HSP EXPRESSION DURING ERYTHROID DIFFERENTIATION

By

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NOMENCLATURE

ATP	adenosine triphosphate
BCA	bicinchoninic acid
BFU-E	burst forming units- erythroid
CFU-E	colony forming units-erythrocytes
CFU-GEMM	colony forming units-granulocytes, erythrocyte, macrophages, megakaryocytes
CHAPS	(3-[(3-cholamidopropyl dimethylammonia]-1-propane sulfonate)
DTT	dithiothreitol
EDTA	ethylenediaminetetra acetic acid
eIF-2	eukaryotic initiation factor-2
FK506	immunosuppressant drug
FKBP	FK506 binding proteins
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEPES	(N-[2-hydroxyethyl] piperazine-N'- [2-ethanesulfonic]) acid
HRI	heme-regulated eIF-2α kinase
hsc	heat shock cognate protein
HSE	heat shock element
HSF	heat shock transcription factor
hsp84	mouse equivalent to hsp90α
hsp86	mouse equivalent to hsp90β
hsp	heat shock protein

IEF	isoelectric focusing
IgG	immunoglobulin G
MOI	multiplicity of infection
p	peptide (when used before a number)
pfu	plaque forming units
PPS	post-polio syndrome
PVDF	polyvinylidene difluoride
PVR	polio virus receptor
RBC	red blood cell
RCM-BSA	reduced carboxy-methylated bovine serum albumin
SDS	sodium dodecyl sulfate
SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	[Tris (hydroxymethyl) aminomethane
VSIEF	vertical separating isoelectric focusing

CHAPTER I

INTRODUCTION

Heat Shock Response

The heat shock response is an adaptive physiological response to elevated temperature observed in nearly all cells. The heat shock response is characterized by shutdown of normal protein synthesis at the initiation level and transcriptional activation of the heat shock genes due to the binding of the positively acting heat shock transcription factor (HSF) to an upstream heat shock element (HSE) (Ang, 1991; Sorger, 1991; Craig, 1991; Mosser, 1990). Transcriptional activation is followed by the selective synthesis of heat shock proteins (hsps) (Morimoto, 1990; Nover, 1990; Lindquist, 1988; Lanks, 1986; Schlesinger, 1990; Ang, 1991). Upon accumulation of hsps, stress induced damage is repaired causing transcriptional and translational rates to return gradually to the prestress states (Matts (2), 1992).

Subsequently the heat shock or stress response was observed to be induced in response to a variety of environmental conditions. These conditions have been grouped into three categories including: 1) environmental stress; i.e. heat shock, exposure to toxic heavy metals; adverse nutritional states, such as amino acid starvation and glucose deprivation; 2) pathophysiological state; i.e. viral and bacterial infection, fever, and inflammation; and 3) normal physiological conditions including cell cycle progression, embryonic development, and differentiation (for reviews; Morimoto, 1990; Watowich, 1990; Morimoto,1992; Lank, 1986; Schlesinger, 1990).

Heat Shock Proteins

Heat shock proteins are coded for by multi-gene families that are highly conserved from bacteria to man (Ernst, 1982). Of particular interest for this work are the hsp70 and hsp90 families. These protein families were thought to have their main functional role in the heat shock response repairing damage to cell structures, and protecting cells from damage of subsequent stress. However, it is now known that heat shock proteins are constitutively expressed in cells and have a more generalized housekeeping role (Jaenicke, 1991, Gething, 1992; Ang, 1991). It is now widely accepted that while protein folding is probably determined only by the primary sequence of amino acids, it must be aided *in vivo* by a set of proteins called chaperones, many of which were previously identified as hsps. The primary function of a chaperone is to prevent non-productive interactions between polypeptides that ensures that proteins are folded or transported properly (Ellis, 1989). The responsibilities of the chaperones have been expanded to include protein transport across biological membranes, protein folding and assembly/disassembly, and renaturation of damaged or aggregated proteins (Georgopoulos, 1990; Ang, 1991; Gross, 1990; Morimoto, 1990; Beckwith, 1990; Wickner, 1991).

Hsp90 and hsp70 have recently been shown to function in association with other proteins (cohorts) in a high molecular weight complex referred to as a chaperone machine (Georgopoulos, 1992). Hsp70 binds to unfolded or partially unfolded peptides and releases them following ATP hydrolysis (Gething, 1992; Ang, 1991). The *E. coli* homologue of hsp70, DnaK, has been shown to be responsible for the negative autoregulation of the heat shock response (Craig, 1991). Hsp 90, which also binds polypeptides and has ATPase activity, is one of the most abundant constitutively expressed stress proteins in the eukaryotic cytoplasm. The effect of the hsp90/hsp70 chaperone machine on protein folding has been most well characterized in studies on the activation of steroid hormone receptors (Johnson (2), 1994). Steroid hormone receptors require the action of the hsp90/hsp70 chaperone machine to be folded and maintained in a form

competent to bind hormone (Johnson (2), 1994) The chaperone machine/steroid hormone receptor complex contains a number of hsp cohorts including p60, FKBP59, FKBP54, immunophilin 40, and p23 (Johnson (2), 1994; Smith (2), 1993). The assembly of the complex and release of active steroid hormone receptor complex requires the presence of hsp70 and the hydrolysis of ATP (Smith, 1992). The functions of the hsp cohorts are not yet well characterized. p60 is homologous to the yeast stress protein STII (Honoré, 1992; Smith (2), 1993). p60 aids in protection from temperature stress in yeast, but its function in mammals is not yet known (Nicolet, 1989). The immunophilins, FKBP59, FKBP54, and FKBP52, have peptidyl prolyl isomerase activity (Schreiber,1991; Trandinh, 1992) and appear to be in complexes individually (Johnson (2), 1994), not simultaneously as a group. Associations of the immunophilins with the chaperone machine appear to be accompanied by the dissociation of hsp70 and p60 (Smith, 1995). p23 appears to be the last hsp cohort to be incorporated into the complex in an ATP dependent reaction (Johnson (2), 1994).

Rabbit Reticulocyte Lysate and Heat Shock Response

The rabbit reticulocyte lysate cell-free system has classically been used as a method to study translational regulation of gene expression. Rabbit reticulocyte lysate has currently been adopted as the system of choice to study chaperone function under quasi-physiologic conditions

Conditions that mimic inducers of the heat shock response in the rabbit reticulocyte lysate activate the heme-regulated eIF-2α kinase (HRI). These inducing conditions include: heat stress, adverse nutritional states (i.e. glucose deprivation or excess ethanol exposure), oxidative stress, and heme deficiency (London, 1987; Hunt ,1979; Jackson, 1991; Wu, 1981; Ernst, 1980; Michelson, 1984; Jackson (3), 1983). HRI is an eIF-2α kinase found in erythroid cells present in the bone marrow and peripheral blood (Crosby, 1994. HRI specifically phosphorylates the α-subunit of eukaryotic initiation factor (eIF-2)

which leads to the inhibition of protein synthesis (London, 1985; Hunt, 1979; Jackson, 1991). In the initiation of translation of proteins, the recycling of eIF-2 requires that the eIF-2 bound GDP, formed at the last step of translation intitation, be exchanged for GTP (Jackson, 1991). The binding of eIF-2B is responsible for catalyzing the exchange of GDP bound to eIF-2 for GTP and the subsequent formation of the eIF-2•GTP•Met-tRNA$_i$ ternary complex. If eIF-2α is phosphorylated the eIF-2B is sequestered in an inactive complex with the eIF-2. This sequestering of eIF-2B inhibits the recycling of eIF-2 and the initiation of translation (London, 1985; Jackson, 1991). HRI has been shown to interact with several hsps such as hsp90, hsp70, and the FKBP59 (Matts (2), 1992). Sensitivity of HRI to activation induced by stress is inversely proportional to hsp expression, in particular hsp70 and FKBP59 (Matts (2), 1992). In addition, the ability of hemin to inactivate HRI upon its addition to heme-deficient lysate has been observed to correlate with the amount hsp90 present (Matts (2), 1992). These observations suggest that the interaction of hsps with HRI play some role in the regulation of HRI activation. This notion is supported by the observation that reduced-carboxymethylated bovine serum albumin (RCM-BSA) binds to hsp70 in hemin-supplemented lysate, blocking the interaction of hsp70 with HRI leading to the activation of HRI and inhibition of protein synthesis (Matts, 1993). In addition, the rate of luciferase renaturation in the reticulocyte lysate is proportional to the amount of hsp70 and hsp90 present and addition of RCM-BSA inhibits the rate of this reaction (Schumaker, 1994). These observations have led to the hypothesis that the binding of hsp70 to HRI inhibits its activation. The activation of HRI is proposed to be regulated by a competition between HRI and nascent or denatured proteins for hsp70 (Matts, 1993). Physiological conditions that lead to the accumulation of denatured or unfolded nascent polypeptides, in complexes with hsp70 would lead to the activation of HRI and the cessation of protein synthesis. Such a mechanism would coordinate the rate of protein synthesis with the ability of the cell to properly transport, process, fold and assemble nascent polypeptides or to renature damaged proteins.

The following events may be expected as an animal recovers from anemia. HRI must be inactivated for maximum accumulation of hemoglobin to occur in reticulocytes. As the reticulocyte becomes a mature red blood cell (RBC), the mitochondria are broken down and hsp levels decline as mRNA turns over. As heme and hsps expression decline, HRI is activated and translation is arrested. The translational machinery is no longer functional and is degraded. During this final phase, it would be expected that the levels of HRI would decrease. The decrease of HRI should correlate with the cells' loss of the ability to translate protein since the cell no longer needs to regulate protein translation. Also since hsps are known to be important in the folding of oligomeric complexes and nascent proteins, a decrease in the levels of chaperone proteins might be expected.

However, the expression of a particular hsp may be maintaineed during maturation of the reticulocyte into a mature erythrocyte, as the hsp may function to protect the erythrocyte from oxidative stress and repair damaged proteins, and these cells can no longer make new proteins. The expression of particular molecular chaperones during the recovery process may give insight into the possible function of the protein *in vivo*. Thus, regulation of hsp expression may give some clues into the role of hsps in the differentiation and maturation of cells.

