furthermore, iron delivered in the form of Fe-SIH leads to an increase in ferritin content in both induced and uninduced cells, while removing iron with the chelator desferrioxamine leads to decreased ferritin content in both cell types. Notably, the ability of Fe-SIH to increase ferritin levels in uninduced cells appears to be greater than in induced MEL cells. This may be due to the fact that in induced cells more of the Fe from Fe-SIH is incorporated into heme and therefore free intracellular iron levels may not be exactly the same in the two groups.

Heme, in addition to its function as the prosthetic group of hemoproteins has also been implicated in a variety of regulatory roles in erythroid cells, including the stimulation of globin mRNA transcription (Fuchs et al., 1981) and translation (Burns and London, 1965), and the inhibition of iron uptake from transferrin (Ponka and Neuwrit, 1967; 1971). In MEL cells heme can potentiate differentiation but cannot by itself initiate terminal erythroid differentiation (Marks and Rifkind, 1978). However, heme synthesis appears to be required to initiate terminal cell division (Mager and Bernstein, 1979). Given these observations, it seemed logical to examine the possible role of heme in the regulation of ferritin levels. The results indicate that heme stimulates ferritin accumulation in both induced and uninduced cells. It is however difficult to determine whether this is a direct effect of heme

itself or results from iron which may be released from heme. When SIH, an iron chelator, is included with hemin, ferritin levels remain constant. This could mean either that heme exerts its effect via iron released from the heme and this iron is subsequently removed by SIH or that the heme moiety itself and SIH, have opposing actions which effectively compete. Recently, Fibach et al. (1987) showed that ^{57}Fe-hemin is able to donate iron to ferritin in MEL cells. However, their data show only the incorporation of ^{57}Fe into ferritin over a period of days, whereas in our studies the stimulation of ferritin accumulation can be measured as early as 2 hours after the addition of hemin. The effect of inhibiting endogenous heme synthesis was also examined. In uninduced cells this resulted in a slight drop in ferritin levels whereas inhibition of heme synthesis in induced cells resulted in ferritin accumulation. Induced cells are taking up large amounts of iron and under conditions of inhibited heme synthesis free intracellular iron would be expected to accumulate and stimulate ferritin synthesis. In conclusion, endogenous "free" heme may play a role in regulating ferritin protein levels in MEL cells.

One final question is why, if heme synthesis is not maximal when iron is provided to induced MEL cells by transferrin, is only 30-40% of the iron taken up by the cells used for heme synthesis? Our results are compatible with the idea that there are two pathways of iron utilization, one leading to heme and another to ferritin, which is prominent in non-erythroid cells. Furthermore, the iron in ferritin will not, in the presence of Fe-Tf, provide iron for heme synthesis. It appears therefore that erythroid differentiation is accompanied by the induction of a new iron pathway which shunts increasing amounts of iron to the mitochondria

for heme synthesis. Only if maximal iron delivery from transferrin can be surpassed and only if this amount surpasses that required for maximal heme syntheis will iron then be incorporated into ferritin. Under normal physiological conditions, however, the tight coordination between transferrin receptor expression, iron uptake from transferrin and intracellular heme levels ensures that during erythroid differentiation iron uptake and heme synthesis will be coupled.

CHAPTER 5

CONTROL OF FERRITIN GENE EXPRESSSION DURING MEL CELL DIFFERENTIATION: ROLE OF IRON AND HEME

PREFACE

Chapter 5 is a continuation of the comphrehensive study of ferritin regulation and the role of ferritin in erythroid iron metabolism initiated in Chapter 4. Here, the regulation of ferritin expresssion at the mRNA level is examined using northern and dot-blot analysis. Ferritin protein and mRNA levels during MEL differentiation, and the effect of iron and heme on ferritin mRNA levels are studied.

ABSTRACT

The regulation of ferritin synthesis was studied in differentiating murine erythroleukemia (MEL) cells. MEL cells were induced to differentiate with dimethylsulfoxide (DMSO) and the relative levels of H and L ferritin subunit mRNA examined by northern analysis. The results show that during DMSO induced differentiation, H ferritin mRNA levels increase geometrically, increasing to approximately 10-fold over control values after 96 h. L ferritin mRNA levels increase biphasically; there is an initial increase after 24 h, followed by a drop at 48 h, after which it subsequently increases again, peaking after 72 h.

The effect of iron and heme on ferritin mRNA levels was examined using dot-blot analysis. The synthetic chelate ferric salicyaldehyde isonicotinoyl hydrazone (Fe-SIH) was used as an iron source. It is an extremely effective iron donor, supplying iron to cells in amounts exceeding those obtained with saturating levels of transferrin. Overnight incubation with Fe-SIH had no effect on ferritin mRNA levels in either induced or uninduced cells. Furthermore, depletion of intracellular iron, using desferrioxamine, was also without effect. To study the effect of heme, MEL cells were induced to differentiate with DMSO, with and without added exogenous hemin, and with and without succinylacetone (SA) an inhibitor of endogenous heme synthesis. The effect of heme and SA was also examined in uninduced cells. In both cases hemin, and SA were added at the time of subculturing and the cells harvested after 4 days. The addition of hemin resulted in increases in both H and L ferritin mRNA levels, in both induced and uninduced cells. Moreover, while SA has no effect in uninduced cells, it partially inhibits the induction of H and L ferritin mRNA levels in DMSO treated cells. These results

indicate that ferritin mRNA levels increase during erythroid differentiation of MEL and that heme may play a role in this process.

INTRODUCTION

Heme, as the prosthetic group of a variety of heme-proteins, plays an important role in a number of cellular functions. These functions include the enzyme activities involved in oxidative phosphorylation, in the splitting of hydrogen peroxide and in the detoxification of chemical agents. In erythroid cells heme serves a specialized function in the transporting of oxygen as part of the hemoglobin molecule.

In addition to its function as a prosthetic group, heme itself has been shown to have various regulatory roles in developing erythroid cells. Heme stimulates both the transcription (Fuchs et al., 1981) and translation (Burns and London, 1965) of globin mRNA, thereby ensuring the coordinate expression of hemoglobin. In murine erythroleukemia (MEL) cells induced to differentiate by dimethlysulfoxide, the addition of exogenous hemin results in a greater than ten-fold increase in globin synthesis as compared to cells treated with DMSO only. In contrast, inhibition of endogenous heme synthesis causes a reduction in globin synthesis to levels comparable to uninduced cells and addition of exogenous hemin relieves this inhibition (Dabney and Beaudet, 1977). However, hemin is not considered to be an inducer of erythroid differentiation since while it can stimulate globin synthesis it does not cause an irreversible commitment and will not cause the onset of terminal cell division. On the other hand, in the presence of a chemical inducer inhibition of endogenous heme synthesis prevents the onset of terminal differentiation and this inhibition can be relieved by the addition of exogenous hemin (Mager and Bernstein, 1979). In conclusion, it seems that heme is required, but not in itself sufficient, for the induction of terminal erythroid differentiation. We therefore wanted to investigate

the role of heme in modulating changes which occur during differentiation of MEL cells, particularly with respect to changes which occur in iron metabolism.

Erythroid differentiation of MEL cells is accompanied by an increase in transferrin receptor expression which in turn results in an increase in total iron uptake. Intracellular iron may either be utilized for heme synthesis or stored in ferritin. Ferritin is an ubiquitous iron storage protein the synthesis of which is stimulated by iron. Iron regulation of ferritin synthesis is thought to occur primarily at the level of translation, since in most cells treatment with actinomycin D does not inhibit the ability of iron to stimulate ferritin accumulation (Zahringer et al., 1976; Munro and Linder, 1978). However, transcriptional regulation of ferritin expression by iron has recently been described in Hela cells (Cairo et al., 1985).

The role, and mechanism of regulation, of ferritin in erythroid cells is not clear. Although some have suggested that ferritin is an intermediate in the pathway of extracellular iron to heme, we previously provided evidence that in MEL cells, when extracellular iron is not limiting, ferritin iron is not used for heme synthesis (Laskey et al., 1987a). Furthermore ferritin synthesis is responsive to iron in these cells since administration of either iron or heme, to induced or uninduced MEL cells, results in an increase in ferritin content (Laskey et al., 1987a).

Recently, it was reported that following induction of MEL cells there is an increase in H and L ferritin mRNA levels (Beaumont et al., 1987). To determine if heme has any role in modulating these changes and to understand better the function of ferritin in MEL cells, we examined changes in ferritin mRNA levels and the effect of iron and heme on

ferritin mRNA levels, prior to and following erythroid differentiation.

MATERIALS AND METHODS

Cells and cell culture. Murine erythroleukemia cells, clone 745A originally isolated by Dr. Charlotte Friend (Friend et al., 1971), were grown in suspension cultures of MEM with 10% fetal calf serum at 37°C in an atmosphere of 95% air and 5% CO_2. Cells were subcultured every 96 h at an initial cell concentration of approximately 5 x 10^4 cells/ml. For induction, 1.5% dimethlysulfoxide (DMSO) was added to the medium at the time of subculturing. After 96 h induced and uninduced cells were collected by centrifugation and washed three times with phosphate buffered saline. Benzidine staining was performed as desribed by Orkin et al. (1975).

Ferritin radioimmunoassay. Ferritin protein levels were measured by a conventional radioimmune assay described previously (Laskey et al., 1987a). Briefly, aliquots of clarified cell lysates, or known standards, were incubated with a constant amount of labelled ^{125}I-ferritin and polyclonal rabbit anti-mouse ferritin antiserum for 1 h at 37°C. Goat anti-rabbit immunobead matrix (Becton Dickinson) was then added and allowed to react for 30 minutes at room temperature. The second reaction was performed in order to precipitate the ferritin antibody complexes, which were then pelleted by centrifugation in an Eppendorf microcentrifuge for 10 minutes.. After removal of the supernatant, the radioactivity associated with these pellets was measured, a standard curve constructed, and the concentration of ferritin in the lysates determined.

RNA isolation. Total cellular RNA was isolated from MEL cells essentially as desribed by Davis et al. (1986). MEL cells were collected by centrifugation and washed with cold, sterile, phosphate buffered saline. Cells were disrupted in an ice cold lysis buffer (140mM NaCl, 1.5

mM $MgCl_2$, 10mM Tris pH 8.4, 0.5% NP-40). The nuclei were discarded following centrifugation in an Eppendorf centrifuge for 10 minutes, and the supernatant collected, phenol extracted, and RNA isolated following ethanol precipitation. To remove any contaminating deoxyribonucleic acids the dried pellet was subsequently resuspended, and the solution digested with ribonuclease-free deoxyribonuclease 1 enzyme, as described by the manufacturer (Boeringer Mannheim). To terminate the reaction the solution was then diluted, phenol extracted, ethanol precipitated, and dried under vacuum. The final pellet was resuspended in water, and its concentration and purity determined by measuring the optical densities at A_{260} and A_{280} as described by Maniatis (1982).

Northern and Dot-blot analysis of H and L ferritin mRNA. Northern analysis was performed essentailly as described by Davis et al. (1986), except that the formaldehyde concentration in the gel and running buffer was increased to 2.2 M. Briefly, RNA samples are denatured by heating them to 65°C in the presence of formamide and formaldehyde, for 10 minutes. They were then cooled, loaded onto a 1% agarose gel, and fractionated by electrophoresis in the presence of formaldehyde. The RNA was then transferred to nitrocellulose paper (Schleicher and Schuell) in 10X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), in a standard procedure described by Maniatis (1982). The filters were then air dried, and baked in a vacuum oven at 80°C for 2 h.

For Dot-blot analysis, a known concentration of total cellular RNA was resuspended in 0.1X SSC, 7.5% (v/v) formaldehyde and heated to 65°C for 10 minutes. The samples were then cooled on ice for 5 minutes and serially diluted in the same buffer. Four microliters of each dilution was then spotted onto nitrocellulose paper which had been previously

soaked for 15 minutes in 1X SSC. The filter was air dried, and then baked in a vacuum oven at 80°C for 2 h.

Nick translation. Recombinant plasmids containing rat H and L ferritin subunit cDNAs were a generous donation of Dr. H. Munro. For nick translation, the cDNA inserts were excized from their respective plasmids by cleavage with the restriction endonuclease PST 1, and electrophoresed in a 1% low melting point agarose gel in 1X TBE buffer (90mM boric acid, 2.5 mM EDTA, 90mM Tris-HCL, pH 8), in the presence of ethidium bromide. The band containing the cDNA probe was visualized under UV light, extracted and the DNA recovered, by first liquifing the gel at 65°C, then extracting twice with phenol and finally, precipitating with ethanol and drying under vacuum.

H and L ferritin cDNAs (0.5 - 1.0 µg) prepared in this manner, were labelled with ^{32}P by nick translation, using a Nick Translation Kit (BRL), to a final specific activity of 1.0-5.0 x 10^7 cpm/µg.

Hybridization conditions. Northern and dot-blot filters were prehybridized in a sealable bag for 4-6 h at 42°C in: 50% formamide, 5X Denhardt's reagent, 100 ug denatured hering sperm DNA, 0.1% SDS, 5X SSPE (1X SSPE is .15 M NaCl, 0.01M $NaH_2PO_4 \cdot H_2O$, 1mM EDTA), and 15% dextran sulfate. Following prehybridization and after first being denatured by heating to 100°C for 10 min, the labelled probe was then added to the hybridization bag. Hybridization was overnight, after which the filters were washed for 2 x 5 minutes in 2X SSC, 0.5% SDS at 25°C; then 2 x 15 minutes in 2X SSC, 0.1% SDS; and finally 2 x 30 minutes 0.1X SSC, 0.5% SDS at 42°C. The filters were then exposed to Fugi NIF-RX film with an intensifying screen for 24 h at -70°C. Films were processed in a Kodak X-Omat. Autoradiograms were scanned with a LKB laser

densitometer.

RESULTS

Analysis of ferritin mRNA levels during differentiation. Total cellular RNA isolated from MEL cells after various times in culture in the presence of dimethylsulfoxide was subjected to Northern analysis, and the relative amounts of H and L ferritin subunit mRNA determined. Figure 1 shows that following DMSO induced differentiation there is a dramatic increase in H ferritin mRNA between 48 and 96 h. This pattern of induction appears very similar to that previously reported for α and β-globin mRNA (Ross et al., 1972). L ferritin mRNA levels also increase following induction; however, this occurs in a biphasic pattern (Figure 2). This suggests that H and L ferritin mRNAs may be regulated differently.

Analysis of ferritin protein levels during differentiation. To determine if ferritin protein levels followed the changes in H and L ferritin mRNA, cells at various stages of differentiation were analysed for total ferritin content by radioimmunoassay. Results from four separate experiments (Figure 3) reveal that, although there is some variation between experiments, ferritin protein levels do not increase following induction.

Effect of iron on ferritin mRNA levels. Though ferritin regulation has long been thought to occur at the level of translation (Zahringer et al., 1976; Munro and Linder, 1978), recent evidence suggests that in Hela cells iron administration results in an increase in H and L ferritin transcription as well as translation (Cairo et al., 1985). We therefore wanted to examine the ability of iron, added in excess or removed by chelation, to affect ferritin mRNA levels in induced and uninduced MEL cells. To deliver iron in excess we took advantage of a chelate developed

Figure 1. Changes in H ferritin subunit mRNA levels during dimethyl sulfoxide induced MEL cell differentiation. MEL cells were cultured in MEM supplemented with 10% fetal calf serum and exposed to 1.5% dimethylsulfoxide (DMSO) for the indicated time intervals (0,24,48,72, and 96, h). Samples of 10 µg of total RNA, from each time interval, were fractionated on 1% agarose gel in 2.2 M formaldehyde and transferred to nitrocellulose paper. In these studies RNA samples were run in duplicate sets on the same gel and two essentially identical filters prepared. Each filter was then hybridized with a nick translated cDNA probe (specific activity 5 x 10^7cpm/µg, 2 x 10^6cpm/ml) for either the H or L ferritin subunit (Figures 1 and 2 respectively). The lower panel in each photograph shows the results as visulaized by autoradiogram. The top panels in both photographs show the ethidium bromide staining of the RNA samples which were run, indicating that all lanes had equal concentration of RNA applied and that there was no RNA degradation.

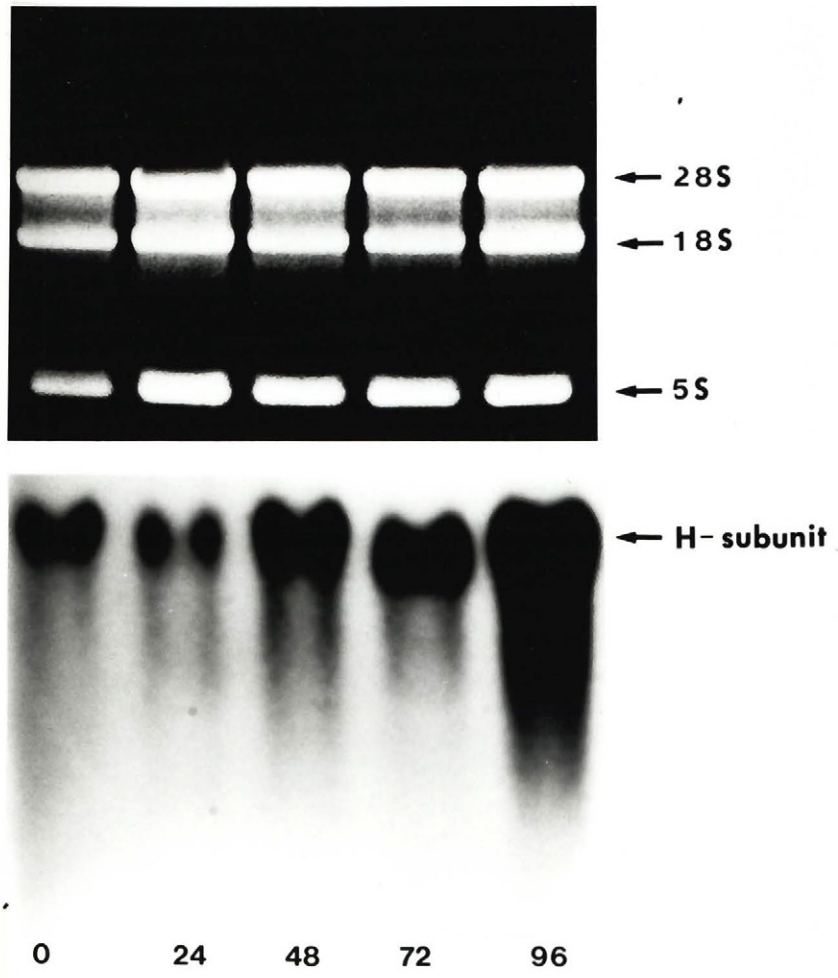

28S
18S
5S
H-subunit
0
24
48
72
96
TIME (h)

Figure 2. Changes in L ferritin subunit mRNA levels during dimethyl sulfoxide induced MEL cell differentiation. MEL cells were cultured in MEM supplemented with 10% fetal calf serum and exposed to 1.5% dimethylsulfoxide (DMSO) for the indicated time intervals (0,24,48,72, and 96 h). Relative L ferritin mRNA levels were measured by Northern analysis. RNA and northern blots were prepared as described in Figure 1.