Erythroid Differentiation and Maturation

Hsp expression is well documented during early embryogenesis, during gametogenesis (Mezger, 1991), and during normal avian erythroid differentiation (Banergi 1987). Transcriptional activation of hsp70 during differentiation in cell culture has been reported in hemin-treated K562 cells (Sistonen, 1992). The human hsp70 gene is expressed at the G1/S boundary of the cell cycle, in response to certain growth factors, hormones, and viral infections (Phillips, 1991; Wu, 1985; Milarski, 1986; Ferris, 1988; Ting, 1989). These observations lead to the possibility that regulated hsp expression may be involved in normal erythroid differentiation.

Erythroid cells are a good model in which to study maturation and differentiation of cells. Erythroid cells have the advantages of specializing in producing only one main product which is hemoglobin (95% of final complement of proteins), having distinct phases of maturation [nucleated, dysfunctional nucleus, expelled nucleus, and finally complete breakdown of translational machinery (Rapoport, 1986)]. The erythrocyte along with most of the other blood cell types is derived from the same common pluripotent stem cell. In normal red blood cell (RBC) differentiation the pluripotent hemopoietic stem cells, which may be the CFU-GEMM (colony forming units- granulocytes, erythrocyte, macrophages megakaryocytes) (Johnson, G., 1977; Hara, 1978; Fauser, 1978), are found in the bone marrow. The CFU-GEMM produces a cell that is fated to be a RBC. This cell is called the BFU-E (burst-forming unit- erythrocyte) and will give rise to the CFU-E (colony forming unit-erythrocyte) cells. The mature BFU-E and CFU-E respond to erythropoietin to produce more RBCs when needed (Rapoport, 1986).

Reticulocyte Maturation

The reticulocyte is the cell type found in the last stage of erythroid differentiation. Reticulocytes have well-defined morphological and biochemical characteristics. The main morphological characteristic is the absence of a nucleus. The mitochondria and ribosomes are still functional; therefore, biochemically it is able to synthesize new proteins as long and messenger RNA is present. Maturation time of a normal reticulocyte is about 3 days with 1 day spent in the blood (Rapoport, 1986). Reticulocytes found in the blood have only 90% hemoglobin and are larger than mature RBC (Killmann, 1964). N-acetyl phenylhydrazine attacks heme proteins such as hemoglobin in a complex reaction sequence. Hemoglobin is denatured, forming aggregates of oxidized and denatured products called Heinze bodies (Winterbourne, 1972). After an application of N-acetyl phenylhydrazine, hemolytic destruction of circulating RBC is seen, and a massive influx of reticulocytes is observed 2 days later (Rapoport, 1986). Reticulocytes produced immediately following an induction

of a hemolytic anemia with N-acetyl phenylhydrazine are doubled in size and are referred to as macroreticulocytes (Rapoport, Guest, and Wing, 1944). One hypothesis about the origin of the macroreticulocytes is that they are the result of skipping one developmental stage in the differentiation sequence and are prematurely released from the bone marrow (Erslev, 1962; Krantz, 1963; Borsook, 1968). The fate of these macroreticulocytes has been reported to be rapid elimination from the peripheral blood before they can mature (Stohlmann, 1961, Neuberger, 1961; Wilner-Zehavi, 1966). The densities of the macroreticulocytes, reticulocytes and mature RBC are different and can be separated by density centrifugation. Therefore, differing ages of reticulocyte and erythrocytes can be studied using peripheral blood from anemic rabbits to study recovery from stress and maturation of reticulocytes to erythrocytes.

K562 Cells

Using the K562 cell line, studies into the differentiation of the erythroid cell beginning with the CFU-GEMM cell type are possible (Sutherland, 1986). The K562 cell line was isolated from a patient with chronic myelogenous leukemia (Gahmberg, 1981). The K562 cells are similar to normal erythroid cells in that they produce and accumulate human and fetal hemoglobins (Anderson, 1979; Rutherford, 1979). K562 cells will divide and remain in approximately the CFU-GEMM stage of growth until induced to differentiate. Many compounds such as hemin can be used to initiate the induction of K562 cells (Monette, 1982).

Recently it has been found that the symptoms of polio virus infection may be reactivated in patients at least 15 years after initial paralytic polio-myelitis (Dalakas, 1995). Post-polio syndrome (PPS) refers to the new neuromuscular symptoms seen in patients years after acute polio infection (Dalakas, 1995). The cause of PPS is not known. Some researchers believe that PPS symptoms may be a manifestation of an ongoing motor neuron dysfunction that begins after the initial acute polio infection (Dalakas, 1995). Other

scientists believe that the polio virus lies dormant in blood cell precursors. The triggering of differentiation of certain blood cells may cause the polio virus to replicate and symptoms occur. Evidence supporting this theory includes that the polio virus receptor (PVR) is expressed on human monocytes and that polio virus replication can be supported by primary human blood cells (Freistadt, 1995). The K562 cells line maybe a good candidate for a model in which to study PPS. Polio virus replicates in the induced K562 cell. It is of interest to analyze what cellular component will cause the polio virus to go into the lytic condition in differentiated cells, but produce a persistent nonlytic infection in uninduced cells. Earlier studies have shown that hsp70 mRNA levels increase in K562 cells when induced with hemin. It is of interest to study regulation of hsp expression during erythroid cell differentiation and maturation as they may be intimately involved in the differentiation of RBCs as well as determining the ability of the transcriptionally inactive cell to withstand oxidative stress, and therefore help determine the level of hemoglobin that will accumulate. In collaboration with Rick Lloyd's lab (OUHSC, Dept. of Microbiology), we investigated the level of hsps expression after 2 days of hemin induction in K562 cells in the presence of polio virus infection.

CHAPTER II

MATERIALS AND METHODS

Materials

Histopaque 1119, 1083, 1077 were obtained from Sigma. Alkaline phosphatase conjugated to goat anti-mouse and goat anti-rabbit IgG were purchased from Jackson Immunoresearch Laboratories. CHAPS for IEF gels was purchased from Fluka. Pharmalytes 4.5-5.4 and 5-6 were obtained from Pharmacia. Antibodies were obtained from the following sources and were used at the following dilutions: 4322 anti-hsp90/hsc70 rabbit antiserum from Dr. Stephen J. Ullrich (NCI, NIH) [1:1000, (Erhart , 1988)]; F5 anti-p60 monoclonal mouse IgG [1:1000 (Smith (2), 1993)] and the FF1 anti-FKBP54 mouse monoclonal IgG [1:1000 (Smith (1)., 1993)] from Dr. David Smith (Univ. of Neb. Omaha); mAbF anti-HRI mouse monoclonal IgG from Dr. Jane-Jane Chen (MIT) [1:1000 (Pal, 1991)]; JJ5 anti-p23 mouse monoclonal IgG from Dr. David Toft (Mayo Med. School) [1:1000 (Johnson (2), 1994)]; anti-eIF-2α mouse monoclonal IgG from Dr. Ed Henshaw [1:1000]; EC1 against FKBP54 mouse monoclonal IgG from Dr.Lee Farber (Med. Col. Ohio, Toledo), [1:200, (Tai, 1986)]; and anti-p84 and anti-p86 anti-hsp90 rabbit polyclonal IgG from Affinity Bioreagents.

Methods

Induction of Anemia

A hemolytic anemia was induced in 10 week old rabbits (rabbits #1 and #2) by subcutaneous injections of N-acetyl phenylhydrazine (50 mg/kg) for 5 days as described

(Hunt, 1972). Anemia was induced in rabbit #3 by daily removal of blood (10-50 ml) from the ear vein as follows: day 1, 50 ml; day 2, 42 ml; days 3-6, 15 ml to maintain hematocrit readings at about 15 %; days 7 and 8, 30 ml and 47 ml, due to an increase in hematocrit to 22%. Removal of blood for the study began on day 9 (day 1 of recovery). Samples (10 ml) were taken for study 1-5, 7 and 9 days of recovery. Control blood was removed two weeks after last sample was taken.

Preparation of Cell Samples

Studies on protein expression in blood cells obtained from rabbit #1 were carried out on samples prepared from lysates of heparinized whole blood. Cells from each bleed were washed free of serum and lysed as previously described (Hunt, 1972).

Studies on protein expression in RBCs obtained from rabbits #2 and #3 were carried out on samples prepared after fractionation of heparinized whole blood on density gradients. RBCs from rabbits #2 and #3's were prepared as follows: Heparinized whole blood was centrifuged for 15 min at 800 rpm. Serum was removed by suction, and the cells were resuspended in equal volume of glucose wash buffer (0.3 M glucose, 14 mM DTT, 2 mM EDTA, 150 mM NaCl, 10 mM HEPES pH 8.0). The blood cells were then passed through a cellulose column to remove leukocytes and platelets as described (Beutler, 1976). The RBCs were then fractionated by centrifugation in a quadruple discontinuous density gradient in Beckman polyallomer (25 x 89 mm) tubes. The quadruple discontinuous density gradients (Bhat, 1993) consisted of 5 ml layers of Histopaque (Sigma) of the following densities: the bottom layer, Histopaque (denstiy =1119); second layer [2:1 of Histopaque 1119: Histopaque 1083; (density = 1.107)]; third layer [1:4 of Histopaque 1119: Histopaque 1083 (density = 1.092)]; and final layer Histopaque 1077 (density = 1077). RBCs were layered over the top of the gradient and gradients were centrifuged at 1600 x g for 30 min at 4°C. Cells were removed from the side of the tube with a 21 gauge needle and washed 2 times with glucose buffer. Samples of the cells were

stained with Brilliant Cresyl Blue as described (Jackson, 1983) to determine the proportion of reticulocytes to erythrocyte in each sample (band). Cells were pelleted, glucose wash buffer was removed and cells were stored at -70°C. Samples were prepared for analysis by lysing the cells in approximately 4 times the pellet volume of 10 mM Tris HCl pH 7.4 and centrifuging cells for 10 min at 14,000 rpm to remove cell debris. The supernatant was removed and pellet discarded. Protein concentrations of all samples were determined by BCA protein assay (Sigma). The amount of lysate needed for each type of protein probed was determined by finding concentration of lysate needed for detection of the protein in a linear range by the appropriate antibody. Standard amounts of hsp90, hsc70, p60, and HRI were used to provide for uniformity between blots in order to allow for differences due to transfer efficiency and detection strength of antibodies.

Western Blots

Samples were denatured in SDS sample buffer containing 55 mg/ml DTT, and separated using SDS-PAGE. Proteins were transferred (Polyblot Transfer System Model SBB-1000) from gels to PVDF membrane (Biorad) at a current density of 2.5 mA/cm^2 for western blot analysis. After transfer, blots were blocked with TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 5% skim milk (TBS/5%sm) for 45 min at room temperature. The primary antibodies at the concentrations noted under "Materials" section were reacted overnight at 4°C or 4 hours at room temperature. Blots were then washed once in TBS, twice in TBS containing 0.5% Tween-20, and then blocked in TBS/5%sm. Primary antibodies were detected by blotting with conjugated alkaline phosphatase goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies [1:5000 in TBS /1%sm] at 20°C for 2 hours. The blots were again washed once in TBS, twice in TBS containing 0.5% Tween-20, and twice in TBS. Blots were incubated in alkaline phosphatase reaction at 35°C for 1 to 5 minutes as described previously (Matts, 1992). For detection of

FKBP59 and FKBP54, the p23 blots were reprobed with the ECI and FFI monoclonal antibodies simultaneously.

Quantifying Proteins

Proteins were quantified using a pdi scanning densitometer. All blots for individual proteins were scanned during the same session on the densitometer, with the optical densities (O.D.) being determined at identical settings. Optical density values for hsp90, hsp70, p60, and HRI were then normalized by using the optical densities of standard proteins at quantities that varied linearly with O.D. The optical densities of each blot was divided by the optical density of the standard protein. Determination of O.D. scanning error was done by comparing two different scans of the same blot. The error was determined to be ± 0.40 O.D. The normalized optical densities were graphed using the KaleidaGraph program.

Isoelectric Focusing Gel

Vertical isoelectric focusing was done essentially as described previously (Maurides, 1989) with some minor modifications. Gels were polymerized using 1.5 mm spacers with a Bio-Rad Mini Protean II electrophoresis unit. Samples consisting of 80 µl of cells were prepared as described (Maurides, 1989). Samples were loaded into 13 mm wells and overlaid 40 µl with overlay solution (8 M urea, 1% pharmalyte 4.5 -5-4 and 1% pharmalyte 5-6). Samples were focused at 25 volts per gel for 16 hours. The final focusing phase for was carried out by increasing the voltage to 150 volts per gel 2 hrs. The proteins were transferred to a PVDF membrane by wet transfer in the Genie Electrophoretic Blotter at a constant voltage of 24 V for 2 hours. Transfer buffer contained 25 mM Tris, 190 mM glycine, 0.35 mM SDS, 20% methanol. The blots were then probed as described above with the anti-eIF-2α monoclonal antibodies.

K562 Cells

Samples of K562 cells were provided by Patti Benton (Dept. Microbiology, University of Oklahoma Health Science Centers). The K562 cells were grown in RPMI-1620 media (Irvine Scientific), 10% bovine calf serum (Hyclone), 1% fetal calf serum (Hyclone), 100 units penicillin per ml, and 100 mg streptomycin per ml (Sigma) at 37°C and in 5% CO_2. Cells were grown to cell densities of 4-8 x 10^5 cells/ml. Hemin (Sigma, Equine) prepared as previously described (Dean, 1981) was added to differentiate cells at concentrations of 0 μM, 25 μM, 50 μM, and 75 μM for 2 days. Cells were then infected with Mahoney strain type 1 polio virus that was purified as previously described (Jones, 1983). The multiplicity of infection (MOI) was 50 plaque forming units (pfu) per cell. Infected cell lysates were prepared at 6 hours post infection. Lysates for protein analysis were prepared by lysis of the pellet containing 2.5 x 10^6 cells in 50 μl of IP buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% NP40. Lysate was added to 100 μl of 2X Laemmli buffer (0.125 M Tris-HCl pH 7.4, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue). Lysates for isoelectric focusing were made by lysing the cells in VSIEF buffer (9.5M urea, 5% CHAPS, 50 mM NaF). Pharmalyte 4.5-5.4 and Pharmalyte 5-6 were added to a concentration of 1% before running vertical isoelectric focusing gel.

CHAPTER III

RESULTS

Rabbit Red Blood Cells

The blood samples to determine the expressions of the proteins in maturing reticulocytes were gathered from three different rabbits. Rabbit # 1 was treated with N-acetyl phenylhydrazine and bled for samples for four consecutive days. The white blood cells were removed by packing the cells by centrifugation. Lysates were made after the removal of buffy coat. Rabbit # 2 was also treated with N-acetyl phenylhydrazine. The white cells were removed by passing blood through a cellulose column. The blood was then put on a quadruple discontinuous gradient and the cells separated by density. The difference in density between the cells in the peripheral blood is the most commonly used marker of the age of the cell (Rapoport, 1986). Density of the cells is based on the condition that hemoglobin content of the cells increases during differentiation and the aging process causes loss of water (Rapoport, 1986). Since the reticulocytes condense upon maturation, the cells with the lowest density represent a pool of the most immature cells. Five bands were clearly visible and were removed by needle and syringe. Figure 1 is a diagram of the banding pattern seen in the quadruple gradients. Day 1 of recovery would have more cells in bands 1 and 2 than subsequent days. The control blood did not contain bands 1 or 2.

Rabbit number 3 was bled from the ear for 8 days prior to start of data collection as described in Materials and Methods and illustrated in figure 2. The hematocrit level for the rabbit was determined daily prior to blood withdraw. The cell populations were fractionated as described for rabbit #2. Staining the cells from the gradients with Brilliant

Fractionated Density Gradient

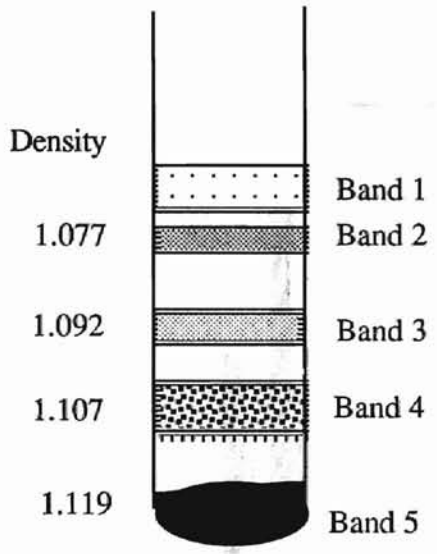

Figure 1. Fractionated density bands of rabbit red blood cells. Cells were separated by using quadruple discontinuous histopaque density gradients. Band 1 represents the most immature red blood cells and band 5 represents the most mature bands. Bands 2-4 represent cells with ages between most immature and mature cells.

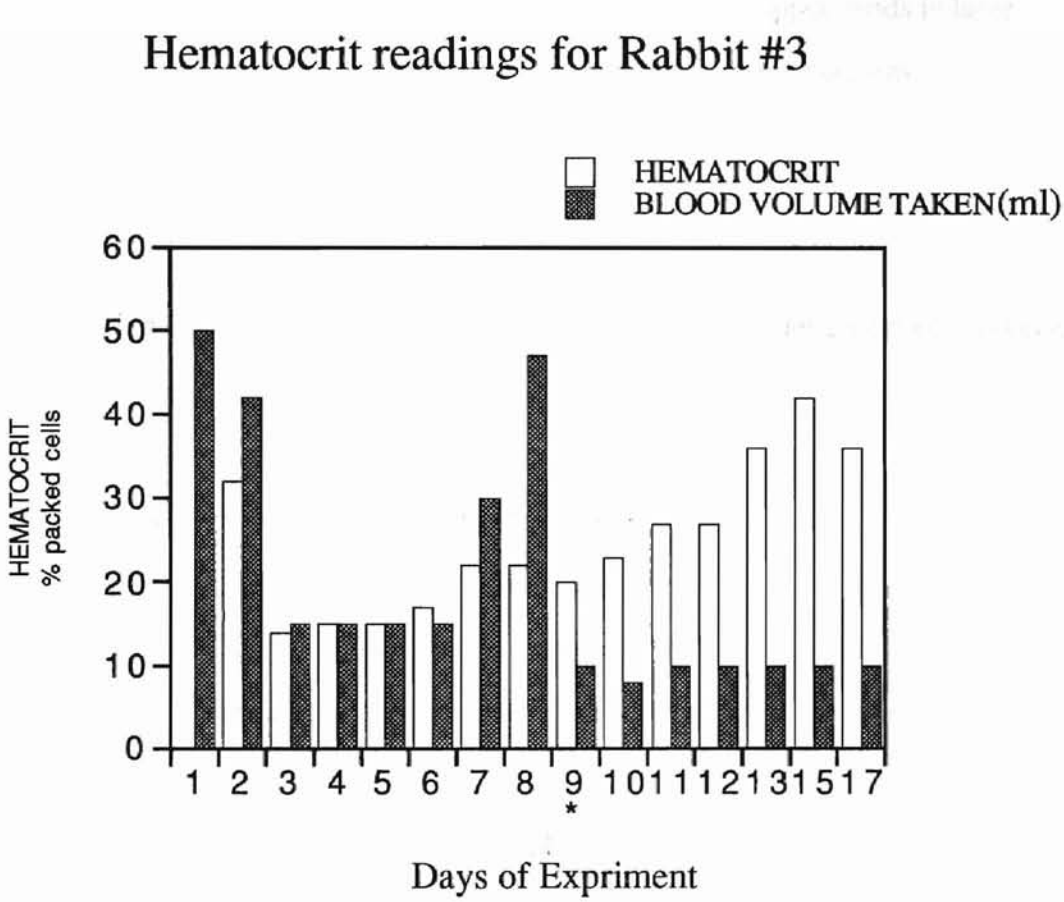

Figure 2. Hematocrit levels and volumes of blood withdrawn from rabbit #3. * indicates day that study on blood samples began.

Cresyl Blue showed that reticulocytes were present in the three upper bands in large quantities. The lower bands had only about 5% reticulocytes which are probably present due to contamination during collection of the fractions.

Expression of HRI

Rabbit #1 was treated with N-acetyl phenylhydrazine. After 2 days of recovery, the rabbit was bled for four consecutive days. The expression of HRI was observed to decrease from day 1 through day 3 followed by a sharp increase on day four. In rabbit #2 (figure 3) this increase was not observed but day four proteins were not examined.