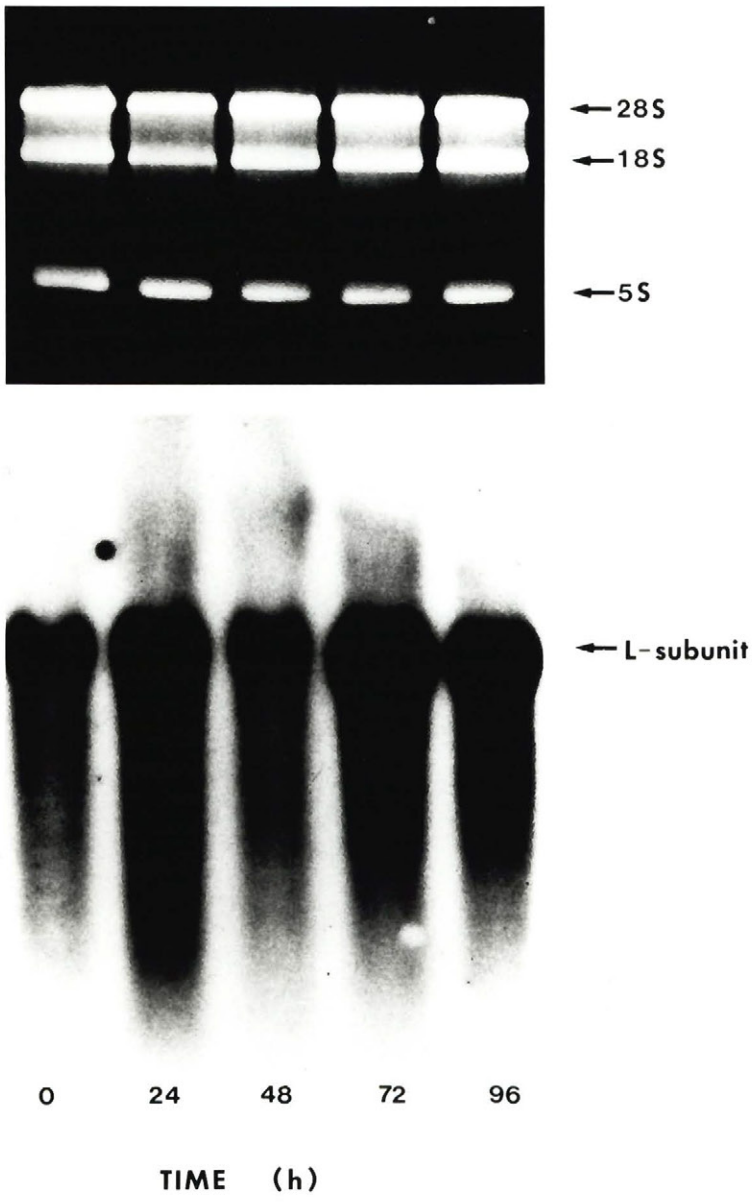
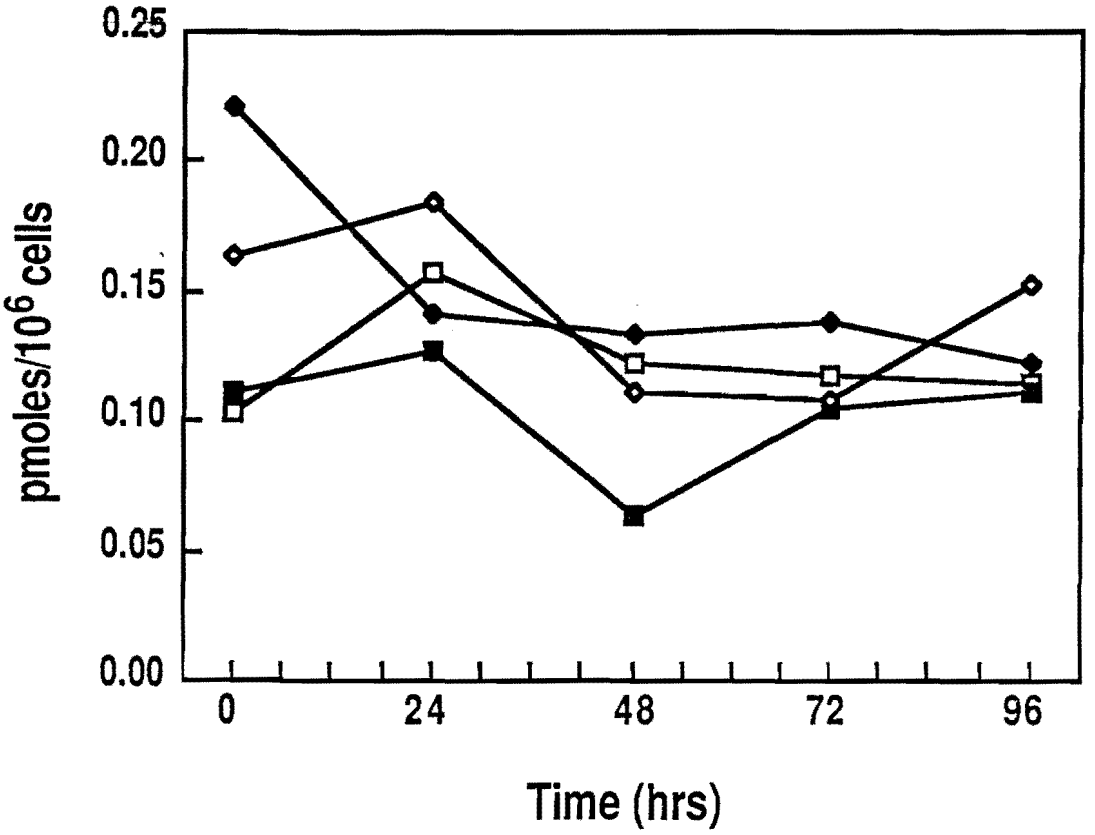

28S
18S
5S
L-subunit
0
24
48
72
96
TIME (h)

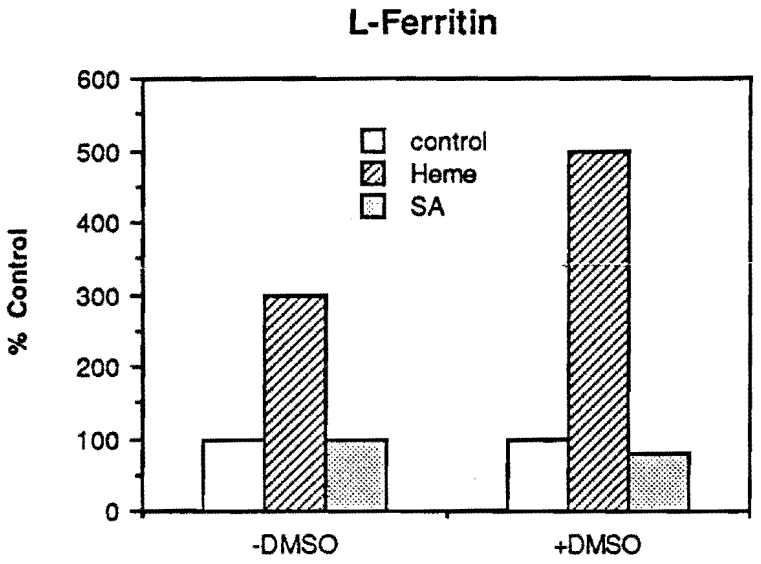
Figure 3. Changes in ferritin protein content following dimethyl sulfoxide (DMSO) induced differentiation in MEL cells. MEL cells were cultured in MEM supplemented with 10% fetal calf serum, and exposed to 1.5% DMSO for the indicated time intervals (0,24,48,72 and 96, h). For each time point cells were collected by centrifugation, washed three times in phosphate buffered saline, and the ferritin content determined by radioimmune assay. Using an estimated molecular weight of 490,000 for mouse ferritin, the results were calculated in pmoles/10^6 cells. The results of four separate experiments are presented.

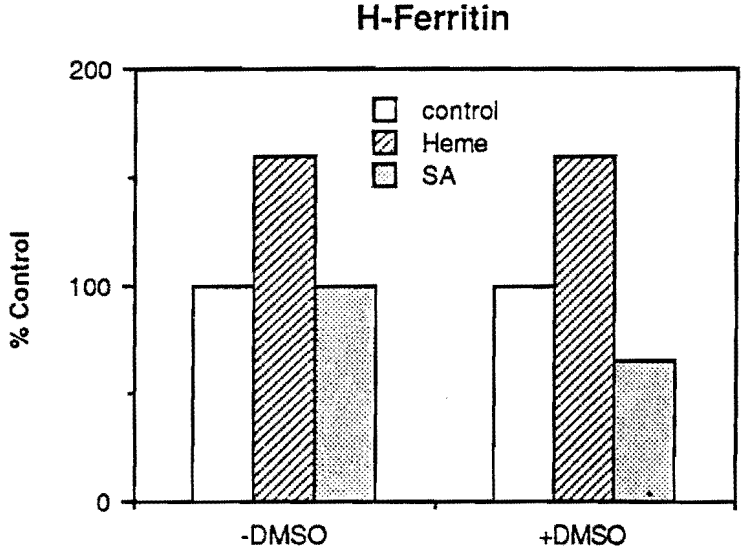

0.25
0.20
0.15
0.10
0.05
0.00
pmoles/10^6 cells
0
24
48
72
96
Time (hrs)

recently in our laboratory, ferric-salicyaldehyde isonicotinoyl hydrazone (SIH). We showed previously that ferric-SIH is able to deliver iron to MEL cells, independently of transferrin and transferrin receptors and in amounts greater than those seen with saturating concentration of Fe-transferrin (Laskey et al., 1986). Moreover, Fe-SIH has been shown to stimulate the accumulation of ferritin protein in these cells (Laskey et al., 1987a). Therefore, using dot-blot analysis, we looked at the ability of Fe-SIH to affect ferritin mRNA levels in induced and uninduced cells. Cells were incubated overnight, in culture, in the presence of Fe-SIH (50 μM), they were then harvested and the relative ferritin mRNA levels determined. The results (data not shown) revealed that iron administration does not affect ferritin mRNA levels in either induced or uninduced cells. Furthermore, in a parallel experiment, removing iron using the chelator desferrioxamine (50 μM), which has previously been shown to lead to a drop in ferritin protein content (Laskey et al., 1987a), did not effect ferritin mRNA levels.

Effect of heme on ferritin mRNA levels. Heme has been shown to have a number of regulatory effects in erythroid cells and was previously shown to increase ferritin protein content in both induced and uninduced MEL cells (Laskey et al., 1987a). To test whether heme has any regulatory effects on ferritin mRNA levels, MEL cells were cultured in the presence or absence of 1.5% DMSO with and without added hemin (50 μM) or with and without added succinylacetone (1mM), an inhibitor of endogenous heme synthesis (Ebert et al., 1979), for 96 h. Added hemin results in an increase in H and L ferritin mRNA in both induced and uninduced cells (Figure 4). Inhibition of endogenous heme synthesis in uninduced cells had no measureable effect. However, this is not unexpected, as endogenous

Figure 4. The effect of heme on H and L ferritin subunit mRNA levels. MEL cells were cultured for four days in MEM supplemented with 10% fetal calf serum, in the presence and absence of 1.5% DMSO, with and without added hemin (50 μM), and with an without added succinyl acetone (SA, .5 mM). After four days, cells were collected by centrifugation, washed three times in sterile phosphate buffered saline, and total RNA extracted. For each experimental condition, 10 μg of total RNA and serial dilutions, were spotted onto nitrocellulose filters, as described in Materials and Methods. Filters were prepared in duplicate and hybridized with ^{32}P-labelled cDNA probes for H and L ferritin subunit mRNA respectively. Specific activity of both probes was 0.5 x 10^8 cpm/μg and hybridization mixtures contained 2 x 10^6 cpm/ml. Autoradiograms of these filters were subsequently scanned with a laser densitometer and arbitrary scanning units calculated for each spot. For any one experimental condition, analysis of these serially diluted spots showed that, within the dilution range used, concentration limits could be selected where the intensity of the RNA spots was proportional to the amount of RNA spotted. In this way the linear range for each experimental condition was established. to compare between groups, a single concentration, which fell within the linear range of all groups compared, was selected and at this concentration, arbitrary scanning units compared. Here the results are expressed as the percent of induced (+DMSO) and uninduced (-DMSO) controls respectively. Presented here are mean values from three separate experiments.


L-Ferritin
600
500
400
300
200
100
0
% Control
control
Heme
SA
-DMSO
+DMSO


H-Ferritin
200
100
0
% Control
control
Heme
SA
-DMSO
+DMSO

heme synthesis in these cells is extremely low (Laskey et al., 1986). In contrast, inhibition of endogenous heme synthesis in cells cultured in the presence of DMSO completely inhibits the accumulation of hemoglobin, as measured by benzidine staining (see Table 1). Moreover, this resulted in a drop in both H and L ferritin mRNA levels as compared to untreated induced cells. These results suggest that endogenous heme levels may play a role in regulating ferritin mRNA accumulation during differentiation.

Table 1. The effect of succinylacetone on benzidine staining of MEL cells

Medium addition	% benzidine positive
none (-DMSO)	≤ 1%
-DMSO + SA (.5mM)	≤ 1%
+DMSO (1.5%)	90%
+DMSO (1.5%) + SA (.5mM)	≤ 1%

MEL cells were subcultured at an initial cell density of approximately 1 x 10^5 in the presence and absence of 1.5% DMSO, with and without succinylacetone (.5mM). After four days of culture under the specified conditions the percentage of benzidine positive cells was determined according to the method of Orkin et al. (1975).

DISCUSSION

An increase in the transcription of H and L ferritin mRNA, resulting in the accumulation of H and L ferritin mRNA, has been observed following differentiation of HL60 cells (Concannon et al., 1985; Chou et al., 1986). Recently, Beaumont et al. (1987) reported that during DMSO induced differentiation of MEL cells, there is an increase in both H and L ferritin mRNA levels. Using isolated nuclei they showed that these increases reflect increases in the rate of H and L ferritin transcription. Together these two reports suggest that the induction of ferritin mRNA may be a general feature of differentiation. Our results in MEL cells confirm and extend the observations of Beaumont et al. (1987). The observed pattern of ferritin mRNA induction, however, varies somewhat between the two laboratories. Our results show that, following DMSO addition, H ferritin mRNA increases in a geometric fashion, reaching a maximum of approximately 10-fold after 96 h, whereas Beaumont et al. (1987) report that H ferritin mRNA increases in a biphasic pattern reaching a maximum of only 3-fold after 96 h. The reason for this discrepancy is not clear, it may be a function of the MEL cell clone, the culture procedures, experimental techniques, or possibly of the cDNA probe used since Beaumont et al used human cDNA probes while we used rat cDNA probes. Secondly, this study confirms the surprising observation that while ferritin mRNA levels increase during induction, ferritin protein levels remain constant.

There are several speculations one can make to explain this phenomenon. It should be noted, for example, that during erythroid differentiation of MEL cells there is a significant decrease (30-40%) in total protein synthesis (Parker and Houseman, 1985). Therefore the observation that

ferritin protein levels remain constant following differentiation may in fact mean that relative ferritin synthesis has increased. In addition, during differentiation of MEL cells there are dramatic increases in the levels of both α and β globin mRNA. Since the translational machinery may be limiting in induced cells (Parker and Houseman, 1985), it is possible that increases in ferritin mRNA are required in order to compete successfully with α and β globin mRNA for translation.

It is well documented that ferritin synthesis is stimulated by iron. In most cells, this is thought to occur at the level of translation, since preincubation of cells with actinomycin D does not inhibit the ability of iron to exert an effect (Zahringer et al., 1976; Munro and Linder, 1978). However, iron regulation of ferritin expression may be more complicated than first suspected. For example, it has been observed in Hela cells that actinomycin D does not inhibit the ability of iron to stimulate ferritin synthesis, suggesting a translational control mechanism (Chu and Fineberg, 1969). However, direct measurements of H and L ferritin mRNA levels show that iron administration results in an accumulation of both H and L ferritin mRNA levels (Cairo et al., 1985). Furthermore, analysis of isolated nuclei show that these increases are due to increases in the rate of transcription (Cairo et al., 1985). We therefore wanted to examine the effect of iron on ferritin mRNA levels in differentiating MEL cells. Contrary to results reported for Hela cells, our dot-blot data show that addition or removal of iron does not affect ferritin mRNA levels in either induced or uninduced MEL cells. These results, together with our previous results showing the ability of iron to induce ferritin protein (Laskey et al., 1987a) support the conclusion that, in erythroid cells, the regulation of ferritin synthesis by iron occurs at the translational

level.

Heme has been shown to have a number of regulatory effects in erythroid cells, including the stimulation of globin mRNA transcription (Ross et al., 1972; Jagus et al., 1981) and translation (Burns and London, 1965; Zucker and Schulman, 1968; Gross and Rabinovitz, 1972). We therefore examined the effect of heme on ferritin mRNA levels. Our results show that adding hemin to either induced or uninduced MEL cells stimulates the accumulation of both H and L ferritin mRNA. Moreover, inhibition of heme synthesis during differentiation of MEL cells prevents the accumulation of H and L ferritin mRNA. This suggests that endogenous "free" heme may play a role in regulating ferritin mRNA levels. Furthermore, the observation that iron does not affect ferritin mRNA levels precludes the possibility that heme is exerting its affect through iron which may, or may not, be released from the heme moiety following catabolism. The exact mechanism of heme action remains to be ascertained, it may act at the level of transcription, mRNA processing and/or mRNA stability. Studies are presently underway in our laboratory to address this question.