Rabbit #2 was also treated with N-acetyl phenylhydrazine but the cells were fractionated by age on density gradients. Figure 4 shows that the HRI levels decrease with days of recovery and by age (density of band). In neither of the N-acetyl phenylhydrazine treated rabbit was HRI found in blood collect after a 2 week recovery.

In order to compare the N-acetyl phenylhydrazine induced anemia to that of an anemia induced by blood loss, rabbit #3 was bled as described in Materials and Methods to an anemic condition indicated by its lower hematocrit level (less the 20 %, see figure 2). Red blood cells were fractionated by density. The expression of HRI seemed to remain high, finally lowering some 9 days after beginning of the study (figure 5). The expression of HRI by age (density of bands) fluctuated markedly. HRI expression was elevated in more mature cells isolated from very anemic stressed rabbit , while HRI expression was low in mature cells isolated from the rabbit whose hematocrit had returned to near normal. The rabbit was more severely anemic after such a long period of continual bleeding (17 days) compared to the N-acetyl phenylhydrazine treated rabbits, and the recovery period from the bleed-induced anemia was much longer. HRI could still be found in control blood taken 2 weeks after last sample, indicating that the rabbit was still anemic, and that its blood containing a significant proportion of young erythrocytes. The presence of young erythrocytes could be tested by increasing the number of different densities on the

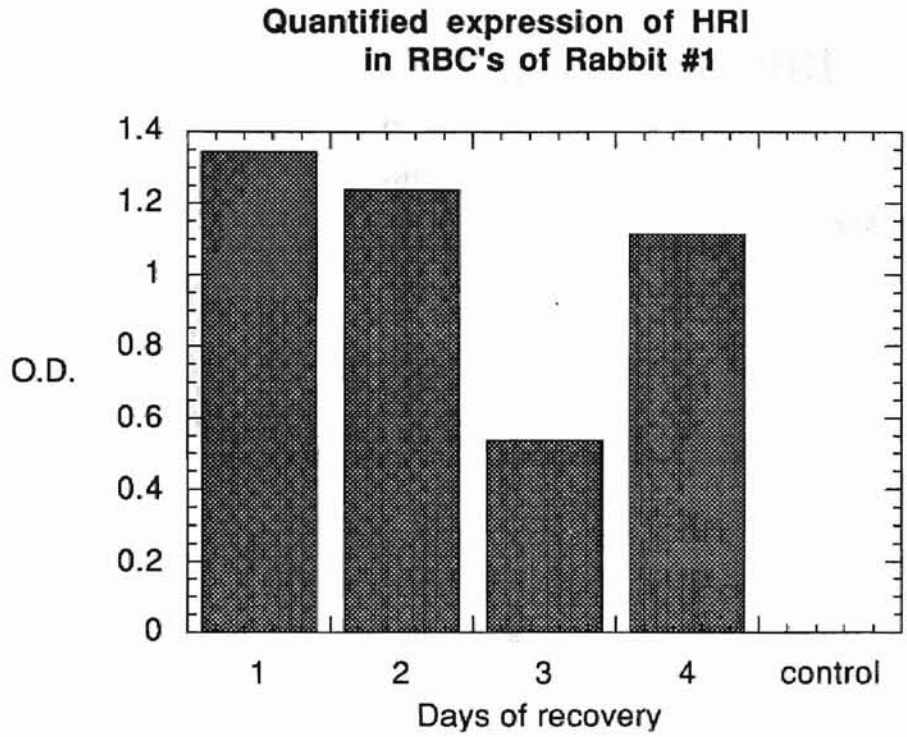

Figure 3. HRI expression in red blood cells of rabbit #1. 30 μg of protein from each sample was used to probe for HRI. Cellular proteins were separated by SDS/PAGE and transferred to PVDF membrane. Western blots were probed with anti mAbF monoclonal antibody, and optical density (O.D.) determined by scanning densitometry.

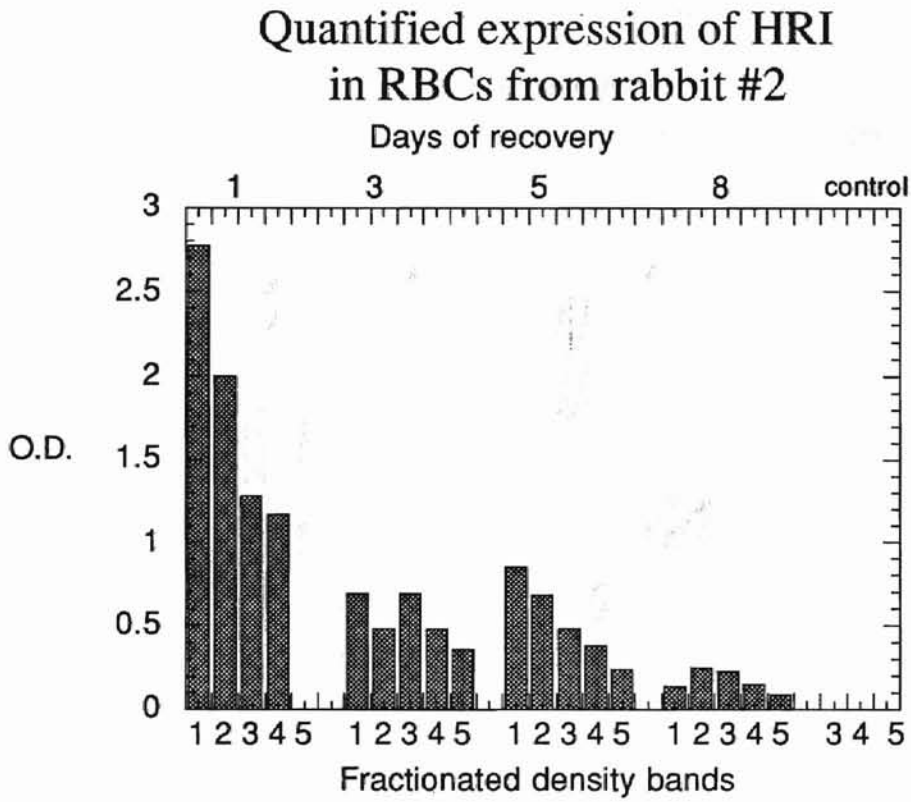

Figure 4 HRI expression in red blood cells from rabbit #2 during recovery from phenylhydrazine-HCl treatment. Fractionated density bands 1-5 on each day represents the band number lowest density to highest during the fractionation of the blood on histopaque discontinuous gradient (see materials and methods). 30 μg of protein from each sample was used to probe for HRI. Cellular proteins were separated by SDS/PAGE and transferred to PVDF membrane. Western blots were probed with anti mAbF monoclonal antibody, and optical density (O.D.) determined by scanning densitometry.

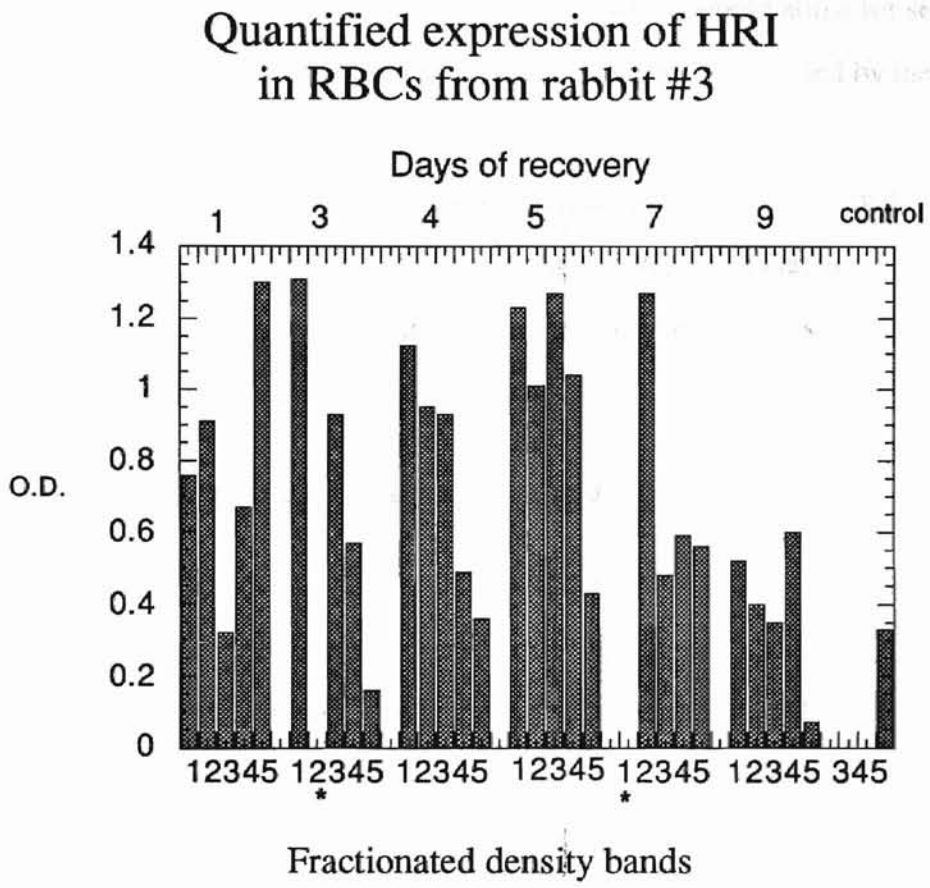

Figure 5. HRI expression quantified from red blood cells fractionated into bands 1-5 from rabbit #3. Day one represents the ninth day that blood was extracted from the rabbit. 30 μg of protein from each sample was used to probe for HRI. Cellular proteins were separated by SDS/PAGE and transferred to PVDF membrane. Western blots were probed with anti mAbF monoclonal antibody, and optical density (O.D.) determined by scanning densitometry.

discontinuous gradient between 1.107 and 1.119, which would allow for separation of erythrocytes by age. It should also be noted that iron from cells lysed by the N-acetyl phenylhydrazine treatment is retained in the animal and reutilized. The bleeding induced anemia, the iron from the withdrawn RBCs is lost. As such these animals suffer from an iron-deficiency that prolongs their recovery period. We noted that this rabbit did not eat or drink normally after 10 days of bleeding. An iron supplement was given only once early during the bleeding period.