CHAPTER 6

EVIDENCE THAT TRANSFERRIN SUPPORTS CELL PROLIFERATION BY SUPPLYING IRON FOR DNA SYNTHESIS

PREFACE

Chapter 6 diverges from the discussion of iron metabolism in erythroid cells to focus on the more general topic of the role of transferrin iron delivery in supporting cell proliferation. As described in Chapter 1, rapidly proliferating cells, both normal and malignant, significantly increase their transferrin receptor numbers and take up considerably more iron. The observation that cells require transferrin for growth has led to debate over whether transferrin functions solely by providing iron or whether it has in addition, a growth factor like activity. Chapter 6 addresses this question taking advantage of the synthetic chelate Fe-SIH to supply iron while blocking transferrin receptors with monoclonal antibodies. In these studies MEL cells and Raji cells were used as model tumor cell lines, while normal human lymphocytes were used as a non-malignant model. Both cell growth and DNA synthesis were examined.

ABSTRACT

Transferrin is essential for cell proliferation and it was suggested that it may trigger a proliferative response following its interaction with receptors, serving as a growth factor. However, since the only clearly defined function of transferrin is iron transport, it may merely serve as an iron donor. To clarify this issue further, we took advantage of an iron chelate, ferric salicylaldehyde isonicotinoyl hydrazone (Fe-SIH) which we developed and previously demonstrated to supply iron efficiently to cells without using the physiological transferrin receptor pathway. As expected, we observed that blocking monoclonal antibodies against transferrin receptors inhibited proliferation of both Raji and murine erythroleukemia cells. This inhibited cell growth was rescued upon the addition of Fe-SIH which was also shown to deliver iron to Raji cells in the presence of blocking monoclonal antibodies. Moreover, blocking anti-transferrin receptor antibodies inhibited ^{3}H-thymidine incorporation into DNA and this inhibition could be overcome by added Fe-SIH. In addition, Fe-SIH slightly stimulated, while SIH (an iron chelator) significantly inhibited DNA synthesis in phytohemagglutinin-stimulated peripheral blood lymphocytes. Taken together, these results indicate that the only function of transferrin in supporting cell proliferation is to supply the cells with iron.

INTRODUCTION

Iron is an essential nutrient required by all eukaryotic cells for growth and survival. For most animal cells, with the possible exception of reticuloendothelial cells, transferrin is the obligatory physiological iron donor (Morgan, 1981). Iron uptake involves the binding of iron-transferrin to specific cell surface transferrin receptors and internalization of the transferrin:receptor complex in acidic endosomes whereupon iron is released. The transferrin:receptor complex is then recycled to the cell surface where apotransferrin is released (Morgan, 1981).

Transferrin receptors are highly expressed on specialized cell types which require iron to fulfill specific functions, such as immature erythroid cells which require large amounts of iron for the formation of hemoglobin. They are also highly expressed on proliferating cells such as neoplastic cells, mitogen-stimulated lymphocytes, and many established cell lines (Larrnick and Creswell, 1979; Galbraith et al., 1980; Trowbridge and Omary, 1981; Hamilton, 1983). T lymphocytes, for example, when stimulated with mitogen increase their transferrin receptor number. This increase precedes the onset of DNA synthesis (Mendelsohn et al., 1983; Neckers and Cossman, 1983).

The correlation between transferrin receptors and cell proliferation may be related to the fact that iron is an essential requirement for the enzyme ribonucleotide reductase (Thelander and Reichard, 1979), whose activity in turn is strongly correlated with the rate of DNA synthesis and is greatly increased during S phase. The enzyme is composed of two non-identical subunits, M_1 and M_2. During the cell cycle M_1 protein levels remain constant, while M_2 protein level fluctuates and, therefore

ribonucleotide reductase activity is regulated by M_2 synthesis and breakdown (Eriksson et al., 1984). Interestingly, it is the M_2 subunit which requires iron for its activity, and recent results (L Thelander, personal communications) have shown a direct effect of iron on the rate of translation of the M_2 subunit. Therefore, it is possible that the regulation of transferrin receptor number during cell proliferation is related to the iron requirement for the M_2 subunit, and that an increase in transferrin receptor number is a neccessary prerequisite for increased M_2 activity.

Monoclonal anti-transferrin receptor antibodies that inhibit transferrin binding to their receptors and consequently iron uptake, have been described for both human (42/6) and murine (R17-208) systems (Trowbridge and Lopez, 1982; Trowbridge et al., 1982). Blocking the transferrin receptor with these iron-depriving monoclonal antibodies leads to an arrest of cell growth and an accumulation of cells in S phase (Trowbridge and Lopez, 1982).

The inability to overcome this growth inhibition by providing iron salts, in some cell systems (Trowbridge and Lopez, 1982; Teatle et al., 1983), has led to the suggestion that the transferrin receptor molecule may have growth related functions other than to provide iron (Trowbridge and Lopez, 1982; May and Cuatrecasas, 1985). However, iron salts may not be effective iron donors when transferrin receptors are blocked. The aim of the present work is to address this question using synthetic iron chelators which bypass the transferrin receptor cycle and yet are able to deliver iron to the cells far above the maximum observed with saturating amounts of Fe-transferrin (Ponka et al., 1979; Ponka and Schulman, 1985a,b; Laskey et al., 1986). Recent evidence (Landschulz et al., 1984;

Brock and Stevenson, 1987) showing the ability of such a chelate, Fe-pyridoxal isonicotinoyl hydrazone, developed in our laboratory (Ponka et al., 1982), to replace Fe-transferrin in serum free medium supports the idea that transferrin promotes cell proliferation solely because of its ability to supply iron. Here we present evidence that iron, provided in the form of a synthetic chelate, can overcome the growth inhibition induced by blocking monoclonal anti-transferrin receptor antibodies and stimulate DNA synthesis. Moreover, our additional studies indicate that iron is also essential for DNA synthesis in mitogen stimulated lymphocytes.

MATERIALS AND METHODS

Cells and Cell Culture. Friend murine erythroleukemia (MEL) cells used were derived from clone 745A originally isolated by Dr. Charlotte Friend (Friend et al., 1971). Raji cells are human B lymphoblasts originally isolated from a patient with Burkitts lymphoma (Pulvertaft, 1965). The Raji and MEL cells were grown in RPMI-1640 and MEM respectively, supplemented with 10% fetal calf serum at 37°C in an atmosphere of 95% air and 5% CO_2. Human peripheral blood lymphocytes were collected from healthy donors by density centrifugation through Ficoll-Paque as described by the manufacturer (Pharmacia), suspended at 1×10^6 cells/ml in RPMI-1640 containing 10% fetal calf serum. Phytohemagglutinin (PHA) was added to selected cultures at a final concentration of 2 µg/ml.

Monoclonal antibodies and their effect on cell growth. Monoclonal antibodies (mAb) against the murine transferrin receptor (R17-208) and human transferrin receptor (42/6) which have been described previously (Trowbridge et al., 1982; Trowbridge and Lopez, 1982), were kindly provided to us by Dr. I. Trowbridge. To measure the effect of these monoclonal antibodies on cell growth, Raji and MEL cells were seeded in triplicate cultures at initial densities of 1×10^5 cells/ml. Samples from control and test cultures were taken and counted daily until the control cultures reached confluence. All points represent averages of triplicates.

Studies with SIH and SIH-Fe. SIH (salicylaldehyde isonicotinoyl hydrazone or 2-hydroxylbenzal isonicotinoyl hydrazone) was prepared as described by Ponka et al. (Ponka et al., 1979). SIH was dissolved in a few drops of 1N NaOH and diluted with medium. To prepare Fe-SIH and

^{59}Fe-SIH, Fe-citrate or ^{59}Fe-citrate (20-fold molar excess of citrate over iron) was added to the SIH solution at a final molar ratio of Fe:SIH of 1:1. The preparation was then adjusted to pH 7.4 and allowed to sit at room temperature for 1 h. Fe-SIH or SIH was sterilized by filtration.

Studies with ^{59}Fe-SIH and ^{59}Fe-transferrin. To prepare ^{59}Fe-transferrin (^{59}Fe-Tf), ^{59}Fe-citrate was mixed with transferrin in a molar ratio of 2 mol Fe:1 mol Tf. After addition of solid $NaHCO_3$ to a final concentration of 0.1 mol/L the pH was adjusted to 7.4 and the preparation kept at room temperature for 3 h. The preparation was then dialyzed extensively against phosphate buffered saline.

To measure iron uptake, Raji cells (1×10^7 cells/ml) were incubated in serum free medium containing either ^{59}Fe-Tf or ^{59}Fe-SIH. At the indicated times cells were collected by centrifugation and washed three times with phosphate buffered saline. ^{59}Fe associated with the cells was then measured with an LKB Compugamma counter.

Studies with ^{125}I-transferrin. To prepare ^{125}I-transferrin, apo-transferrin was saturated with unlabelled iron as its citrate complex (1:20) and labelled with ^{125}I using lactoperoxidase coupled to CNBr activated Sepharose 4B (Pharmacia) according to the prcedure described by David (David, 1972). To measure transferrin binding, resting and PHA stimulated lymphocytes were prepared as described above and suspended in 0.25 ml samples containing constant amounts of ^{125}I-transferrin and increasing amounts of unlabelled transferrin, with final concentrations of 2.5 to 200 μM. After incubation for 60 minutes at 37°C, the cells were washed at 4°C and counted. Receptor numbers were determined by Scatchard analysis (Scatchard, 1949)

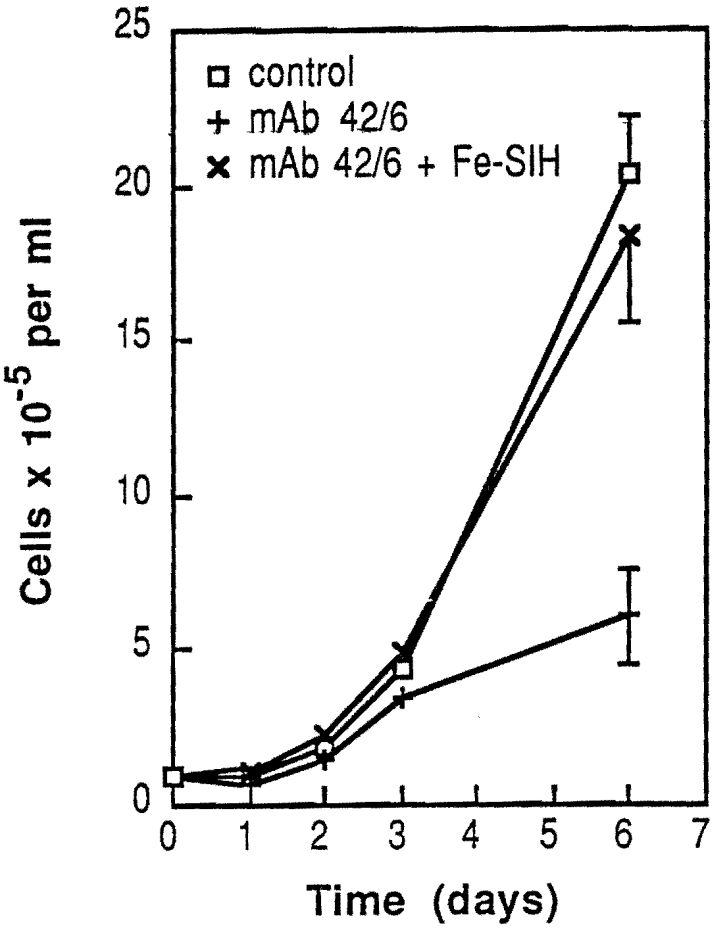
Tritiated thymidine uptake. Cells were incubated under the specified experimental conditions for 66 h, and then divided into triplicate samples of 300,000 cells each and placed in microtiter plates. One μCi of ^{3}H-thymidine was then added and the incubation continued for 2-4 h, after which the plates were frozen and later thawed and the cell remnants harvested onto methyl cellulose filters. The filters were then washed three times with H_2O, and counted following drying.

RESULTS

The role of iron in mAb 42/6-induced growth inhibition. Monoclonal antibody (mAb) 42/6 is an anti-human-transferrin-receptor antibody which inhibits iron uptake from transferrin (Trowbridge and Lopez, 1982). Figure 1 shows that Raji cells grown in the presence of mAb 42/6 (10 µg/ml) exhibit a marked decline in growth rate after 72 h, reaching a maximum inhibition of 75% after six days of culture, (when control cultures reached maximum cell densities). If the inhibition of growth was due to iron deprivation, addition of utilizable chelated iron should reverse 42/6 -induced inhibition. In previous studies this has been tested with iron salts but conflicting results were reported (Trowbridge and Lopez, 1982; Teatle et al., 1985). It is possible that the uptake of iron salts is mediated by the transferrin:transferrin receptor pathway and thus would have to utilize a small proportion of transferrin receptors which may not have been blocked by the antibody. Alternatively, iron salts, regardless of their entry pathway, may only enter in amounts less than those seen with saturating levels of Fe-transferrin. If this were the case the ability of iron salts to overcome the growth inhibition due to mAb 42/6 may depend on the iron requirements of specific cell types. To overcome this ambiguity we have used a synthetic iron chelate Fe-salicylaldehyde isonicotinoyl hydrazone (Fe-SIH) which bypasses the transferrin:transferrin receptor pathway and delivers iron in amounts above those seen with saturating levels of Fe-transferrin (Laskey et al., 1986). As seen in Figure 1 5µM Fe-SIH reverses the 42/6-induced inhibition, suggesting that iron availability is the limiting factor for cell growth in 42/6 treated cells.

To assess whether this observation was cell type specific, we examined

Figure 1. Growth of Raji cells in the presence of monoclonal antibody 42/6 with or without 5 μM Fe-SIH. Raji cells were subcultured at 1 x 10^5 cells/ml in RPMI-1640 supplemented with 10% fetal calf serum. The effect of monoclonal antibody 42/6 (final concentration 10 μg/ml) and 42/6 with 5 μM Fe-SIH on growth was recorded. Each point represents averages of triplicate samples.


25
20
15
10
5
0
0 1 2 3 4 5 6 7
Time (days)
Cells x 10-5 per ml
control
mAb 42/6
mAb 42/6 + Fe-SIH

the ability of Fe-SIH to overcome antibody induced growth inhibition in murine eythroleukemia cells. For this R17-208 (Trowbridge et al., 1982), a monoclonal antibody directed against the murine transferrin receptor which inhibits iron uptake from transferrin, was used. As seen in Figure 2, four days of culture with 5 μg/ml R17-208 caused a 50% inhibition of MEL cell growth which was overcome by the addition of Fe-SIH (5 μM).

^{59}Fe uptake from ^{59}Fe-transferrin and ^{59}Fe-SIH in the presence and absence of mAb 42/6. To clarify further the effect of mAb 42/6 on iron uptake by Raji cells, we examined the effect of mAb 42/6 (10 μg/ml) on ^{59}Fe uptake from both saturating levels of ^{59}Fe-Tf (12.5 μM) and ^{59}Fe-SIH (5 μM) (Fig. 3). As previously reported for murine erythroleukemia cells (Laskey et al., 1986), Fe-SIH can also provide iron for Raji cells with an efficiency comparable, or even higher, than that observed with Fe-Tf. Furthermore, while mAb 42/6 significantly inhibits (63%) iron uptake from Fe-Tf, it has no effect on iron delivery from Fe-SIH. These results confirm that mAb 42/6 limits iron uptake from transferrin and demonstrates that Fe-SIH can be used as an iron source in the presence of blocking antibody against the transferrin receptor.

Fe availability and DNA synthesis. To test whether Fe availability limits DNA synthesis we also examined the effect of the monoclonal antibody (mAb) and Fe levels on DNA synthesis in Raji cells. As compared to control cells, those treated with mAb 42/6 show an approximate 50% decrease in the rate of ^{3}H-thymidine incorporation (Table 1). Desferrioxamine, an iron chelator, also causes a similar drop in ^{3}H-thymidine incorporation suggesting that in both cases Fe supply may be limiting DNA synthesis. Furthermore, ^{3}H-thymidine incorporation returns to normal levels when 42/6 treated cells are supplied with Fe-SIH.

Figure 2. Growth of murine erythroleukemia (MEL) cells in the presence and absence of monoclonal antibody R17-208 with or without Fe-SIH. MEL cells were subcultured at an initial cell density of 1 x 10^5 cells/ml in MEM supplemented with 10% fetal calf serum. The effect of monoclonal antibody R17-208 (final concentration 5 µg/ml) and R17-208 with 5 µM Fe-SIH on the growth of MEL cells was recorded. Each point represents an average of triplicate samples.

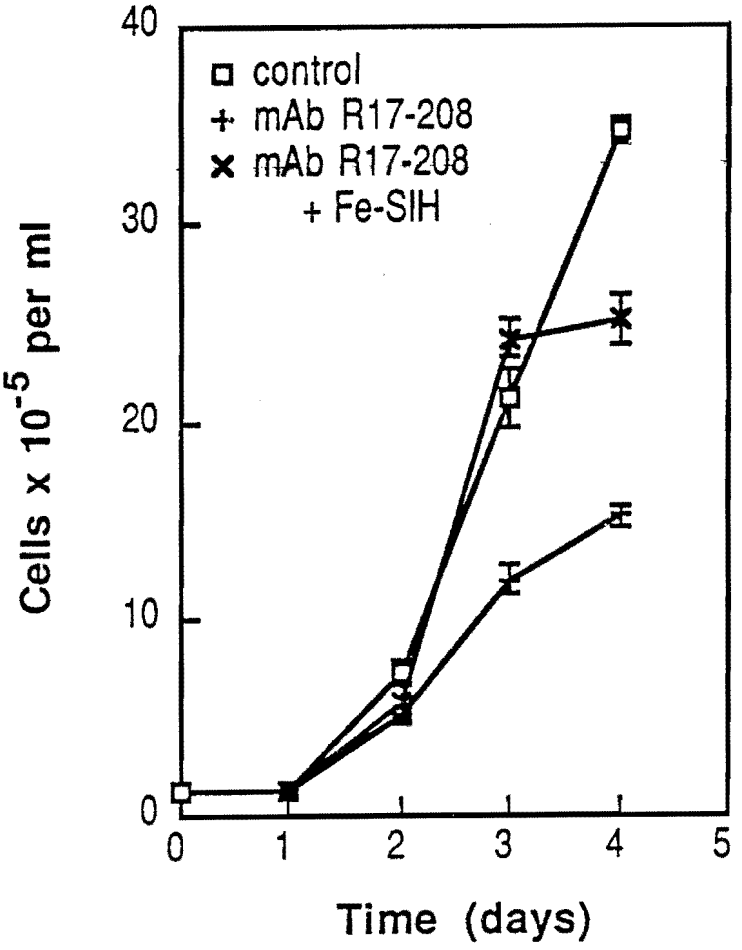

40
30
20
10
0
0
1
2
3
4
5
control
mAb R17-208
mAb R17-208
+ Fe-SIH
Cells x 10-5 per ml
Time (days)

Figure 3. Iron uptake from ^{59}Fe-Tf (A) or ^{59}Fe-SIH (B) in the presence and absence of mAb 42/6. Raji cells were harvested by centrifugation and washed three times in phosphate buffered saline. The cells were then preincubated for 30 min at 37^{o}C in the presence or absence of mAb 42/6 (10 µg/ml). ^{59}Fe-Tf (12.5 µM) or ^{59}Fe-SIH (5 µM) was then added and the samples incubated for the indicated time period, after which time they were washed and the cell associated radioactivity determined. Each point represents the average value of duplicate samples.

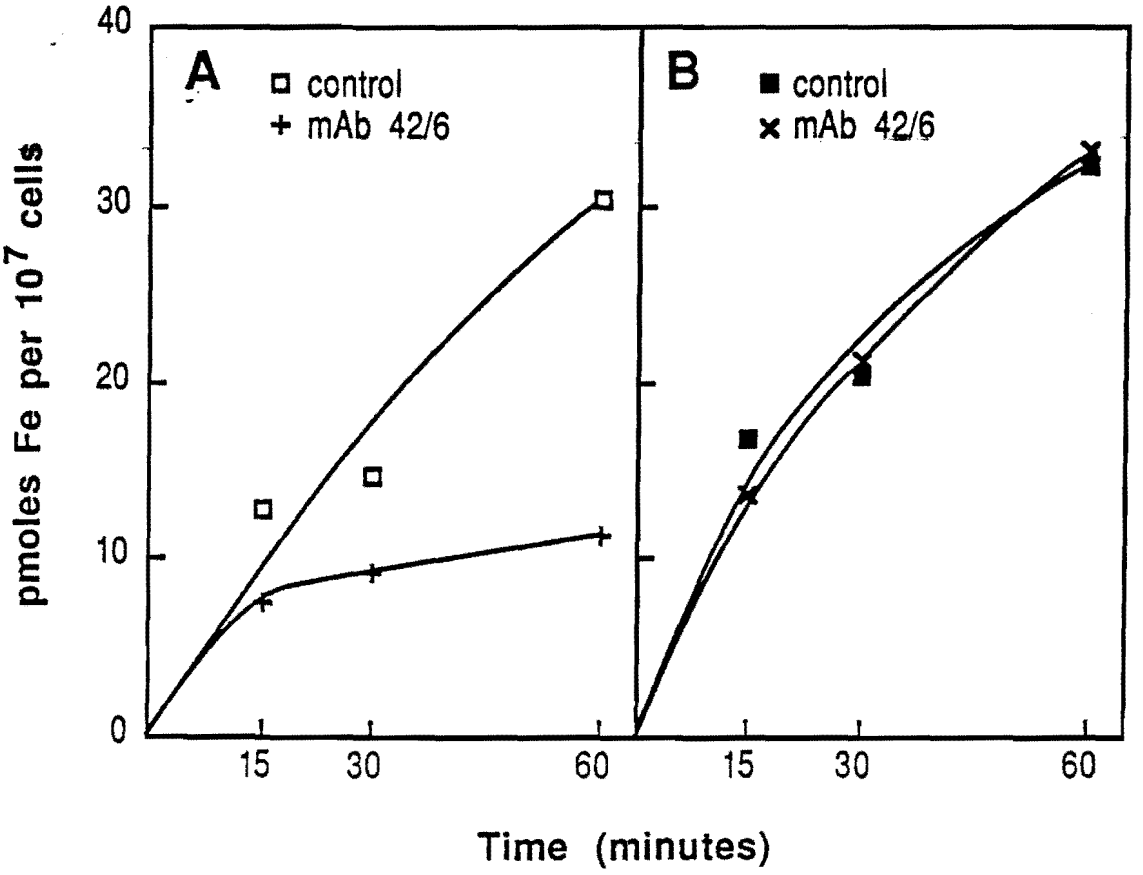

A
control
mAb 42/6
B
control
mAb 42/6
pmoles Fe per 10^7 cells
0
10
20
30
40
15
30
60
15
30
60
Time (minutes)

Table 1. The effect of mAb 42/6 and iron availability on the incorporation of ^{3}H-thymidine into DNA of Raji cells.

Additive	^{3}H-thymidine incorporation	% control
Nil (control)	17663 ± 3686	100
Desferrioxamine (1mM)	6600 ± 1430	37
42/6	9778 ± 889	55
42/6 + 2.5 μM Fe-SIH	18009 ± 2878	102
42/6 + 5.0 μM Fe-SIH	16876 ± 1028	95
2.5 μM Fe-SIH	16002 ± 4801	91
5.0 μM Fe-SIH	18959 ± 2452	107

Raji cells wer grown under the indicated conditions for three days. Triplicate samples of 300,000 cells/ml were then aliquoted into microtiter plates, 1 μCi of ^{3}H-thymidine was added and the incubation continued for 4 h. The plates wer frozen, subsequently thawed, and harvested onto methyl cellulose filters which were then washed and counted. Results are expressed as the mean of triplicate samples ± S.D., the units are cpm/300,000 cells.

Interestingly, in control cells treated with Fe-SIH there is no significant change in the rate of ^{3}H-thymidine incorporation suggesting that under normal tissue culture conditions Fe is not limiting for DNA synthesis.

Fe availability and DNA synthesis in resting and proliferating lymphocytes. To ascertain whether similar observations could be made with non-malignant hematopoietic cells, we examined the iron requirements of mitogen stimulated peripheral blood lymphocytes. As previously reported (Hamilton, 1982), there is a large increase in transferrin receptors following PHA stimulation (Figure 4). Given that this increase in transferrin receptor number has been shown to be an essential prerequisite for DNA synthesis (Neckers and Cossman, 1983), we wanted to examine the effect of iron availability on DNA synthesis in resting and PHA stimulated lymphocytes.

DNA synthesis, as measured by ^{3}H-thymidine incorporation, was examined in resting and PHA stimulated lymphocytes which had been grown for 66 h with and without SIH (which chelates and removes iron) or Fe-SIH (which delivers iron). Figure 5 shows that in resting lymphocytes increasing or decreasing the cellular iron levels appeared to have no effect on DNA synthesis. Intracellular iron stores may be sufficient to sustain the very low levels of DNA synthesis in these cells.Following PHA stimulation there is a dramatic increase in the rate of thymidine incorporation, which can be completely inhibited by the removal of intracellular iron with SIH. In contrast, DNA synthesis can be stimulated in PHA treated lymphocytes with 2.5 μM Fe-SIH, but iron levels beyond this concentration are toxic. Together these results suggest that the increase in transferrin receptor expression in proliferating cells is

Figure 4. Binding of ^{125}I-transferrin to resting and PHA stimulated lymphocytes. To measure transferrin binding at each concentration of transferrin the cells were incubated with a constant amount of ^{125}I-transferrin and increasing amounts of unlabelled transferrin. Each sample was incubated for 60 min at 37^{o}C, then washed three times with cold phosphate buffered saline, and the cell bound ^{125}I-radioactivity measured. Specific transferrin binding was calculated by taking the difference between total and non-specific binding curves (not shown). Non-specific binding was calculated from the slope of the curve between 140 and 200 nM. Specific binding curves for resting and PHA stimulated lymphocytes are presented.

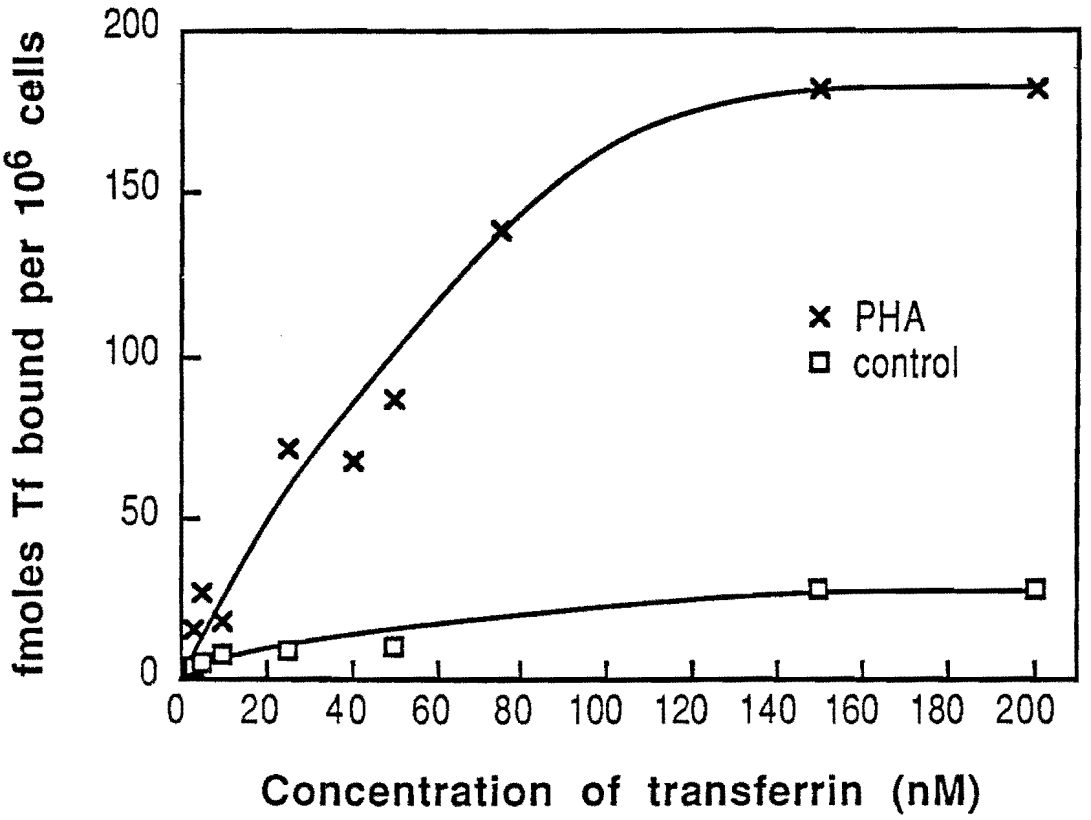
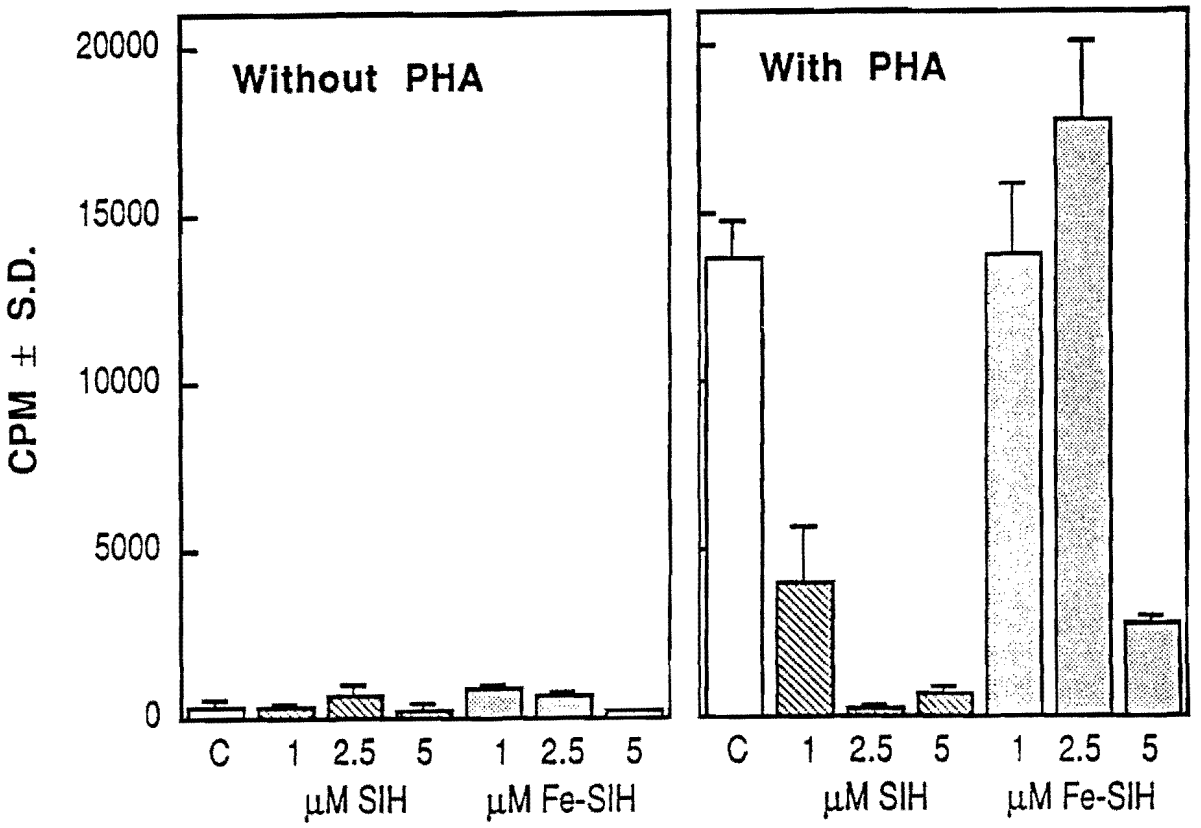

fmoles Tf bound per 10^6 cells
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
200
PHA
control
Concentration of transferrin (nM)

Figure 5. The influence of SIH and Fe-SIH on ^{3}H-thymidine uptake by resting and PHA stimulated peripheral blood lymphocytes. Resting and PHA stimulated lymphocytes were grown under the indicated experimental conditions for 66 h, then divided into microtiter plates in triplicate samples of 300,000 cells/ml. One μCi of ^{3}H-thymidine was then added and the incubation allowed to continue for 2-4 h. After incubation, the plates were frozen and later thawed and the cell remnants harvested onto methly cellulose filters which were washed and counted. The data is expressed as counts per minute x 10^{3} per 300,000 cells ± S.D. .


Without PHA
With PHA
CPM ± S.D.
20000
15000
10000
5000
0
C
1
2.5
5
1
2.5
5
µM SIH
µM Fe-SIH

neccessary to provide iron for DNA synthesis.

DISCUSSION

Many cellular reactions, including electron transport and DNA synthesis are iron dependent. Physiologically iron delivery to cells is mediated by the iron carrier transferrin, which delivers iron following its binding to specific cell surface transferrin receptors. In addition, transferrin has been identified as an obligatory growth factor for in vitro culture systems (Barnes and Sato, 1980).

Transferrin receptors are highly expressed on both specialized cell types, such as erythroblasts (Laskey et al., 1986; Sawyer and Krantz, 1986), and on proliferating cells, such as mitogen stimulated lymphocytes (Larrick and Creswell, 1979). Transferrin receptor expression is correlated with the cell proliferative capacity, increasing during exponential growth and decreasing when cells are in the plateau phase (Testa et al., 1986; Musgrove et al., 1986). Theoretically, this increase in transferrin receptor expression in proliferating cells should reflect an increased iron requirement. It has been suggested that this increase in iron uptake may be required for the formation and/or function of the enzyme ribonucleotide reductase, the rate limiting enzyme of DNA synthesis (Thelander et al., 1983; Hedley et al., 1985) the activity of which is strongly correlated to the rate of DNA synthesis, greatly increasing during S phase. This variation in enzyme activity is regulated by the de novo synthesis and breakdown of of one of its two subunits, M_2 which requires iron for its activity. Recently iron has been shown to have a direct effect on the rate of translation of the M_2 subunit (L. Thelander, 1987).

The exact role, however, of transferrin iron and transferrin receptor expression in this process is not clear. Although blocking transferrin

receptors with monoclonal antibodies leads to the arrest of cell growth and the accumulation of the cells in S phase (Trowbridge and Lopez, 1982), conflicting evidence as to the mechanisms of this inhibition has been reported. In previous studies for example, addition of exogenous iron salts was able to reverse growth inhibition of some cells but not others (Trowbrige and Lopez, 1982; Taeltle et al., 1985). This has led to the suggestion that transferrin may have a growth factor like activity, in addition to that of supplying iron. We have addressed this question using a new and more effective iron chelator, salicyaldehyde isonicotinoyl hydrazone (SIH).

We have shown that the inhibition of Raji cell growth by the monoclonal antibody 42/6 could be effectively overcome by supplying iron in the form of Fe-SIH. As described earlier (Laskey et al., 1986) and confirmed in this study (Figure 3), Fe-SIH is an effective iron donor which bypasses the transferrin:transferrin receptor pathway supplying iron in amounts comparable to those seen in the presence of saturating amounts of Fe-transferrin. Similarly, inhibition of MEL cell growth by R17-208 was overcome using Fe-SIH suggesting that in the presence of monoclonal antibody it is iron availablilty which limits proliferation. These results, together with the observation that in serum free medium transferrin can be replaced by a Fe-PIH (Landschulz et al., 1984; Brock et al., 1987), suggest that transferrin supports cell proliferation solely by supplying iron.