Effects of anemia on expression of hsp in erythroid cells

The reticulocytes in the whole blood of an anemic rabbit express heat shock proteins. These heat shock proteins may serve as protection against oxidative stress brought about by the anemia. Also immature reticulocytes are still translating large quantities of proteins. The heat shock proteins would be required to chaperone nascent protein folding and maintain protein function in enucleated red blood cells during recovery from anemia.

Hsp90 is constitutively expressed in large amounts in the eukaryotic cytoplasm (Lindquist, 1988). Hsp90 is thought to act as a general chaperone, and has been demonstrated to be required for renaturation of denatured luciferase (Schumacher, 1994). Hsp90 proteins are essential for viability (Lindquist, 1988), and for the recovery from stress, presumably due to its requirement as a chaperone in the proper folding of certain cellular proteins. Therefore, we expected that the levels of hsp90 family proteins would increase during the induction of anemia. As the reticulocyte matures it loses its ability to make new proteins as the ribosomal machinery is destroyed. It was of interest then whether hsp90 proteins might be necessary for maintaining some critical RBC function or whether hsp90 would disappear. Thus, studying the levels of hsp90 during recovery from anemia and maturation of the reticulocyte into RBC may help in determining the role of hsp90 in the mature RBC as well as during the recovery from anemia.

Figure 6 shows the levels of hsp90 in RBC found throughout 4 days of recovery from N-acetyl phenylhydrazine induced anemia in rabbit #1. Rabbit #1 levels of hsp90 showed a 4 fold increase on day 2 of recovery then declined to levels that were not detectable in the control lysate (figure 6). Rabbit #2 was used to investigate the expression of hsp90 with respect to different maturity levels of RBCs during recovery from N-acetyl phenylhydrazine treatment. Figure 7 shows that hsp90 expression was greatly increased by induction of anemia, evidenced by comparisons of hsp90 expression day 1 of recovery to day 8 and control. It was quite evident that levels of hsp90 dramatically decreased during maturation of RBC, both with respect to day of recovery and age of the cell. Comparing hsp90 expression in cells fractionated by density for each day, the decrease in the expression of hsp90 is quite obvious.

Cells from rabbit #3 were used to analyze hsp90 expression in response to anemia induced by blood loss. Figure 8 shows the changes in hsp90 during recovery from bleeding induced anemia. Figure 8 indicates that the hsp90 levels greatly decreased after day 2 of recovery, after which hsp90 expression was maintained at a relatively constant amounts. Figure 8 does indicate that in general the most immature (less dense) cells express a higher amount of hsp90 compared to older (most dense) cells present on a single day. It is important to note the bands 1 and 2 disappear as the rabbit recovers from the anemia, and on days 7 and 9 fractions 4 and 5 of the density gradient contained most of the cells. While the total amount of hsp90 present in the whole blood on a given day was not determined (figure 8), figures 6 and 7 indicate that the expression of hsp90 should become progressively lower in the later days of recovery.

Another constitutively expressed heat shock protein of major importance, is hsc70 (Welch, 1982). Expression of hsc70 in reticulocytes from rabbits treated with N-acetyl-phenylhydrazine was examined with respect to the maturity of the cells determined by their density. The 4322 anti hsp70/90 antisera is able to detect both the constitutive (hsc) and heat inducible (hsp) forms of hsp70. Western blots of samples prepared from

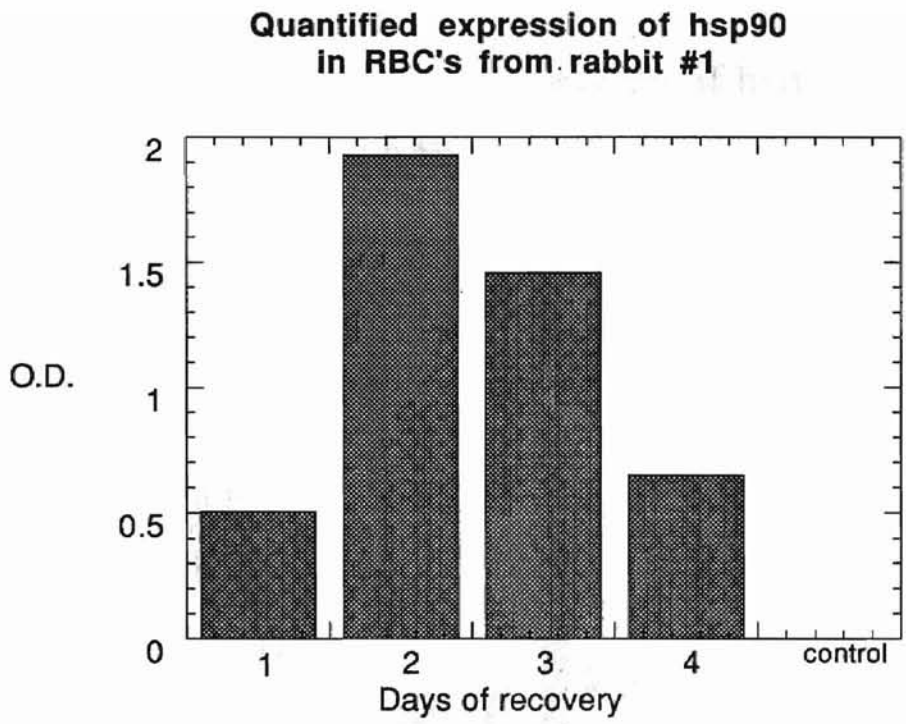

Figure 6. Quantified expression of hsp 90 in red blood cells from rabbit #1. 10 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by 4332 polyclonal rabbit IgG antibody, and optical density (O.D.) was determined by scanning densitometry.

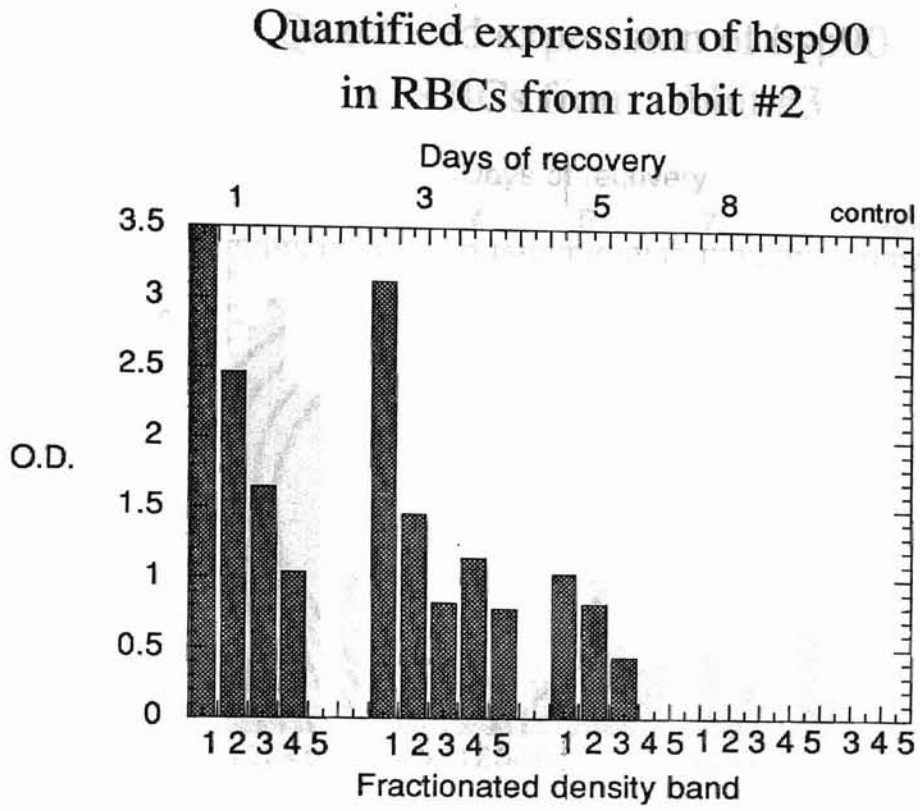

Figure 7. Quantified expression of hsp 90 in red blood cells from rabbit #2. Fractionated density bands 1 to 5 represent the lightest density (most immature cells) to heaviest density (most mature cells). 20 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by F5 antibody (for p60 detection) then probed by 4332 polyclonal rabbit IgG antibody. The optical density (O.D.) was determine by scanning densitometry.

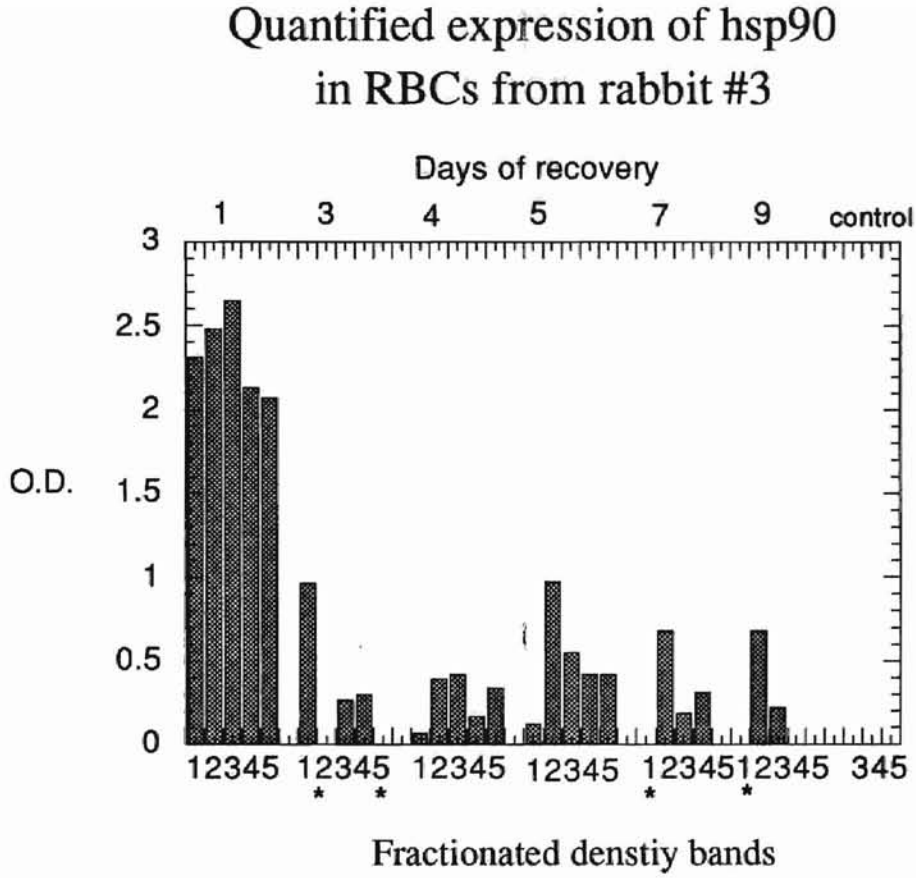

Figure 8. Quantified expression of hsp 90 in red blood cells from rabbit #3. Fractionated density bands 1-5 represent the lightest to heaviest cell bands. * bands indicate the cells for this density were not available. 10 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by 4332 polyclonal rabbit IgG antibody, and optical density (O.D.) was determined by scanning densitometry.

cells isolated from rabbit #1-#3 indicated that hsc70 was present, while hsp70 was not. Figure 9 shows that hsc70 increased at least 4 fold during N-acetyl phenylhydrazine induced anemia. On day 2 a ten fold increase was observed. Interestingly there is a decrease followed by an increase on hsc70 levels on day four which corresponds with the increase of HRI as described previously (figure 9).