It seems likely, therefore, that iron availability limits DNA synthesis in proliferating cells. In experiments with both normal mitogen stimulated lymphocytes and the Raji cell line, removal of iron with either desferrioxamine or SIH, both iron chelators, results in a dramatic decline

in DNA synthesis as measured by ^{3}H-thymidine incorporation. In contrast, SIH and SIH-Fe appeared not to affect DNA synthesis in resting lymphocytes suggesting that iron only becomes limiting once the cells have received the mitogenic signal for division. Neckers and Cossman (1983) have previously shown that the increase in transferrin receptor expression in mitogen stimulated T lymphocytes is dependent on the prior expression of IL-2 receptors which act as a signal to stimulate DNA synthesis. Blocking transferrin receptors with monoclonal antibodies, however, eliminates the mitogenic capacity of Il-2 (Neckers and Cossman, 1983). In their study however, they did not examine the mechanism by which transferrin exerted its effect. This present study suggests that the induction of transferrin receptors in proliferating cells, both normal and malignant, is required to supply iron for DNA synthesis. This effect may occur at the level of ribonucleotide reductase, the rate limiting enzyme of DNA synthesis, since iron is essential for the activity of this enzyme and both iron uptake and the acitivity of this enzyme fluctuate in a parallel fashion during the cell cycle.

CHAPTER 7

GENERAL DISCUSSION

1. MEL CELLS AS A MODEL FOR STUDY

The objective of this research was to investigate changes which occur in iron metabolism during erythroid development and their significance with respect to the program of erythroid differentiation. It was also hoped, in a broader context, to contribute to a better understanding of the role of iron and physiological iron delivery in the regulation of cell proliferation.

Normal erythroid development occurs over a period of 3-5 days, during which time the immature erythroid element matures through a series of recognizable developmental stages - from the earliest stem cell ultimately to the mature erythrocyte. This is an extremely complex process which involves the selective turning on and off of groups of genes and gene products. It is thought to be primarily under the humoral control of erythropoietin (Babior and Stossel, 1984) although other growth factors (such as IL-3) and the stromal environment present in the bone marrow are also extremely important to normal development (Wheaton and Dexter, 1986). Moreover, the signals for proliferation and differentiation must be carefully balanced, and responsive to physiological need if normal erythropoiesis is to be effective. The observation that the phenotypic abnormality in acute leukemias is the inability of early hematopoietic elements to undergo differentiation, underlies the importance of understanding these phenomena. The study of normal erythroid differentiation therefore provides insight into both normal blood physiology and malignancy.

The study of normal erythroid development is difficult, because of the inability to obtain uniform cell populations of erythroid precursors which would be synchronized with respect to the stage of erythroid development.

One erythroid cell which has been extensively studied is the reticulocyte. Reticulocytes can be collected from the peripheral blood of anemic animals and partially purified from other blood elements by centrifugation in dextran gradients. Study of these cells has provided considerable information about heme and globin synthesis, their regulation and co-ordinate expression. However, reticulocytes appear late in erythroid differentiation and, lacking nuclei, cannot be used for studying transcriptional events. To study less mature erythroid cells, cell lines capable of undergoing erythroid differentiation in vitro, such as the human erythroleukemic cell line k562 (Lozzio and Lozzio, 1975) and murine erythroleukemia cells (MEL) (Friend et al., 1971), have been used. Systems such as these offer several distinct advantages: they are easy to maintain and manipulate in culture; they provide a homogeneous population; they can be induced, with chemical agents, to undergo erythroid differentiation as a synchronized population; and it is relatively easy to obtain genetic variants useful for study. In this study the virus transformed murine erythroleukemic cell line was used to study changes in iron metabolism during erythroid differentiation, in particular with respect to the regulation of heme and ferritin synthesis.

To ascertain whether MEL cells were a good model for the study of erythroid iron metabolism, in Chapter 2, the characteristics of dimethylsulfoxide (DMSO) induced erythroid differentiation in MEL cells were studied. The cell line studied was originally derived from MEL cell clone 745, first isolated by Dr. Charlotte Friend (Friend et al., 1971). Before attempting to manipulate the experimental model, it was first necessary to characterize the differentiation pattern of this particular clone under the experimental conditions used in this laboratory. It was

observed that cells which were allowed to grow in medium containing 1.5% DMSO matured from the stage of erythroblast to normoblast. This maturation was characterized by the accumulation of hemoglobin and could be measured by benzidine staining. In accordance with previously reported data, it was observed that following DMSO induction there was an approximate 10-fold increase in the levels of both α and β-globin mRNA (Ross et al., 1972; Boyer et al., 1972). In addition, a 3-fold increase in transferrin receptor expression (Hu et al., 1977; Wilczynska et al., 1980) and a 6 to 10-fold increase in total cellular iron uptake (Glass et al., 1978), presumably mediated by the increase in transferrin receptor number, was observed. The rate of heme synthesis, as measured by ^{59}Fe incorporation, also increased. In 1976 Sassa reported that following DMSO addition to MEL cells, there is a sequential induction of the enzymes of the heme biosynthetic pathway. Later, Grandchamp et al. (1985) showed that the induction of at least two of these enzymes (porphobilinogen deaminase and uroporphyrinogen decarboxylase) is accounted for by a coordinate increase in their respective mRNAs. Together, these observations show that during chemically induced differentiation of MEL cells, a number of genes and gene products are activated to produce the final phenotype which is characterized by the accumulation of large amounts of hemoglobin. More importantly, this pattern of gene activation and expression in many ways parallels those changes seen in normal erythropoiesis.

MEL cells are however, a transformed cell line and changes in iron metabolism have been reported to occur following transformation. Malignant cells, for example, are known to have higher that usual numbers of transferrin receptors and a larger total cellular iron uptake (Huebers

and Finch, 1987). This increase in iron uptake appears to be required to support DNA synthesis in rapidly dividing cells (Mendelsohn et al., 1983; Neckers and Cossman, 1983; Laskey et al., 1987c). Furthermore, malignant cells have been shown to have higher than average levels of ferritin (Drysdale et al., 1977). This may be related to the increase in iron uptake or may serve an as yet undefined function in these cells.

As a result, results obtained from this system must be interpreted cautiously. The basal level of iron metabolism in the uninduced cell may, or may not, be somewhat higher than would be found in the normal pronormoblast (the physiological equivalent). However, chemical induction does result in the regulated and coordinate expression of hemoglobin, and transferrin receptor expression and iron uptake both increase as would be expected, in order to support heme synthesis. Therefore, it is argued that, although the basal level of iron metabolism may be higher than normal, this system is a good model for the study of changes which occur in iron metabolism, gene activity and the regulation of gene expression during erythroid development.

2. IRON METABOLISM AND THE REGULATION OF HEME SYNTHESIS.

It has long been recognized that the biosynthesis of heme in both erythroid and non-erythroid cells is subject to negative feedback, but in erythroid cells there is controversy as to where this occurs. In Chapter 3, the postulate that the regulation of heme synthesis is distinctly different in erythroid and non-erythroid cells was tested. In non-erythroid cells such as hepatocytes and bacteria heme controls the synthesis and activity of ALA synthase (Burnham and Lascelles, 1963; Granick et al., 1975), the first and rate limiting enzyme (Granick and Urata, 1963) in the heme biosynthetic pathway, thereby regulating heme

formation. However, a growing body of evidence suggests that ALA synthase activity does not control heme synthesis in erythroid cells (Ponka and Neuwirt, 1970; Ponka et al., 1973; Woods, 1973; Sassa, 1976; Malik et al., 1979a,b; Sassa, 1980; Ponka and Schulman, 1985a,b). In reticulocytes heme has been shown to control the cellular acquisition of iron from transferrin (Ponka and Neuwirt, 1969; Ponka et al., 1974; Schulman et al., 1974), and recent evidence (Ponka and Shulman, 1985a,b) suggests that, at least in rabbit reticulocytes, the rate of iron uptake from transferrin limits the rate of heme formation. To see if these observations were true of less mature erythroid cells and to see if the regulation of heme synthesis changes during erythroid development, MEL cells were studied, prior to and following differentiation. The results suggest that in undifferentiated MEL cells, which synthesizes little if any hemoglobin, ALA synthase is rate limiting for heme synthesis. However, following DMSO addition and the induction of the heme pathway enzymes, the rate of iron delivery from transferrin rather than ALA synthase activity limits and controls the rate of heme synthesis.

While these data provide a strong argument for this hypothesis, the regulation of heme synthesis in erythroid cells remains an area of ongoing controversy. Earlier studies in MEL cells had shown that there is a sequential induction of the enzymes of the heme biosynthetic pathway and that hemoglobin did not appear until after ferrochelatase induction (Sassa, 1976), suggesting that ferrochelatse is limiting. Furthermore, cell lines which do not induce ferrochelatase do not differentiate and will not synthesize hemoglobin (Sassa et al., 1978). However a recent report of Beaumont et al. (1984) suggests that this argument may not be true, since they show ferrochelatase being induced as early as ALA

synthase during differentiation of MEL cells.

Another approach to testing whether ferrochelatase is rate limiting is to examine the effects of exogenously supplied protoporphyrin on the total amount of cellular heme produced. It is assumed that if the early porphyrin biosynthetic enzymes were limiting the addition of protoporphyrin would bypass these steps and supply sufficient porphyrin for heme biosynthesis. Using such an approach, Fadigan and Dailey (1987) have recently provided evidence, directly in conflict with our earlier data, that ferrochelatase is rate limiting in induced MEL cells. Their data show that supplementation with protoporphyrin did not alter heme biosynthesis until 48 h post induction, suggesting that iron insertion was limiting up to that time, and that 72 h post induction cultures, supplied with varying concentrations of protoporphyrin, had cellular heme levels directly proportional to the amount of protoporphyrin. These results are in direct conflict with our data showing that the addition of ALA to uninduced cells was able to stimulate heme synthesis (as measured by ^{59}Fe incorporation into heme). One criticism of the results of Fadigan and Dailey is that protoporphyrin was supplied in culture over extensive time periods. It is possible that this addition affected the pattern of heme biosynthetic enzyme induction. If their results are valid it should have been shown that in short term incubations, addition of protoporphyrin would stimulate, or fail to stimulate, heme production. By testing induced and uninduced cells in this manner the possibility that protoporphyrin administration in some way affected heme enzyme induction or activity would have been excluded.

3. IRON METABOLISM AND THE REGULATION OF FERRITIN SYNTHESIS.

In order to better understand iron metabolism and the regulation of

intracellular iron distribution in developing erythroid cells, iron distribution and the regulation of ferritin synthesis in differentiating MEL cells were examined. The results, presented in Chapter 4, show that following differentiation there is an increase in total iron uptake and that the majority of this newly incorporated iron is shunted to the heme fraction. Only when iron is supplied in excess of that which is seen with saturating concentrations of Fe-transferrin is the iron then directed to ferritin. This suggests that transferrin receptor expression induction and heme synthesis induction are closely coupled to ensure that iron in excess of that required for heme synthesis is not taken up. Furthermore, when extracellular iron is not limiting, iron stored in ferritin is not used for heme synthesis. These results, together with previous observations that ferritin iron is not used for hemoglobin synthesis in reticulocytes (Ponka et al., 1982), and is not an intermediate in the utilization of iron for heme synthesis in newt erythropoietic cells (Grasso et al., 1984), suggest that ferritin functions primarily as an iron store rather than as an intermediate in the pathway of iron to heme biosynthesis.

In rabbit reticulocytes up to 90% of the incoming iron will be utilized for heme synthesis (Ponka and Schulman, 1985a,b). In differentiatied MEL cells, after one hour of incubation with ^{59}Fe-transferrin, 1/3 of the iron is found in the heme fraction, 1/3 in the ferritin fraction and 1/3 in the stromal fraction. The stromal bound iron most likely represents hemosiderin, which is insoluble, although a certain proportion may also arise from trapped mitochondria and therefore some heme iron may also be present in this fraction. Why the distribution of iron into the heme fraction is so low compared to reticulocytes is uncertain. It is possible that this occurs because these cells are less

mature, and that a similar distribution would be found in normal cells at an equivalent stage of development. This is supported by the observation of Konijn et al. (1979) who studied normal erythroid precursor cells from rabbit bone marrow and showed that undifferentiated cells synthesize 12 times more protein and 20 times more ferritin than reticulocytes; and Ali et al. (1983) who measured ferritin concentrations in human erythroblasts isolated from the bone marrow and found concentrations approximately 2,000 times higher than in mature erythrocytes. A second alternative is that high intracellular ferritin levels and high ferritin iron content, are a function of the transformed phenotype. Mattia et al. (1986) recently provided evidence that in K562 cells, the proportion of iron which goes to ferritin is a function of cellular ferritin content. They loaded K562 cells with ^{59}Fe over a period of hours, and found that after 1 h approximately 30% of the iron was recovered in the ferritin fraction (notably, this result is similar to our results with MEL cells). As the incubation continued, however, iron in the ferritin fraction represented increasing proportions of the total and this in turn was shown to reflect changes in total ferritin content. If this is true in all cell types, and if indeed transformed cells, MEL cells in particular, are expressing higher than normal levels of ferritin (Drysdale et al., 1977) then this may explain the iron distribution disparity between induced MEL cells and reticulocytes. Further studies are needed to clarify these observations. Nevertheless, the observation that following induction there is a shunting of iron to heme and that ferritin iron is not utilized for heme synthesis when extracellular iron sources are present, should be valid.

In most cells ferritin synthesis is regulated by iron. This is also true for MEL cells. Iron administration is able to stimulate ferritin

accumulation in both induced and uninduced cells. Heme will also stimulate ferritin accumulation, although it is difficult to ascertain whether this is an effect of the heme moiety itself or of iron, which may be released from heme.

The regulation of ferritin biosynthesis at the transcriptional level was studied in Chapter 5 and the results showed that following DMSO induction there is an increase in both H and L ferritin mRNA levels. H ferritin levels increase in a geometric fashion accumulating dramatically between 48-96 h. After 96 h in culture in the presence of DMSO H ferritin mRNA levels are approximately 10-fold over uninduced levels. L ferritin mRNA levels also increase but in a biphasic pattern reaching a maximun of approximately 5-fold over uninduced levels. These results are confirmed by a recent report of Beaumont et al, (1987) who provide additional evidence that these increases are due to increases in the rates of H and L ferritin gene transcription .

Surprisingly, though ferritin mRNA levels increase ferritin protein levels do not change following induction. The reason for this discrepancy is not clear but the fact that this observation is reported by two independent groups (Beaumont et al., 1987; Laskey et al., 1987b) suggests that the data are solid. It is possible that since total RNA and total protein synthesis drop during differentiation, the fact that ferritin content remains the same means that relative synthesis has increased. It may also be that higher levels of ferritin mRNA are required to compete successfully with the increasing quantities of α and β globin mRNA which appear following induction. These two explanations are of course not mutally exclusive. Peto (Peto et al., 1986), Beaumont (Beaumont et al., 1987) and this author (unpublished data) have observed

that following differentiation of MEL cells, there is no change in the rate of ^{35}S incorporation into immunoprecipitable ferritin protein. It seems likely therefore that the relative proportion of ferritin to total protein increases following induction because of the ability of increasing levels of ferritin mRNA to compete successfully for diminishing translational machinery. No change in ^{35}S incorporation would be expected under these conditions. If ferritin mRNA levels did not increase then ferritin levels would be expected to fall. One may conclude then that the maintenance of cellular ferritin levels is important during erythroid development.

In attempting to understand the transcriptional regulation of ferritin, the ability of iron to alter ferritin mRNA levels was tested. Cairo et al. (1985) have previously shown that in Hela cells iron administration stimulates both the transcription and translation of ferritin mRNA, suggesting that iron regulation of ferritin synthesis may be more complex than first suggested (Zahringer et al., 1976). However, in MEL cells neither iron administration nor removal alter ferritin mRNA levels, implying that iron regulation of ferritin synthesis occurs at the translational level. Together these results suggest that there are cell specific variations in ferritin regulation.

Heme has been shown to have a number of regulatory functions in erythroid cells and so the ability of added hemin or inhibition of endogenous heme synthesis , to alter ferritin mRNA levels was also examined. Hemin administration resulted in increases in H and L ferritin mRNA levels in both induced and uninduced cells. Since iron had been shown not to alter ferritin mRNA levels in these cells, the possibility that heme was exerting this effect through release of iron was excluded.

Inhibition of heme synthesis in uninduced cells , which synthesize very little heme, had no significant effect, as would be expected. However, inhibition of heme synthesis during differentiation, inhibited mRNA induction. To observe this effect, succinylacetone (SA, the heme synthesis inhibitor) had to be added at the time of subculturing. If SA was added after 72 hours no significant drop in ferritin mRNA was observed. It is concluded therefore, that inhibition of heme accumulation is the critical factor. Interestingly, SA administration also results in an increase in ferritin protein levels in induced cells. This may be explained by the observation that inhibition of endogenous heme synthesis in induced cells which are taking up large amounts of iron, results in the accumulation of intracellular, non-heme iron which would in turn stimulate ferritin synthesis. Further studies need to be done to determine the mechanism of heme action. In light of the data, the most plausible explanation is that heme is involved in the transcriptional activation of H and L ferritin genes during differentiation; however, it may alternatively or additionally, affect ferritin mRNA processing and/or stability.

These studies suggest that free heme plays an important role in the differentiation process. As previously documented, heme acts to ensure the coordinate expression of hemoglobin by stimulating globin mRNA transcription and translation, and by regulating its own synthesis through feedback inhibition. Evidence is provided that, in erythroid cells, this regulation may occur at the level of iron delivery from transferrin rather than ALA synthase. Furthermore, heme may regulate ferritin gene expression during erythroid development.