Anemia was also induced in rabbit #2 by N-acetyl phenylhydrazine, but cell lysates were examined by maturity level of the cells. Maturity was determined by the density of the cell. Figure 10 shows the trends of expressing hsc70 for a given day of recovery. The peak seemed to go from band 1 (most immature cells) in day 1 to band 5 (most mature cells) in the 8th day. Particularly interesting are levels of hsc70 present in cells isolated on day 5,8 and control. In the most immature cells (lightest density), hsc70 expression was maximal during the early phase in recovery. Subsequently, cells of medium density expressed the highest amounts of hsc70. Late in recovery, the most mature (high density) cells expressed highest amounts of hsc70. This change in the hsc70 expression profile would appear to correspond to the same population of cells as they mature to RBCs. As expected band 3, which is normally found in the blood stream corresponds to the last stage of reticulocyte maturation, has slightly higher levels of hsc70. This profile probably indicates that hsc70 expression decreased with age of the reticulocyte, but also with respect to the degree of anemia (days of recovery) present in the animal.

Blood samples from rabbit #3, which was made anemic by the draining of blood, were also analyzed for changes in hsc70 expression.(figure 11). Hsc70 expression increased until day four of recovery. Hsc70 expression then decreased to control levels day 7 and day 9. Hsc70 was found to be constitutively expressed in the control cells (control, figure 11). In the control blood, the most mature band (band 5) showed the highest levels of hsc70 of the three density bands. This cells population are mature cells form early stages of recovery and it follows that they would have had higher hsc70 levels left when they matured into erythrocytes.

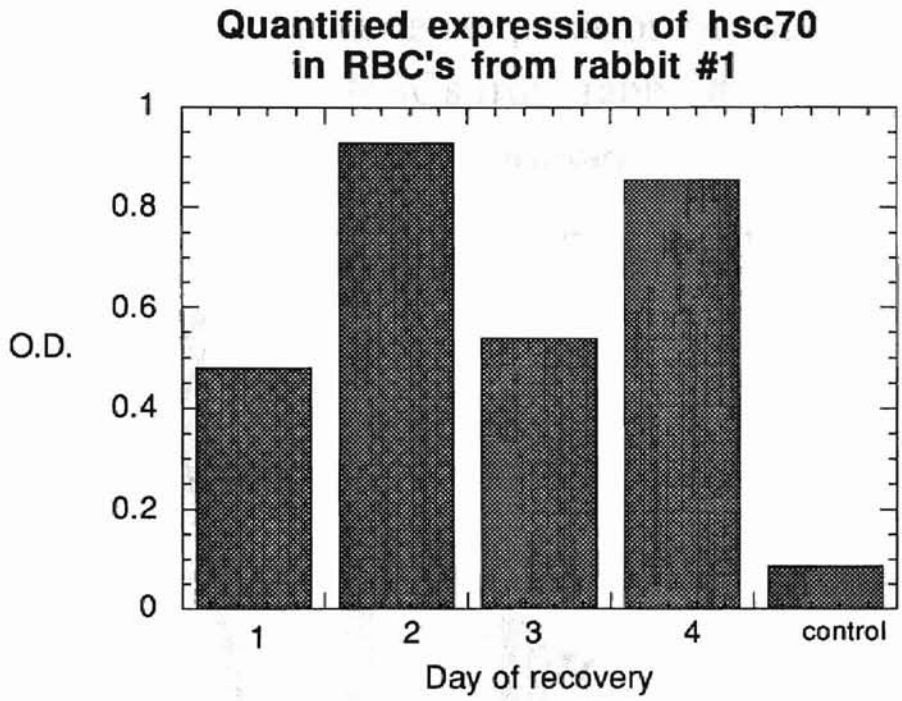

Figure 9. Quantified expression of hsc70 in red blood cells from rabbit #1. Days of recovery indicate time when blood was removed for purpose of studying protein levels. 5 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by 4332 antibody. The optical density (O.D) was determined by scanning densitometry.

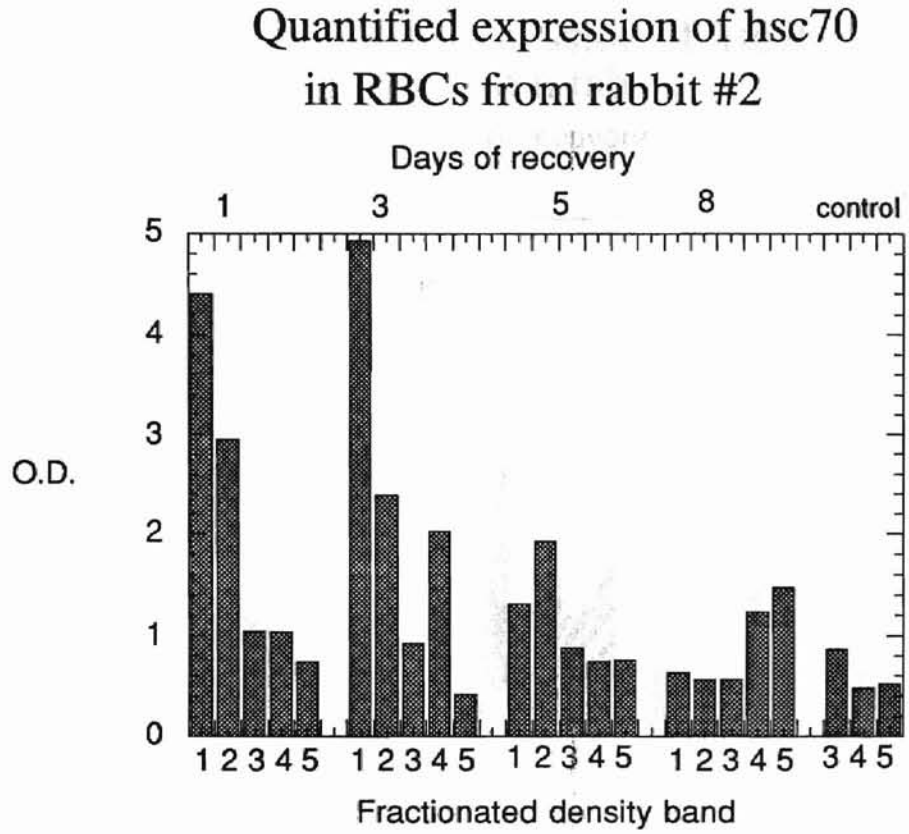

Figure 10. Quantified expression of hsc70 in red blood cells from rabbit #2. Fractionated density bands 1-5 indicate the density of the fractionated whole cells by histopaque lightest to heaviest. 5 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membrane. Western blots were probed by 4332 antibody. The optical density (O.D) was determined by scanning densitometry.

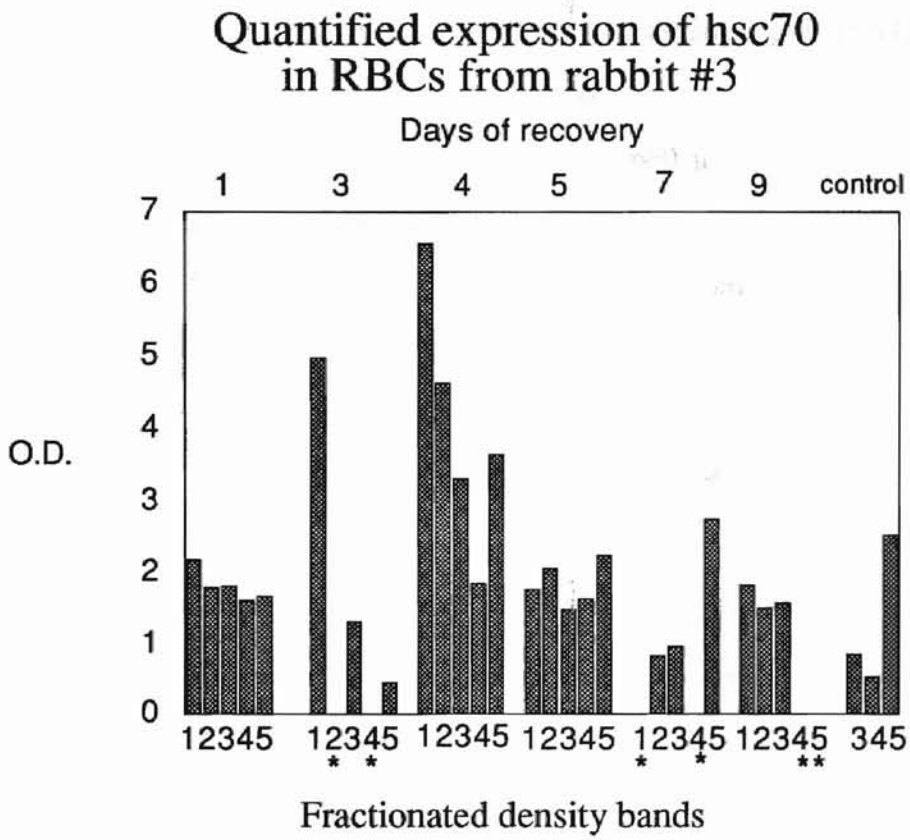

Figure 11. Quantified expression of hsc70 in red blood cells from bleed induced anemic rabbit #3. Days of recovery begin when the blood was drawn for analysis on the 9th day of continual bleeding. 5 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by 4332 antibody. The optical density (O.D) was determined by scanning densitometry.