4. THE ROLE OF TRANSFERRIN IN SUPPORTING CELL PROLIFERATION.

The primary function of transferrin is thought to be the transport of iron. Iron is required by all cells for growth and survival since many cellular reactions, including electron transport and DNA synthesis, are iron dependent. However, the observation that transferrin was an absolute requirement in serum free media (Barnes and Sato, 1980) and that transferrin receptors are highly expressed on proliferating cells led some investigators to suspect that transferrin may have growth related functions independent of its ability to deliver iron. To test this possibility experiments were performed to attempt to segregate these two functions, and their respective effects on proliferation. One such experimental design was to supply iron in the form of low molecular weight chelates while blocking transferrin binding with monoclonal antibodies (mAb) directed against the transferrin receptor. Unfortunately, these experiments gave conflicting results, since in some cell systems iron supplied in such a form was able to support growth while in others it was not, suggesting a requirement for transferrin (Trowbridge and Lopez, 1982; Teatle et al., 1983; May and Cuatrecasas, 1985). However, iron chelates such as these may not supply iron in amounts which would be comparable to transferrin-Fe delivery. In Chapter 6 this question is addressed, taking advantage of a new iron chelator, salicylaldehyde isonicotinoyl hydrazone (SIH) (Ponka et al., 1979). This chelator is very effective in donating iron to a variety of cell types, and can deliver iron independently of transferrin and transferrin receptors and in amounts greater than those seen with saturating concentrations of Fe-transferrin (Laskey et al., 1986). Our data show that iron, supplied in the form of Fe-SIH, is able to overcome the growth inhibition induced by treating cells with blocking

monoclonal antibodies against the transferrin receptor.

The results presented in Chapter 6 suggest that the acquisition of iron by proliferating cells is necessary in order to support DNA synthesis since removal of iron (either by using a chelator, or by blocking transferrin receptors with blocking monoclonal antibodies) inhibits DNA synthesis while addition of iron stimulates DNA synthesis. This is true of both malignant and non-malignant cells which are actively dividing. Indirectly these data support the conclusion that the primary function of ferritin is iron storage, since it appears that even in short term incubations, such as those described here, the iron which is in ferritin (both Raji and lymphocytes, have measurable ferritin levels), is not available to support DNA synthesis. Iron is required for the synthesis and activity of ribonucleotide reductase, which is the rate limiting enzyme of DNA synthesis (Thelander and Reichard, 1979). Recent evidence suggests that newly incorporated iron is required to support the function of this enzyme. This may explain the observation that both ribonucleotide reductase activity and transferrin receptor expression both increase just prior to S phase during cell division. Taken together these results suggest that the only function of transferrin in supporting cell growth is to supply cells with iron. Furthermore, acquisition of iron from extracellular sources appears to be required in order to support DNA synthesis suggesting that ferritin iron is insufficient or unavailable under these conditions.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The following novel findings and observations have been demonstrated in this thesis:

1. The synthetic chelate ferric salicyaldehyde isonicotinoyl hydrazone (Fe-SIH) can deliver iron to induced and uninduced MEL cells without the involvement of transferrin and transferrin receptors and to a much greater extent than from saturating concentrations of transferrin. Iron taken up in this form can be utilized for heme synthesis.

2. Increasing iron delivery above the levels obtained with saturating concentrations of transferrin, using Fe-SIH, stimulated heme synthesis in induced but not uninduced MEL cells. The ability of Fe-SIH to stimulate heme synthesis could be detected as early as 24-48 h following dimethylsulfoxide (DMSO) addition. Fe-SIH also stimulated globin synthesis above levels seen with saturating concentrations of transferrin, in induced cells.

3. Exogenous ALA stimulated heme synthesis, as measured by ^{59}Fe incorporation from transferrin, in uninduced cells but not in induced cells. This implies that ALA synthase is rate limiting for heme synthesis in uninduced cells only. The results of experiments using ^{59}Fe-SIH suggest that ALA synthase becomes rate limiting in induced cells only when the transferrin/transferrin receptor pathway is bypassed to supply cells with non-limiting amounts of iron.

Together, these results support the hypothesis that some step(s) in the pathway of iron from extracellular transferrin to protoporphyrin, rather than ALA synthase, limits and controls the rate of heme and possibly hemoglobin synthesis in erythroid cells.

3. Chase experiments showed that hemoglobin synthesizing MEL cells, incubated in the presence of saturating concentrations of transferrin, do not use ferritin iron for heme synthesis.

4. H and L ferritin subunit mRNA levels both increase during MEL cell differentiation; however, ferritin protein levels remain constant.

5. Iron administration, using Fe-SIH, stimulates ferritin protein accumulation in both induced and uninduced MEL cells, although for the same concentration the response is somewhat greater in uninduced cells. Fe-SIH has no effect on either H or L ferritin subunit mRNA levels.

6. Removal of iron with desferrioxamine results in a decrease in ferritin content in both induced and uninduced MEL cells, but has no effect on either H or L ferritin subunit mRNA levels.

Together, these results indicate that in differentiating MEL cells iron regulates ferritin synthesis at the level of translation.

7. Overnight exposure to exogenous hemin stimulated ferritin protein

accumulation in both induced and uninduced cells. Addition of hemin at the time of subculturing, resulted in an increase in both H and L ferritin mRNA levels in induced and uninduced cells.

8. Short term inhibition of heme synthesis, with succinyl acetone, has no effect on ferritin protein levels in uninduced cells but increases ferritin content in induced cells. When succinyl acetone is added to MEL cells at the time of subculturing and the cells harvested after four days, it partially inhibits the induction of H and L ferritin mRNA levels shown to occur in DMSO treated cells.

Together, these results suggest that heme may regulate ferritin synthesis, and may play a role in the induction of ferritin mRNA which occurs during MEL differentiation.

9. Fe-SIH was shown to effectively deliver iron to Raji cells in the presence of blocking monoclonal antibodies (mAb) to the transferrin receptor.

10. Blocking transferrin receptors with mAbs was shown to inhibit the proliferation of both Raji and MEL cells. This inhibited cell growth was reversed by Fe-SIH. Furthermore, in Raji cells, mAbs were shown to inhibit DNA synthesis, as measured by ^{3}H-thymidine incorporation into DNA, and this effect could be overcome by added Fe-SIH. Fe-SIH also stimulated DNA synthesis in PHA stimulated lymphocytes, whereas removing iron with the chelator SIH inhibited DNA synthesis.

These results suggest that the sole function of transferrin in supporting cell proliferation is to supply iron, and that an incoming source of iron is required to support DNA synthesis in rapidly dividing cells.

APPENDIX

Ferritin radioimmunoassay.

This section describes in detail the development of the ferritin radioimmunoassay (RIA) which was used in these studies. As described in Chapter 4, the assay is a conventional RIA in which an antigen competes with labelled antigen for a limited number of available antibody binding sites. To develop this assay it was neccessary first to isolate and purify mouse ferritin and then to use this antigen to raise antibodies in rabbits.

Ferritin purification.

Ferritin was purified from the livers of iron injected mice. Mice (25g) were injected intraperitoneally with 1mg of iron-dextran on three successive days, left untreated for the next two, and injected once again on the sixth day. The mice were sacrified on the seventh day by decapitation and their livers were removed.

The liver tissue was homogenized in four volumes of 1 mM β-mercaptoethanol (BME). It was then heated quickly (reaching temperature in 1 min) to 70^{o}C, for 10 minutes. The homogenate was then centrifuged at 19,000 x g for 20 minutes and the supernatant collected. While most other proteins coagulate at 70^{o}C, ferritin is stable and will therefore remain in the supernatant fraction.

The remaining protein in the supernatant fraction was precipitated with ammonium sulfate (313 mg/ml in 1 mM BME, stirring for three hours at 4^{o}C), and the solution then centrifuged at 48,000 x g for 20 minutes at 4^{o}C. Following centrifugation the pellet was collected, redissolved in potassium phosphate pH=6.8, 1mM BME and dialysed overnight against the same buffer. This was then centrifuged at 44,000 x g, for 10 minutes at 4^{o}C and the supernatant fraction collected.

Ferritin in the final supernatant was precipitated by centrifugation at 100,000 x g, for 3 h, at 4°C, and the pellet resuspended in 20 mM phosphate, 1mM BME.

Homogeneity after these procedures was assessed by SDS-PAGE performed on a 6-22% gradient gel.

Purified mouse ferritin prepared in this manner was subsequently used for a variety of purposes. It was a) used to raise antibodies in rabbits (described below), b) labelled with ^{125}I, using lactoperoxidase coupled to CNBr activated Sepharose 4B according to the procedure described by David (1972), for use in the RIA and c) it was used as standards in the creation of the standard curve for the RIA.

Antibody Production.

Antibodies to mouse ferritin were raised in rabbits by subcutaneous injections of mouse ferritin (0.5-1.0 mg), mixed 1:1 with Freund's adjuvant. This procedure was repeated weekly for three weeks. After the fourth week animals were bled by cardiac puncture and the serum collected, and frozen. Aliquots of these serum samples were tested by ELISA for their reactivity with the antigen mouse ferritin. The sample with the highest reactivity was selected for use in these studies. To ensure that this reaction is specific for ferritin, cell lysates were incubated with either, rabbit anti-mouse-ferritin antiserum or with normal rabbit serum and the immunoprecipitates anaylsed by SDS-PAGE. The results of these tests show that there is selective precipitation of ferritin when the antiserum is used. The antiserum was not processed further.

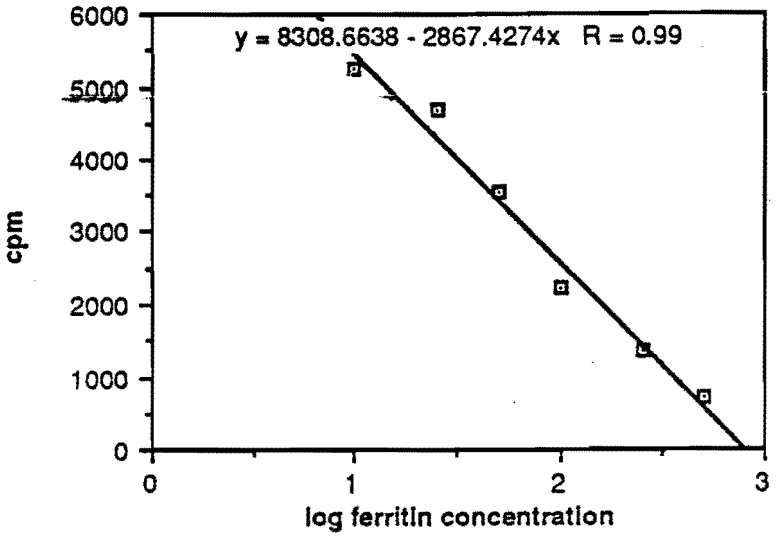
Assay performance.

The ferritin RIA was performed using the purified mouse ferritin as a standard and ^{125}I-mouse-ferritin as the labelled antigen. The standards,

labelled antigen and antiserum were all diluted in buffer A (0.05 M Tris, 0.2 M NaCl, 0.05 M lysine, 0.05 M leucine, 1% BSA and 0.1% Triton X-100, pH 7.2), which is the reaction buffer for the RIA. To use and analyse the assay a standard curve is constructed. Ferritin standards ranging from 0-500 ng, and unknown samples are mixed with a constant amount of ^{125}I ferritin and a constant amount of polyclonal rabbit anti-mouse-ferritin antiserum and incubated for 1 h, at 37^{o}C. After one hour goat anti-rabbit antibody coupled to a solid matrix is added to precipitate the ferritin-antibody complexes. This reaction is allowed to continue for 30 minutes at room temperature, after which the solution is centrifuged and the immune complex precipitated. The radioactivity associated with the pellet is measured and a standard curve constructed. From this curve the concentration of ferritin unknowns can be calculated. Table 1 shows typical data for the RIA standard curve, these data are plotted in Figure 1.

Note: In these experiments the matrix used was either the goat anti-rabbit immunobead matrix supplied by Biorad or the goat anti-rabbit matrix which is supplied with the Ferritin Immunoradiometric Assay Kit (human) supplied by Becton Dickenson. Both were equally effective in precipitating the antigen-antibody complexes and were therefore used interchangeably depending upon availability.

Figure 1. Ferritin RIA, standard curve. Graphic representation of the data presented in Table 1.



The ferritin concentration is usually plotted on the x-axis and is either plotted on semi-log paper or the data are transformed and plotted as the log of the ferritin concentration. The cpm (specific binding) recovered in the pellet are plotted on the y-axis; these data are not transformed. The accuracy of the standard curve was assessed by calculating the coefficient of linear regression(r). One can also calculate the equation of the line which subsequently allows for mathematical derivation of the ferritin concentrations in the unknown samples. A standard curve was routinely run with each experiment, the "r" values were always .85 or better.

REFERENCES

Abramson, S., Miller, R.G. and Phillips, R.A. (1977). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J. Exp. Med. 145:1567-1579.

Ali, F.M., May, A., Jones, B.M., and Jacobs, A. (1983). Enrichment of erythroblasts from human bone marrow using complement-mediated lysis: measurement of ferritin. Brit. J. Haemat. 53:227-235.

Arosio, P., Adelman, T.G., and Drysdale, J.W. (1978). On ferritin heterogeneity. Further evidence for heteroploymers. J. Biol. Chem. 253:4471-4458.

Aviv, H., Voloch, Z., Bastos, R., and Levy, S. (1976). Biosynthesis and stability of globin mRNA in cultured erythroleukemic Friend cells. Cell 8:495-503.

Babior, B.M., and Stossel, T.P. (1984). Hematology: A Pathophysiological Approach., Churchill Livingstone, New York.

Barnes, D., and Sato, G. (1980) Methods for growth of cultured cells in serum free medium. Anal. Biochem. 102:255-270.

Battersby, A.R., Fookes, C.J.R., Matcham, G.W.J., and McDonald E. (1979). Order of assembly of the four pyrrole rings during biosynthesis of the natural porphyrins. J. Chem. Soc. Chem. Commun. 539-541

Baynard, S.H., Stammers, D.K., and Harrison, P.M. (1978). Electron density map of apoferritin at 2.8 A^{o} resolution. Nature 271:282-284.

Beaumont,C., Deybach, J.C., Grandchamp, D., Da Silva, V., de Verneuil, H., and Nordmann, Y. (1984). Effects of succinylacetone on dimethylsulfoxide mediated induction of heme pathway enzymes in mouse Friend virus-transformed erythroleukemia cells. Exp. Cell Res. 154:474-484.

Beaumont, C., Jain, S.K., Bogard, M., Nordmann, Y., and Drysdale, J. (1987). Ferritin synthesis in differentiating Friend erythroleukemia cells. J. Biol. Chem. 262:10619-10623.

Beck, G., Bollock, C., and Beck, J.D. (1974). Synthesis of ferritin in cultured hepatoma cells. FEBS Lett., 47:314-317.

Bomford, A., Colon-Hollongshead, C., and Munro, H.N. (1981). Adaptive reponse of rat tissue isoferritins to iron administration. J. Biol. Chem. 256:948-955.

Borova, J., Ponka, P., and Neuwrit, J. (1973). Study of intracellular iron distribution in rabbit reticulocytes with normal and inhibited heme synthesis. Biochim. Biophys. Acta, 320:143-156.

Bothwell, T.H., Charlton, R.W., Cook, J.D., and Finch, C.A. (1979). Iron Metabolism in Man. Blackwell Scientific Pub., Oxford, U.K. .

Boyd, D., Jain, S., Crampton, J., Barret, K., and Drysdale, J. (1984). Isolation and characterization of a cDNA clone for human ferritin heavy chain. Proc. Natl. Acad. Sci. USA 81:4751-4755.

Boyd, D., Vecoi, C., Belcher, D., Jain, S.K., and Drysdale, J.W. (1985). Structure and functional relationships of human ferritin H and L chains deduced from cDNA clones. J. Biol. Chem. 260:11755-11761.

Boyer, S.H., Wuu, K.D., and Noyes, A.N. (1972). Hemoglobin biosynthesis in murine virus induced leukemia cells in vitro: structure and amounts of globin chains produced. Blood 40:823-835.

Brock, J.H., and Stevenson, J. (1987). Replacement of transferrin in serum-free cultures of mitogen-stimulated lymphocytes by a lipophilic iron chelator. J. Immuno. Lett. 15:23-27

Brown, A., Leibold, E., Munro, H. (1983). Isolation of cDNA clones for the light subunit of rat liver ferritin: Evidence that the light unit is encoded by a multigene family. Proc. Natl. Acad. Sci. 80:1265-1269.

Brown, J.P., Henwick, R.M., Hellstrom, I., Hellstom, K.E., Dodlittle, R.F., and Dreyer, W.J. (1982). Human melanoma-associated antigen p97 is structurally related to transferrin. Nature 296:171-173.

Broxmeyer, H.E., Bognacki, J., Ralph, P., Dorner, M.H. Lu, L., and Castro-Malaspina, H. (1982). Monocyte-macrophage-derived acidic isoferritins: normal feedback regulators of granulocyte-macrophage progenitor cells in vitro. Blood 60:595-607.

Bunn, H.F. and Forget, B.G. (1986). Hemoglobin: Molecular Genetic and Clinical Aspects., W.B. Saunders Pub., Philadelphia, Pennsylvania.

Burnham, B.F., and Lascelles, J. (1963). Control of porphyrin biosynthesis through a negative-feedback mechanism. Biochem. J. 87:462-472.

Burns, G.P., and London, I.M. (1965). Effect of hemin on the synthesis of globin. Biochem. Biophys. Res. Commun. 18:236-242.

Cairo, G., Bardella, L., Schiaffonati, L., Arosio, P., Levi, S., and Bernelli-Zazzera, A. (1985). Multiple mechanisms of iron-induced ferritin synthesis in HeLa cells. Biochem. Biophys. Res. Commun. 133:314-321.

Caskey, J.H., Jones, C., Miller, Y.E., and Seligman, P.A. (1983). Human ferritin gene is assigned to chromosome 19. Proc. Natl. Acad. Sci. USA 80:482-486.

Charlwood, P.A. (1971). Differential sedimentation-velocity and gel filtration measurements on human apo-transferrin and iron-transferrin. Biochem. J. 125:1019-1026.

Chasteen, N.D. (1983). The identification of the probable locus of iron and anion binding in the transferrins. Trends Biochem. Sci. 8:272-295.

Chou, C., Gatti, R.A., Fuller, M.L., Concannon, P., Wong, A., Chada, S., Davis, R.C., and Salser, W.A. (1986). Structure and expression of ferritin genes in a human promyelocytic cell line that differentiates in vitro. Mol. Cell. Biol. 6:566-573.