The expression of hsp90/hsp70 associated cohorts was also examined. P60 is a hsp90/70 associated cohort with homology to the yeast stress protein STII (Honoré, 1992; Smith (2), 1993). In yeast, p60 aids in protection from temperature stress, but its function in mammalian cells is not known (Nicolet, 1989). p60 along with hsp90 and hsp70 have been shown to exist in a chaparone complex (Smith (2), 1993). Figure 12 shows the expression of p60 found in RBC's during the recovery from N-acetyl phenylhydrazine anemic rabbit #1. The trend indicates that p60 expression consistently decreases, and that p60 is found in peripheral blood of non-anemic rabbits.

Rabbit #2 was used to analyze the expression of p60 during maturation from reticulocytes to mature red blood cells in cells fractionated on the basis of density (figure 13). Of interest is the observation that the overall expression of p60 in RBC's increased at day five. The high expression of p60 in immature cells (bands 1 & 2, day 5) results in subsequent high expression of p60 in more mature cells (bands 3-5, day 8). This increase was also observed in cells isolated from rabbit #3 (figure 14).

Expression of p60 in fractionated cells isolated from rabbit #3 levels is shown in figure 14. The expression of p60 was also found to be highest in cells isolated on the 5th day (figure 14). In each experiment (figures 12-14), p60 was found to be expressed in mature red blood cells (erythrocytes). As mentioned previously, the function of p60 is not entirely understood, but our observations suggest it is may be important throughout the red blood cell's lifetime.

Additional hsp90/70 associated cohorts were also studied. These proteins, which include p23, the immunophillins FKBP59 and FKBP54, form a novel chaperone complex with hsp90 and hsp70 in rabbit reticulocyte lysate (Johnson (2), 1994). p23 expression decreases with respect to days of recovery from the anemia (rabbit #1, figure 15) and with age (density) of the cell (rabbit #2, figure 16), similar to observations made for HRI and hsp 90 expression (figures 3 & 6). Similar to hsp90, no p23 was observed to be present in control cells. This trend is not unexpected, since p23 is found in a complex with hsp90.

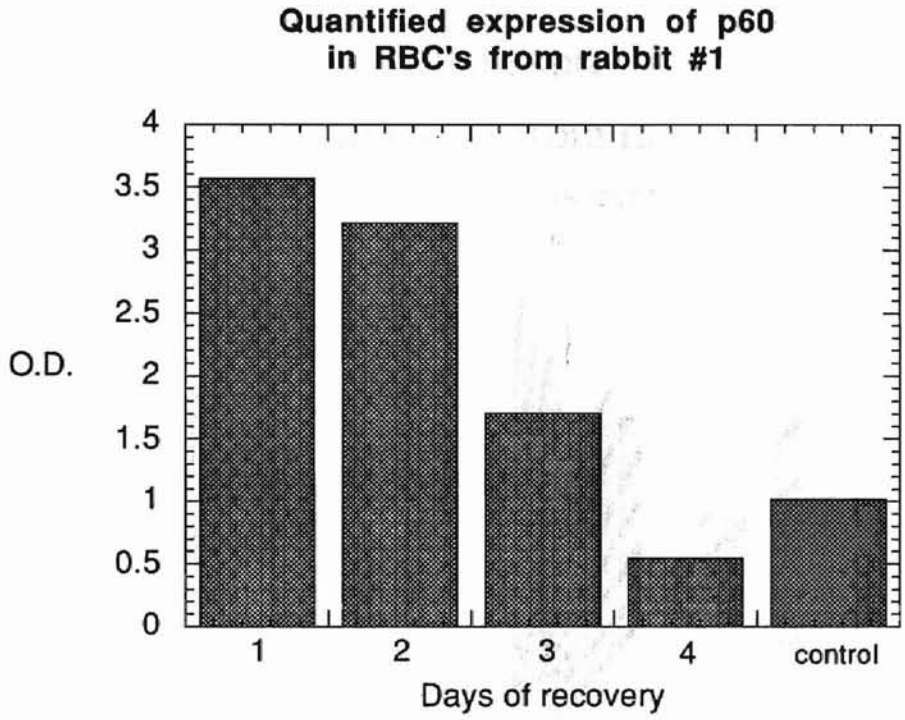

Figure 12 The quantified expression of p60 in red blood cells from N-acetyl phenylhydrazine-HCl induced anemic rabbit #1. 20 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by F5 monoclonal mouse IgG antibody. The optical density (O.D) was determined by scanning densitometry.

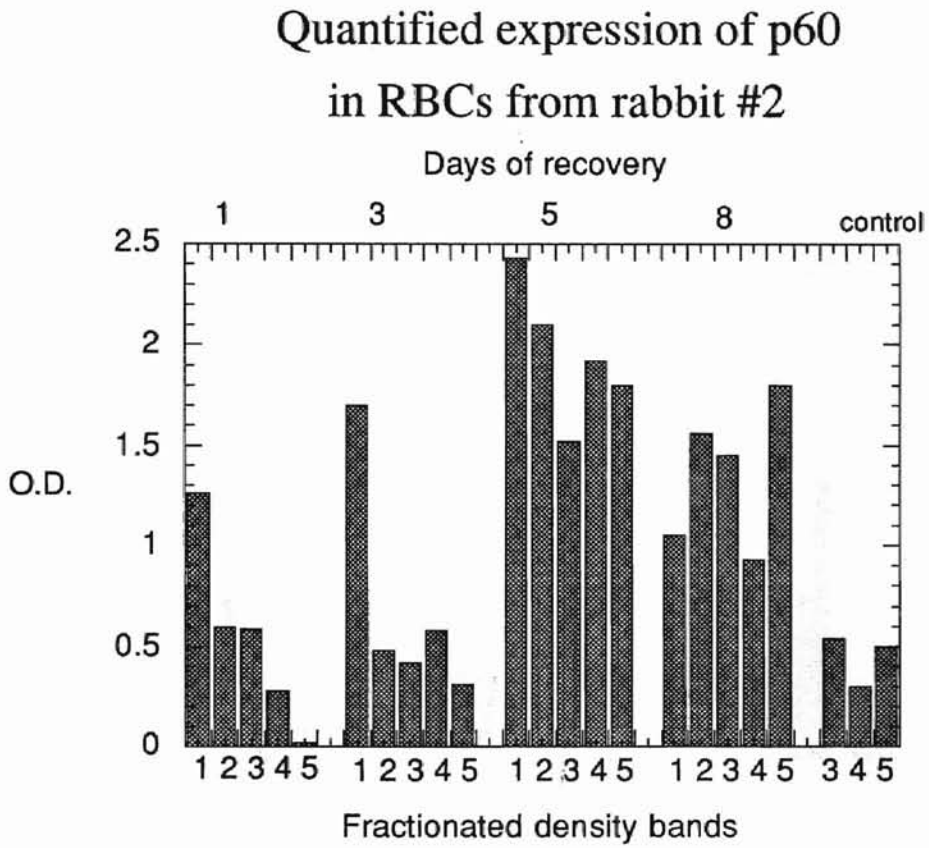

Figure 13 Quantified expression of p60 in red blood cells from rabbit #2. Fractionated density bands 1-5 indicate the density of the fractionated whole cells by histopaque (lightest density to heaviest density). 20 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by F5 monoclonal mouse IgG antibody. The optical density (O.D) was determined by scanning densitometry.

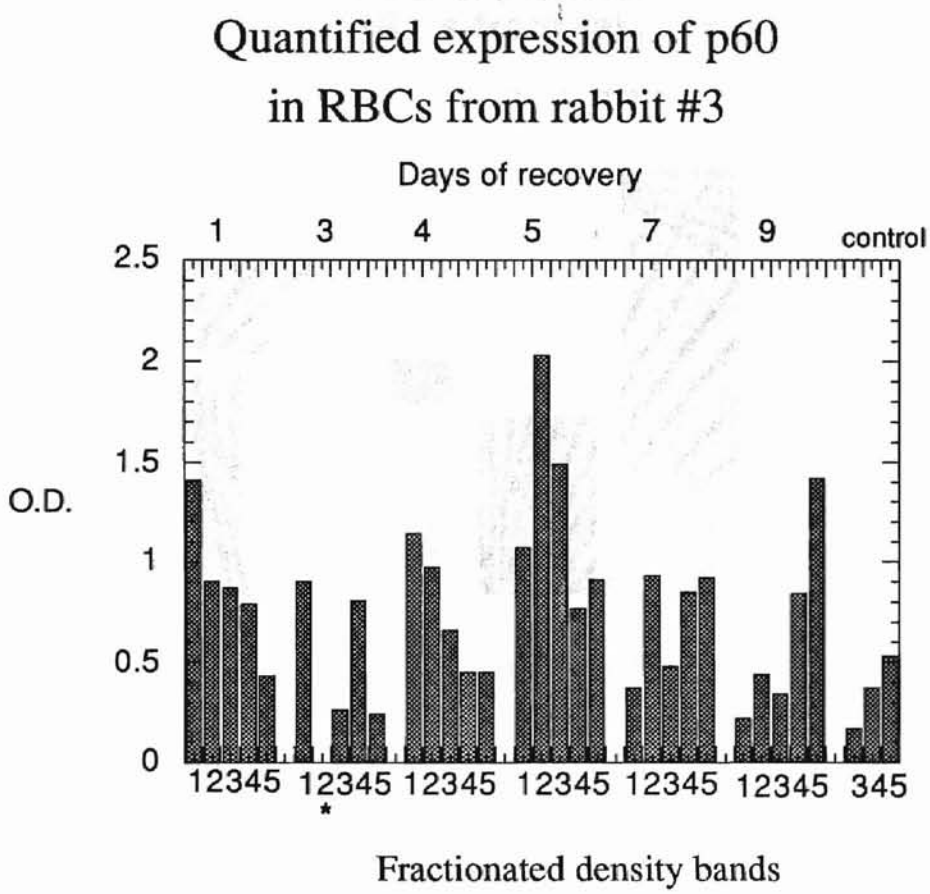

Figure 14. Quantified expression of p60 in red blood cells from the bleed induced anemic rabbit #3. Days of recovery begin when the blood was drawn for analysis on the 9th day of continual bleeding. 20 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by F5 monoclonal mouse IgG antibody. The optical density (O.D) was determined by scanning densitometry.