Concannon, P., Teraoka, N., Nelson, R., and Salser, W.A. (1985). HL-60 cells regulate the expression of transfected genes during terminal cell differentiation. In: Leukemia: recent advances in biology and treatment. Gale, R.P., and Golde, D.W. (ed.), Alan R. Liss Inc., New York, New York, pp. 73-89.

Coppola, J.J., and Cole, M.D. (1986). Constitutive c-myc oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. Nature 320:760-763.

Constanzo, F., Colombo, M., Staempfli, S., Santoro, C., Marone, M., Frank, R., Delius, H., and Cortese, R. (1986). Structure of gene and pseudogenes of human apoferritin H. Nucleic Acids Res. 14:721-736.

Crichton,R.R., and Roman, F. (1978). A novel mechanism for ferritin iron oxidation and deposition. J. Mol. Catal. 7:75-82.

Curry, J.L., and Trentin, J.J. (1967). Hematopoietic spleen colony studies: I. Growth and differentiation. Dev. Biol. 15:395-413.

Curtis, P. (1980). Globin mRNA in Friend cells: its structure, function and synthesis. Biochim. Biophys. Acta 605:347-364.

Dabney, B., and Beadet, A. (1977). Increase in globin chains and globin mRNA in erythroleukemia cells in response to hemin. Arch. Biochem. Biophys. 179:106-112.

David, G.S. (1972). Solid state lactoperoxidase: A highly stable enzyme for simple, gentle iodination of proteins. Biochem. Biophys. Res. Commun. 48:464-471.

Davis, L.G., Dibner, M.D., and Battey, J.F. (1986). Basic Methods in Molecular Biology. Elsevier Sci. Pub., New York, New York.

Dick, J.E., Magli, M.C., Huszar, D., Phillips, R.A., and Bernstein, A. (1985). Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hematopietic system of W/W^{V} mice. Cell 42:71-79.

Dick, J.E., Magli, M.C., Phillips, R.A., and Bernstein, A. (1986). Genetic manipulation of hematopoietic stem cells with retrovirus vectors. Trends. Genet. 2:165-170.

Dickerson, R.E. and Geis, I. (1983). Hemoglobin: Structure, Function, Evolution and Pathology. Benjamin/Cummings Pub., Menlo Park, California.

Dickey, L.F., Sreedharan, S., Theil, E.C., Didsbury, J.R., Wang, Y., and Kaufman, R.E. (1987). Differences in the regulation of messenger RNA for housekeeping and specialized-cell ferritin. J. Biol. Chem. 262:7901-7907.

Dorner, M.H., Silverstone, A.E., DeSosta A., Munn, G., and De Sousa, M. (1983). Relative subunit composition of the ferritin synthesized by selected human lymphomyeloid cell populations. Exp. Hematol. 11:866-872.

Drysdale, J.W., and Munro, H.N. (1966). Regulation of synthesis and turnover of ferritin in rat liver. J. Biol. Chem. 241:3630-3637.

Drysdale, J.W., and Shafritz, D.A. (1975). In vitro stimulation of apoferritin synthesis by iron. Biochim. Biophys. Acta 383:97-105.

Drysdale, J.W., Adelman, T.G., Ariso, P., Casareale, D., Fitzpatrick, P., Hazard, J.T., and Yokota, M. (1977). Human isoferritins in normal and disease states. Semin. Hematol. 14:71-78.

Drysdale, J.W. (1977a). Ferritin phenotypes: Structure and metabolism. In: "Iron Metabolism". Ciba Found. Symp. 51:41-57.

Drysdale, J.W., Jain, S.K., Boyd, D., Barrett, K.J., Vecoli, C., Belcher, D.M., Beaumont, C., Worwood, M., Lebo, R., McGill, J., and Crampton, J. (1985). Human ferritins: Genes and proteins. In: Proteins of Iron Storage and Transport. Spik, G., Montreuil, J., Crichton R.R., and Mazurier, J. (ed.), Elsevier Science Pub., New York, New York, pp. 343-347.

Ebert, P.S., Wars, I., and Buell, D.N. (1976). Erythroid differentiation in cultured Friend leukemia cells treated with metabolic inhibitors. Cancer Res. 36:1809-1813.

Ebert, P.S., Hess, R.A., Frykholm, B.C., and Tschudy, D.P. (1979). Succinylacetone, a potent inhibitor of heme biosynthesis: Effect on cell growth, heme content and δ-aminolevulinic acid dehydrolase activity of malignant murine erythroleukemia cells. Biochem. Biophys. Res. Commun., 88:1382-1390.

Eriksson, S., Graslund, A., Skog, S., Thelander, L., and Tribukait, B. (1984). Cell cycle-dependent regulation of mammalian ribonucleotide reductase. J. Biol. Chem. 259:11695-11700.

Fadigan, A., and Dailey, H.A. (1987). Inhibition of ferrochelatase during differentiation of murine erythroleukemia cells. Biochem. J. 243:419-424.

Fibach, E., Reuben, R.C., Rifkind, R.A., and Marks, P. (1977). Effect of hexamethylene bisacetamide on the commitment to differentiation of murine erythroleukemia cells. Cancer Res. 37:440-444.

Fibach, E., Konijn, A.M., Bauminger, R.E., Ofer, S., and Rachmilewitz, E.A. (1987). Effect of extracellular hemin on hemoglobin and ferritin content of erythroleukemia cells. J. Cell. Phys. 130:460-465.

Finch, C.A., Deubelbeiss, K., Cook, J.D., Eschbach, J.W., Harker, L.A., Funk, D.D., Marsaglia, G., Hillman, R.S., Slichten, S., Adamson, J.W., Ganzoni, A., and Giblelt, E.R. (1970). Ferrokinetics in man. Medicine, 49:17-53.

Friend, C. (1957). Cell-free transmission in adult swiss mice of a disease having the characteristics of a leukemia. J. Exp. Med. 105:307-318.

Friend, C., Patuleia, S., and deHarven, E., (1966). Erythrocytic maturation in vitro of murine (Friend) virus-induced leukemia cells. Natl. Cancer Inst. Monogr. 228:505-520.

Friend, C., Scher, W., Holland, J.G., and Sato, T. (1971). Hemoglobin synthesis in murine-virus induced leukemic cells in vitro: Stimulation of erythroid differentiation by dimethylsulfoxide. Proc. Natl. Acad. Sci. USA 68:378-382.

Frydman, R.B. and Feinstein, G. (1974). Studies on porphobilinogen deaminase and uroporphyrinogen III cosynthase from human erythrocytes. Biochem. Biophys. Acta 350:358-373.

Fuchs, O., Ponka, P., Borova, J., Neuwrit, J., and Travnicek, M. (1981). Effect of heme on globin messenger RNA synthesis in spleen erythroid cells. J. Supramol. Struc. Cell Biochem. 15:73-81.

Galbraith, R.M.P., Werner, P., Arnaud, P., and Galbraith, G.M.P. (1980). Transferrin binding to peripheral blood lymphocytes activated by phytohemagglutinin involves a specific receptor. Clin. Invest. 16:1135-1143.

Ganong, W.F. (1983). review of Medical Physiology. 11th ed., Lange Medical Pub., Los Altos, California.

Glass, J., Nunez, M., Fisher, S., and Robinson, S. (1978). Transferrin receptors, iron transport and ferritin metabolism in Friend erythroleukemia cells. Biochim. Biophys. Acta 542:154-162.

Goldwasser, E. (1975). Erythropoietin and the differentiation of red blood cells. Symposium on Blood Cell Differentiation, Federation Proc. 34:2285-2292.

Gorinsky, B.C., Horsburgh, P.F., Lindley, D.S., Moss, M., and Watson, J.L. (1979). Evidence for the bilobe nature of diferric rabbit plasma transferrin. Nature 281:157-158.

Goubin, G., Goldman, D.S., Luce, J., Neiman, P.E., and Cooper, G.M. (1983). Molecular cloning and nucleotide sequencing of a transforming gene detected by transfection of chicken B-cell lymphoma DNA. Nature 302:114-119.

Grandchamp, B., Beaumont, C., de Verneuil, H., and Normand, Y. (1985). Accumulation of porphobilinogen deaminase, uroporphyrinogen decarboxylase, and alpha and beta globin mRNAs during differentiation of mouse erythroleukemic cells. J. Biol. Chem. 260:9630-9635.

Granick, S. (1943) Ferritin. Occurance and immunological properties. J. Biol. Chem. 149:157-167.

Granick, S., and Urata, G. (1963). Increase in activity of δ-aminolevulinic acid synthetase in liver mitochondia induced by feeding of 3,5-dicarbethoxy-1-4-dihydrocollidine. J. Biol. Chem. 238:821-827.

Granick, S., Sinclair, P., Sassa, S., and Frieninger, G. (1975). Effects by heme, insulin, and albumin on heme and protein synthesis in chick embryo liver cells cultured in a chemically defined medium, and a spectrofluorometric assay for porphyrin composition. J. Biol. Chem. 250:9215-9225.

Granick, S., and Sassa, S. (1978). Hemin control of heme biosynthesis in mouse Friend virus-transformed erythroleukemia cells in culture. J. Biol. Chem. 253:5402-5406.

Grasso, J., Hillis, T., and Mooney-Frank, J. (1984). Ferritin is not a required intermediate for iron utilization in heme synthesis. Biochim. Biophys. Acta 797:247-255.

Green, R., Charlton, R., Seftel. H., Bothwell, T., Mayet, F., Finch, C.A., and Layrisse, M. (1968). Body iron excretion in man. Am. J. Med. 45:336-353.

Greenman, J., and Jacobs, A. (1975). The effect of iron stores on iron absorption in the rat. Gut 16:613-616.

Gross, M., and Rabinovitz, M. (1972). Control of globin synthesis in cell-free preparations of reticulocytes by formation of a translational repressor that is inactivated by hemin. Proc. Natl. Acad. Sci. USA 69:1565-1568.

Gusella, J., Geller, R., Clarke, B., Weeks, V., and Houseman, D. (1976). Commitment to erythroid differentiation by Friend erythroleukemia cells: a stochastic analysis. Cell 9:221-229.

Gusella, J.F., Wells, S., and Tsiftsoglou, A.S. (1980). Hemin does not cause commitment of murine erythroleukemia cells to terminal differentiation. Blood 56:481-487.

Halliwell, B., and Gutteridge, J.M.C. (1984). Oxygen toxicity, oxygen radical, transition metals and disease. Biochem. J. 219:1-14.

Hamilton, T.A., Wada, H.G., and Sussman, H.H. (1979). Identification of transferrin receptors on the surface of human cultured cells. Proc. Natl. Acad. Sci. USA 76:6406-6410.

Hamilton, T.A. (1983). Receptor-mediated endocytosis and exocytosis of transferrin in concanavalin A stimulated rat lymphoblasts. J. Cell. Phys. 114:222-228.

Harrison, P.M., Clegg, A.A., and May, K. (1980). Ferritin structure and function. In: Iron in Biochemistry and Medicine II. Jacobs, A., and Worwood, M., (ed.), Academic Press, New York, New York. pp 131-171.

Harrison, P.M., Artymiuk, P.J., Ford, G.C., Lawson, D.M., Smith, J.M.A., White, J.L., and Trelly, A. (1987). The structure and function of ferritin. In: Proc. Eight Int. Conf. on Proteins of Iron Transport and Storage, May 10-14, 1987, Montebello, Quebec, pp. 6.

Hazard, J.T., and Drysdale, J.W. (1975). Ferritinaemia in cancer. Nature 265:755-756.

Hedley, D., Rugg, C., Musgrove, E., and Taylor, I. (1985). Modulation of transferrin receptor expression by inhibitors of nucleic acid synthesis. J. Cell. Phys. 124:61-66.

Hersko, C. (1977). Storage iron regulation. Prog. Haemtol. 10:105-148.

Higuchi, M., and Bogorad, L. (1975). The purification and properties of uroporphyrinogen I synthase and uroporphyrinogen III cosynthase. Interactions between enzymes. Ann. N.Y. Acad. Sci. 244:401-418.

Hoffman, L.M., and Ross, J. (1980). The role of heme in the maturation of erythroblasts. The effects of inhibition of pyridoxal metabolism. Blood 55:762-771.

Hu, H.Y., Gardner, J., Aisen, P., and Skoultchi, A.I. (1977). Inducibility of transferrin receptors of Friend erythroleukemia cells. Science 197:559-561.

Huebers, H.A., and Finch, C.A. (1987). The physiology of transferrin and transferrin receptors. Phys. Rev. 67:520-582.

Ibraham, N.G., Friedland, M.L., and Levere, R.D. (1983). Heme metabolism in erythroid and hepatic cells. In: Progress in Hematology, Vol XIII, E.B. Brown, (ed.), Grune and Stratton, New York, pp. 75-130.

Jagus, R., Anderson, .F., and Safer, B. (1981). The regulation of initiation of mammalian protein synthesis. In: Progress in Nucleic Acid Research and Molecular Biology, Vol. 25, W.E. Cohn, (ed.), Academic Press, New York, pp. 128-185.

Jandl, J.H., and Katz, J.H. (1963). The plasma-to-cell cycle of transferrin. J. Clin. Invest. 42:314-326.

Jarritt, P.H. (1976). Effect of iron on sedimentation-velocity and gel filtration behavior of transferrins from several vertebrates. Biochim. Biophys. Acta 453:332-343.

Kappas, A., Sassa, S., and Anderson, K.E. (1983). The porphyrias. In: The metabolic basis of inherited disease. 5th ed., Stanbury, J.B., Wyngaarden, J.B.W., Freidrickson, D.S., Goldstein, J.C., and Brown, M.S., (ed.), McGraw-Hill, New York, New York.

Klausner, R.D., Casey, J.L., Hentze, M.W., Di Jeso, B., Rouault, T.A., Caughman, A., Dancis, A., and Harford, J.B. (1987). Molecular aspects of the regulation of the human cellular iron metabolism. In: Proc. Eighth Int. Conf. on the Proteins of Iron Storage and Transport. May 10-14, 1987, Montebello, Quebec, pp. 108.

Kohgo, Y., Yokota, M., and Drysdale, J.W. (1980). Differential turnover of rat liver isoferritins. J. Biol. Chem. 255:5195-5200.

Konijn. A., Hersko, C., and Izak, G. (1979). Ferritin synthesis and iron uptake in developing erythroid cells. Amer. J. Hematol. 6:373-379.

Krantz, S.B., and Jacobson, L.O. (1970). Erythropoietin and the regulation of erythropoiesis. University of Chicago Press, Chicago.

Kuhn, L.C., McClelland, A., and Ruddle, F. (1984). Gene transfer, expression and molecular cloning of the human transferrin receptor gene. Cell 37:95-103.

Landschulz, W., Theslell, I., and Ekblom, P. (1984). A lipophilic iron chelator can replace transferrin as a stimulator of cell proliferation and differentiation. J. Biol. Chem. 98:596-601.

Larrick, J.W., and Creswell, P. (1979). Transferrin receptors on human B and T lymphoblastoid cell lines. Biochim. Biophys. Acta 583:483-490.

Laskey, J.D., Ponka, P., and Schulman, H.M. (1986). Control of heme synthesis during Friend cell differentiation: Role of iron and transferrin. J. Cell. Phys. 129:185-192.

Lee, J.C.K., Lee, S.S.C., Schlesinger, K.J., and Richter, G.W. (1975). Production of ferritin by rat hepatoma cells in vitro: demonstration of protein subunits and ferritin by immunoflorescence. Am. J. Pathol. 80:235-243.

Lehninger, A.L. (1975). Biochemistry, 2nd ed., Worth Pub., New York, New York.

Leibold, E., Aziz, N., Brown, A., and Munro H. (1984). Conservation in rat liver of light and heavy subunit sequences of mammalian ferritin. J. Biol. Chem. 259:4327-4334.

Lestas, A.N. (1976). The effect of pH upon human transferrin: selective labelling of the two iron-binding sites. Brit. J. Haemt. 32:341-350.

Levine, E.Y. (1968). Uroporphyrinogen III cosynthase in bovine erythropoietic porphyria. Science 161:907-909.

London, I.M., Burns, G.P., and Karibian, D. (1964). The regulation of hemoglobin synthesis and pathogenesis of some hypochromic anemias. Medicine 43:789-802.

Louache, F., Pelosi, E., Titieux, M., Pelschle, C., and Testa, U. (1985). Molecular mechanisms regulating the synthesis of transferrin receptors and ferritin in human erythroleukemic cell lines. FEBS Lett. 183:223-227

Lowenhaupt, K., and Lingrel, J.B. (1979). Synthesis and turnover of globin mRNA in murine erythroleukemia cells induced with hemin. Proc. Natl. Acad. Sci. 76:5173-5177.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-275.

Lozzio, C.B., and Lozzio, B.B., (1975). Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood 45:321-334.

Macara, I.G., Hoy, T.G., and Harrison, P.M. (1972). The formation of ferritin from apoferritin: kinetics and mechanism of iron uptake. Biochem. J. 126:151-162.

Macara, I.G., Hoy, T.G., and Harrison, P.M. (1973). The formation of ferritin from apoferritin: catalytic action of apoferritin. Biochem. J. 135:343-348.

MacGillivary, R.T.A., Mendez, E., and Brew, K. (1977). Structure and evolution of serum transferrin. In: Proteins of Iron Metabolism. Brown, E.B., Aisen, P., Feilding, J., and Crichton, R.R. (ed.), Grune and Straton, New York, New York. pp. 133-141.

Mager, D., and Bernstein, A. (1979). The role of heme in the regulation of the late program of Friend cell erythroid differentiation. J. Cell. Phys. 100:467-480.

Malik, Z., and Djaldetti, M. (1979). δ-Aminolevulinic acid stimulation of porphyrin and hemoglobin synthesis by uninduced Friend erythroleukemia cells. Cell Differ. 8:223-233.

Malik, Z., Bessler, H., and Djaldetti, M. (1979a). The role of hemin in the regulation of heme synthesis by fetal mouse liver erythroblasts in culture. Exp. Hematol. 7:183-188.

Malik, Z., Halbrecht, I., and Djaldetti, M. (1979b). Regulation of hemoglobin synthesis, iron metabolism, and maturation of Friend erythroleukemia cells by δ-aminolevulinic acid and hemin. Differentiation 13:71-79.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Sring Harbor, New York.

Mann, K.G., Fish, W.W., Cox, A.C., and Tanford, C. (1970). Single chain nature of human serum transferrin. Biochemistry 9:1348-1354.

Marks, P., and Rifkind, R. (1978). Erythroleukemic differentiation. Ann. Rev. Biochem. 47:419-448.

Marks, P.A., Sheffery, M., and Rifkind, R.A. (1987). Induction of transformed cells to terminal differentiation and the modulation of gene expression. Cancer Res. 47:659-666.

Martinez-Medellin, J., and Schulman, H.M. (1972). The kinetics of iron and transferrin incorporation into rabbit erythroid cells and the nature of stromal-bound iron. Biochim. Biophys. Acta 264:272-282.

Mattia, E., Rao, K., Shapiro, D., Sussman, H.H., and Klausner, R.D. (1984). Biosynthetic regulation of the human transferrin receptor by desferrioxamine in K562 cells. J. Biol. Chem. 259:2689-2692.

Mattia, E., Josic, D., Ashwell, G., Klausner, R., and van Reswoude, J. (1986). Regulation of intracellular iron distribution in K562 human erythroleukemia cells. J. Biol. Chem. 261:4587-4593.

May, W.S., and Cuatrecasas, P. (1985). Transferrin receptor: its biological significance. J. Membr. Biol. 88:205-215.

Mazur, A., and Carleton, A. (1963). Relation of ferritin iron to heme synthesis in marrow and reticulocytes. J. Biol. Chem. 238:1817-1824.

Mazurier, J., Metez-Boutique, M., Jolies, J., Spik, G., Montreuil, J., and Jolles, P. (1983). Human lactotransferrin: Molecular, functional, and evolutionary comparisons with human serum transferrin and hen ovotransferrin. Experimentia 39:135-141.

McClelland, A., Kuhn, L.C., and Ruddle, F.H. (1984). The human transferrin receptor gene: genomic organization and the complete primary structure of the receptor deduced from a cDNA sequence. Cell 39:267-274.

Mendelsohn, J., Trowbridge, I., and Castagnola, J. (1983). Inhibition of human lymphocyte proliferation by monoclonal antibody to transferrin receptor. Blood 62:821-826.

Meinkoth, J., and Wahl, G. (1984). Hybridization of nucleic acids immobilized on solid supports. Analyt. Biochem. 138:267-284.

Mintz, B., Anthony, K., and Litwin, S. (1984). Monoclonal derivation of mouse myeloid and lymphoid lineages from totipotent hematopoietic stem cells experimentally engrafted in fetal hosts. Proc. Natl. Acad. Sci. USA 81:7835-7839.

Morgan, E.H. (1981). Transferrin biochemistry, physiology and clinical significance. Mol. Aspects Med. 4:1-123.

Munro, H., and Linder, M. (1978). Ferritin: Structure, biosynthesis and role in iron metabolism. Physiol. Rev. 58:317-396.

Munro, H.N., Leibold, E.A., Vass, J.K., Aziz, N., Rogers, J., Murray, M., and White, K. (1985). Ferritin gene structure and expresssion. In: Proteins of Iron Storage and Transport. Spik, G., Montreuil, J., Crichton, R.R., and Mazurier, J., (ed.), Elsevier Sci. Pub., New York, New York, pp. 331-341.

Munro, H.N., Leibold, E., Aziz, N., Murray, M., White, K., and Rogers, J. (1987). The ferritin gene family: Expression and regulation. In: Proc. Eighth Int. Conf. on the Proteins of Iron Transport and Storage, May 10-14, Montebello, Quebec, pp. 52.

Musgrove, E., Rugg, C., Taylor, I., and Hedley, D. (1984). Transferrin receptor expression during exponential and plateau phase growth of human tumor cells in culture. J. Cell. Phys. 118:6-12.

Neckers, L.M., and Cossman, J. (1983). Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc. Natl. Acad. Sci. USA 80:3494-3498.

Neilands, J.B. (1981). Microbial iron compounds. Ann. Rev. Biochem. 50:715-731.

Neuwrit, J., Ponka, P., and Borova, J. (1969). The role of heme in the regulation of δ-aminolevulinic acid and heme synthesis in rabbit reticulocytes. Eur. J. Biochem., 9:36-41.

Newman, R., Schneider, C., Sutherland, R., Vodinelich, L., and Greaves, M. (1982). The transferrin receptor. Trends Biochem. Sci. 7:397-400.

Ofer, S., Fibach, E., Kessel, M., Bauminger, E.R., Cohen, S.G., Eikelboom, J., and Rachmilewitz, E.A. (1981). Iron incorporation into ferritin and hemoglobin during differentiation of murine erythroleukemia cells. Blood 58:255-262.

Orkin, S.H., Haros, F.I., and Leder, P. (1975). Differentiation in erythroleukemic cells and their somatic hybrids. Proc. Natl. Acad. Sci. USA 72:98-102.

Orkin, S.H., and Swerdlow, P.S. (1977). Globin RNA synthesis in vitro by isolated erythroleukemic cell nuclei: direct evidence for increased transcription during erythroid differentiation. Proc. Natl. Acad. Sci. 74:2475-2479.

Park, I., Schaeffer, E., Alessandro, S., Baralle, F.E., and Cohen, G.N. (1985). Organization of the human transferrin gene: direct evidence that it originated by gene duplication. Proc. Natl. Acad. Sci. USA 82:3149-3153.

Parker, D., and Housman, D. (1985). Regulation of protein synthesis and accumulation during murine erythroleukemia cell differentiation. J. Biol. Chem. 260:604-609.

Paterniti, J.R., Jr., and Beattie, D.S. (1979). δ-Aminolevulinic acid synthetase from rat liver mitochondiria: Purification and properties. J. Biol. Chem. 254:6112-6118.

Pecoraro, V.L., Harris, W.R., Carrano, C.J., and Raymond, K.N. (1981). Siderophilin metal coordination. Difference ultraviolet spectroscopy of di-, tri- and tetravalent metal ions with ethylenebis ((O-hydroxyphenyl)-glycine)). Biochem. 20:7033-7039.

Peto, T.E.A., Rutherford, T.R., Thompson, J.L. and Weatherall, D.J. (1983). Iron metabolism in murine erythroleukemia cells. Brit. J. Haemat. 54:623-631.

Peto, T.E.A., and Thompson, J.L. (1986). Characterization of ferritin in murine erythroleukemia cells. Biochim. Biophys. Acta 881:79-86.

Ponka, P., and Neuwirt, J. (1969). Regulation of iron entry into reticulocytes. I. Feedback inhibitory effect of heme on iron entry into reticulocytes and heme synthesis. Blood 33:690-707.

Ponka, P., and Neuwirt, J. (1970). The use of reticulocytes with high non-haem iron pool for studies of haem synthesis. Brit. J. Haematol. 19:593-604.

Ponka, P., and Neuwirt, J. (1971). Regulation of iron entry into reticulocytes. II. Relationship between hemoglobin synthesis and entry of iron into reticulocytes. Biochim. Biophys. Acta 230:381-392.

Ponka, P., Neuwirt, J., and Borova, J. (1973). The use of exogenous δ-aminolevulinic acid for the studies of the regulation of haem synthesis in rabbit reticulocytes. Biochim. Biophys. Acta 304:123-131.

Ponka, P., and Neuwirt, J. (1974). Annotation: Haem synthesis and iron uptake by reticulocytes. Brit. J. Haematol. 28:1-5.

Ponka, P., Borova, J., Neuwirt, J., Fuchs, O., and Necas, E. (1979). A study of intracellular iron metabolism using pyridoxal isonicotinoyl hydrazone and other synthetic chelating agents. Biochim. Biophys. Acta 586:278-297.

Ponka, P. and Wilczynska, A. (1981). Iron chelating potential of pyridoxal isonicotinoyl hydrazone and structurally related compounds. In: Development of Iron Celators for Clinical Use. Martell, A.E., Anderson, W., and Badman, D.G.,(ed.), Elsevier Sci. Pub., New York, New York.

Ponka, P., Wilczynska, A., and Schulman, H.M. (1982). Iron utilization in rabbit reticulocytes: A study using succinyl-acetone as an inhibitor of heme synthesis. Biochim. Biophys. Acta 720:96-105.

Ponka, P., and Schulman H.M. (1985a). Regulation of heme synthesis in erythroid cells: Hemin inhibits transferrin iron utilization but not porphyrin synthesis. Blood 65:850-857.

Ponka, P., and Schulman, H.M. (1985b). Acquisition of iron from transferrin regulates heme synthesis. J. Biol. Chem. 260:14717-14721.

Popovic, W.J., and Adamson, J.W. (1979). Erythropoietin assay: present status of methods, pitfalls, and results in polycythemic disorders. CRC Crit. Rev. Clin. Lab. Sci. 10:57-87.

Primosigh, J.V., and Thomas, E.D. (1968). Studies on the partition of iron in bone marrow cells. J. Clin. Inv. 47:1473-1482.

Puro, P.G., and Richter, G.W. (1971). Ferritin synthesis by free and membrane bound (poly) ribosomes of rat liver. Proc. Soc. Exptl. Biol. Med. 138:399-403.

Queensbury, P., and Levitt, L. (1979). Hematopoeitic stem cells II. New Eng. J. Med. 301:819-821.

Ramsay, R.G., Ikeda, K., Rifkind, R.A. and Marks, P.A. (1986). Changes in gene expression associated with induced differentiation of erythroleukemia: proto-oncogenes, globin genes, and cell division. Proc. Natl. Acad. Sci. USA 83:6849-6853.

Rao, K., Mattia, E., Shapiro, D., Bridges, K., and Klausner, R. (1985). Effects of alterations in cellular iron on biosynthesis of the transferrin receptor in K562 cells. Molec. and Cell. Biol. 5:595-600.

Rao, K., Harford, J.B., Rouault, T., McClelland, A., Ruddle, F., and Klausner, R.D. (1986). Transcriptional regulation by iron of the gene for the transferrin receptor. Molec. and Cell. Biol. 6:236-240.

Reeves, W.B., and Haurani, F.I. (1980) Cinical applicability and usefulness of ferritin measurements. Ann. Cli. Lab. Sci. 10:529-535.

Reuben, R., Rifkind, A., and Marks, P. (1980). Chemically induced murine erythroleukemic differentiation. Biochim. Biophys. Acta 605:325-346.

Rice D.W., Ford, G.C., White, J.L., Smith, J.M.A., and Harrison, P.M. (1983). Recent advances in the three dimensional structure of ferritin. In: Structure and Function of Iron Storage and Transport Proteins. Urushizaki, I., Aisen, P., Listowsky, I., and Drysdale, J.W. eds., pp. 11-16.

Rogers, T.B., Gold, R.A., and Feeney, R.E. (1977). Ethoxyformylation and photooxidation of histidines in transferrins. Biochem. 16:2299-2305.

Ross, J., Ikawa, Y., and Leder, P. (1972). Globin messenger-RNA induction during erythroid differentiation of cultured leukemia cells. Proc. Natl. Acad. Sci. USA 69:3620-3623.

Ross, J., and Sautner, D. (1976). Induction of globin mRNA accumulation by hemin in cultured erythroleukemic cells. Cell 8:513-520.

Rouault, T., Rao, K., Harford, J., Mattia, E., and Klausner, R. (1985). Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. J. Biol. Chem. 260:14862-14866.

Rutherford, T., Thompson, G., and Moore, M. (1979). Heme biosynthesis in Friend erythroleukemia cells. Proc. Natl. Acad. Sci. USA 76:833-836.

Santoro, C., Marone, M., Ferrone, M., Costanzo, F., Colombo, M., Manganti, C., Cortese, R., and Silengo, L. (1986). Cloning of the gene for human L apoferritin. Nucl. Acids Res. 14:2863-2876.

Sassa, S. (1976). Sequential induction of heme pathway enzymes during erythroid differentiation of mouse Friend leukemia virus-infected cells. J. Exp. Med. 143:305-315.

Sassa, S.(1980). Control of heme biosynthesis in erythroid cells. In: In Vivo and In Vitro Erythropoiesis: The Friend System, G.B. Rossi, ed. Elsevier/North Holland, Amsterdam, pp. 219-228.

Sawyer, S.T., and Krantz, S.B. (1986). Transferrin receptor number, synthesis, and endocytosis during erythropoietin-induced maturation of Friend virus-infected erythroid cells. J. Biol. Chem. 261:9187-9195.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51:660-672.

Schlacbach, M.R., and Bates, G.W. (1976). The synergistic binding of anions and Fe^{3+} by transferrin. Implications for the interlocking site hypothesis. J. Biol. Chem. 250:2181-2188.

Schneider, C., Kurkinen, M., and Greaves, M. (1983). Isolation of cDNA clones for the human transferrin receptor. EMBO J. 2:2259-2263.

Schulman, H.M., Martinez-Medellin, J., and Sidloi, R. (1974). The reticulocyte-mediated release of iron and bicarbonate from transferrin: Effect of metabolic inhibitors. Biochim. Biophys. Acta 343:529-534.

Schulman, H.M. Wilczynska, A., and Ponka, P. (1983). Intracellular transferrin and transferrin receptors in reticulocytes. In: Stucture and Function of Iron Storage and Transport Proteins. I. Urushizaki, ed. Elsevier, Amsterdam, pp. 305-310.

Schulman, H.M., Wilczynska, A., and Ponka, P. (1981). Transferrin and iron uptake by human lymphoblastoid and K-562 cells. Biochem. Biophys. Res. Commun. 100:1523-1530.

Sherton,C.C., and Kabat, D. (1976). Changes in RNA and protein metabolism preceding the onset of hemoglobin synthesis in cultured Friend leukemia cells. Dev. Biol. 48:118-131.

Siimes, M.A., and Dallman, P.R. (1974). New kinetic role for serum ferritin in iron metabolism. BN4rit. J. Haematol. 28:7-18.

Siminovitch, L., McCulloch, E.A., and Till, J.E. (1963). The distribution of colony forming cells among spleen colonies. J. Cell. Comp. Phys. 62:327-336.

Sirivach, S., Frieden, E., and Osaki, S. (1974). The release of iron from horse spleen ferritin by reducing flavins. Biochem. J. 143:311-315.

Speyer, B., and Feilding, J. (1979). Ferritin as a cytosolic iron transport intermediate in human reticulocytes. Brit. J. Haemat. 42:255-267.

Spiro, T.G., and Saltman, P. (1969). Polynuclear complexes of iron and their biological implications. Struc. and Bonding 6:116-156.

Stamatoyannopoulos, G. et al. (1987). The Molecular Basis of Blood Diseases. Stamatoyannopoulos, G., Nienhuis, A.W., Leder, P., and Majerus, P.W. (ed.), W.B. Saunders Pub., Philadelphia, Pennsylvania.

Taetle, R., Honeysett, J.M. and Trowbridge, I. (1983). Effects of anti-transferrin receptor antibodies on growth of normal and malignant myeloid cell. Int. J. Cancer 32:343-349.

Taetle, R., Rhyner, K., Castagnola, J., to, D., and Mendelsohn, J. (1985). Role of transferrin, Fe, and transferrin receptors in myeloid leukemia cell growth. J. Clin. Inv. 75:1061-1067.

Teale, F.W. (1959). Cleavage of the haem-protein link by acid methylethylketones. Biochim. Biophys. Acta 35:543.

Teraoka, S., Concannon, P., Chou, C., Fuller, M., Chada, S., Wong, A., Wright, J., Kania, K., Snyder, C., Hwa, J., Davis, R., Nelson, R., Thomason, A., Salser, W. (1985). Gene regulation during myeloid differentiation: Characterization of novel ferritin genes and reintroduction of their genomic clones into a cell line that differentiates in vitro. In: Proteins of Iron Storage and Transport. Spik, G., Montreuil, J., Crichton, R.R., and Mazurier, J., (eds.), Elsevier Sci. Pub., New York, New York, pp. 349-356.

Testa, U. (1985) Transferrin receptors: structure and function. Curr. Top. Hematol. 5:127-161.

Testa, E., Testa, U., Samoggia, P., Salvo, G., Camagna, A., and Peschle, C. (1986). Expression of transferrin receptors in human erythroleukemic lines: regulation in the plateau and exponential phase of growth. Cancer Res. 46:5330-5334.

Theil, E. (1987). Ferritin: Stucture, gene regulation, and cellular function in animals, plants and microorganisms. Ann. Rev. Biochem. 56:289-315.

Thelander, L., and Reichard, P. (1979). Reduction of ribonucleotides. Ann. Rev. Biochem. 48:133-158.

Thelander, L., Graslund, A., and Thelander, M. (1983). Continual presence of oxygen and iron required for mammalian ribonucleotide reductase: possible regulation mechanism. Biochem. Biophys. Res. Commun. 110:859-865.

Thelander, L. (1987). Ribonucleotide reductase. In: Proc. of the Eighth Int. Conf. on the Proteins of Iron Transport and Storage. May 10-14, 1987, Montebello, Quebec, pp. 58.

Till, J.E., and McCulloch, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14:213-222.

Worwood, M. (1977). The clinical biochemistry of iron. Seminars in Hematology. 14:3-27.

Worwood, M., Brook, J.D., Cragg, S.J., Hellkuhl, B., Jones, B.M., Perera, P., Roberts, S.H., and Shaw, D.J. (1985). Assignment of human ferritin genes to chromosomes 11 and 19q13.3----19qter. Hum. Genet. 69:371-374.

Yeoh, G.C.T., and Morgan, E.H. (1979). Dimethlysulfoxide induction of transferrin receptors on Friend erythroleukemia cells. Cell. Differ. 8:331-343.

Zahringer, J., Baliga, B.S., and Munro, H.N. (1976). Novel mechanism for translational control in regulation of ferritin synthesis by iron. Proc. Natl. Acad. Sci. USA 73:857-861.

Zail, S.S., Charlton, R.W., Torrance, J.D., and Bothwell, T.H. (1964). Studies on the formation of ferritin in red cell precursors. J. Clin. Inv. 43:670-680.

Zucker, W.V., and Schulman, H.M. (1968). Stimulation of globin-chain initiation by hemin in the reticulocyte cell-free system. Proc. Natl. Acad. Sci. USA 59:582-589.