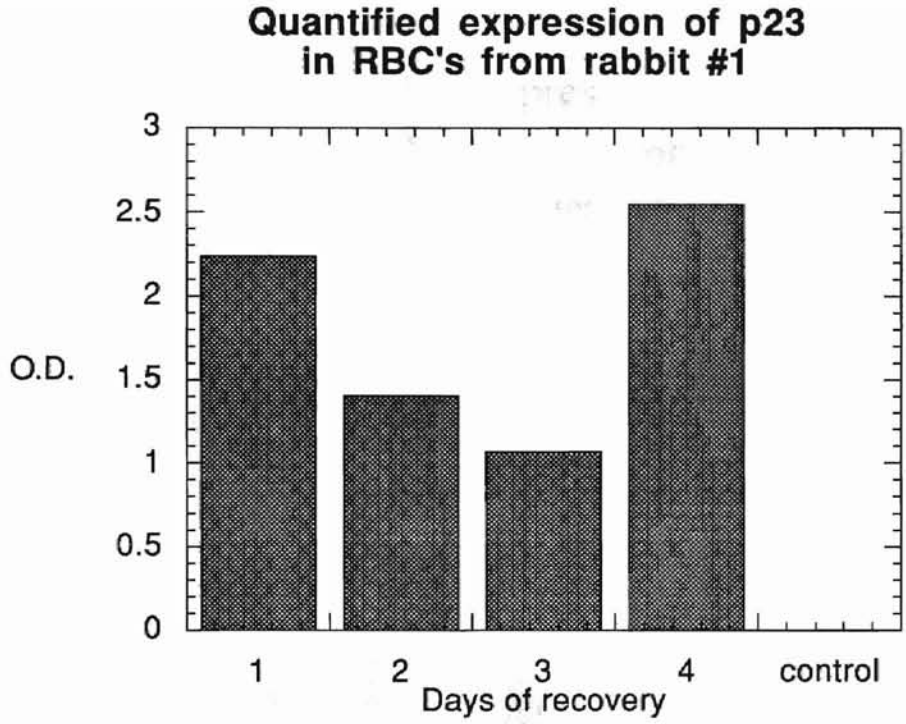

Figure 15. The quantified expression of p23 in red blood cells from rabbit #1. 100 μg protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes Western blots were probed by JJ 5 monoclonal mouse IgG antibody. The optical density (O.D) was determined by scanning densitometry.

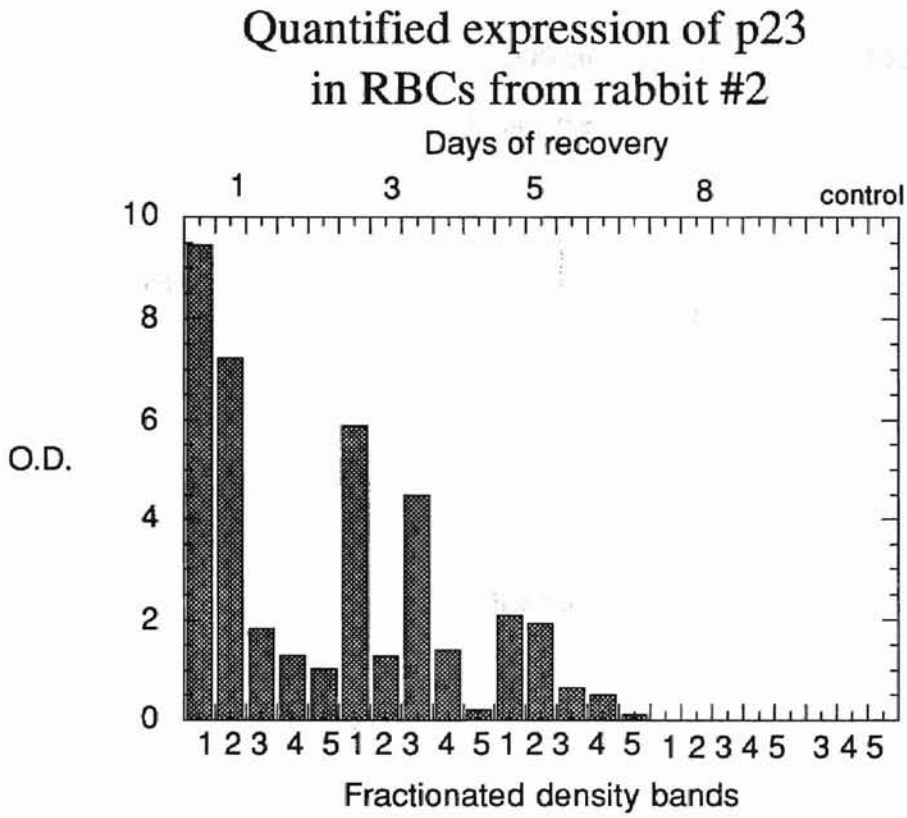

Figure 16. Quantified expression of p23 in red blood cells from rabbit #2. Fractionated density bands 1-5 indicate the density of the fractionated whole cells by histopaque (lightest density to heaviest density). 100 μg protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. Western blots were probed by JJ 5 monoclonal mouse IgG antibody. The optical density (O.D) was determined by scanning densitometry.

The expressions of the immunophilins, FKBP59, and FKBP54 in cells isolated from rabbit #2 and fractionated by density were analyzed (figures 17 and 18 respectively). Similar to the trends observed for HRI, hsp90 and p23, expression of FKBP59 and FKBP54 decreased as cells matured until they were no longer detected in the same concentration of lysate.

Expression of hsps and associated cohorts in differentiating K562 cells: Effects of polio virus infection

K562 cells have been used as a model system to study gene expression in differentiating RBC's. Hemin-induced differentiation of K562 causes transcriptional and activation of the gene encoding hsp70 (Sistonin, 92). Polio virus can mount a lytic infection in differentiating, but not in undifferentiated K562 cells. K562 cells were induced to differentiate by different levels of hemin, and the effect of polio virus infection on the expression of hsp90, hsp70, hsc70, p60, and phosphorylation levels of eIF-2α during early differentiation were examined..

Effects of Differentiation on Expression of Hsps in K562 Cells

Two forms of hsp90, α & β, are constitutively expressed in cells. It may be possible that the increased of one family member is masked by the decreased expression of a different family member when using antibodies such as 4332. 4332 antiserum does not differentiate between the two isoforms, since the antibody's epitopes are found in a conserved region within the hsp90 family. In order to provide a clearer view of the levels of hsp90, antibodies specific for the α and β forms of hsp90, anti-p84 and anti-p86 were used to probe the western blots. Figures 19 & 20 depict the expression of hsp90α and hsp90β found in K562 cells. The high level of hemin (75 μM) appeared to cause an increase in both hsp90α (figure 19), and hsp90β (figure 20). While the lower levels of hemin (25 μM and 50 μM) appears to suppress hsp90α expression (figure 19), only the

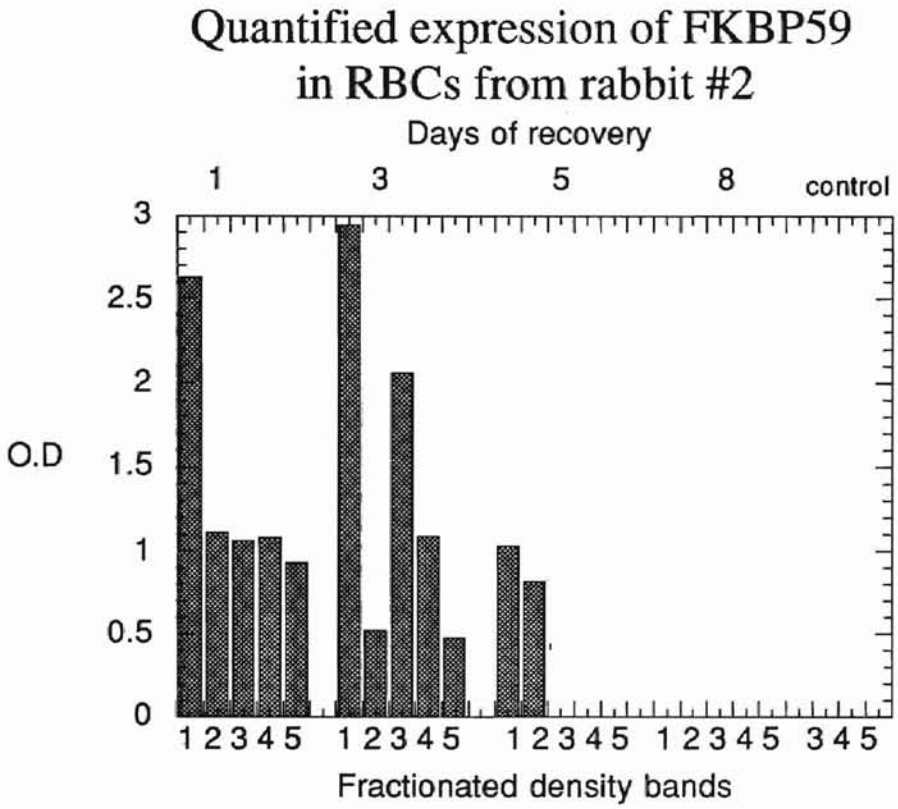

Figure 17. Quantified expression of FKBP59 in red blood cells from rabbit #2. Fractionated density bands 1-5 indicate the density of the fractionated whole cells by histopaque lightest to heaviest. 100 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. After probing for p23 with the JJ 5 antibody, western blots were probed for FKBP59 and FKBP54 with the EC1 polyclonal rabbit antibody and the FF1 polyclonal rabbit IgG antibody simultaneously using the p23 blots.. Optical density (O.D.) was determined by scanning densitometry.

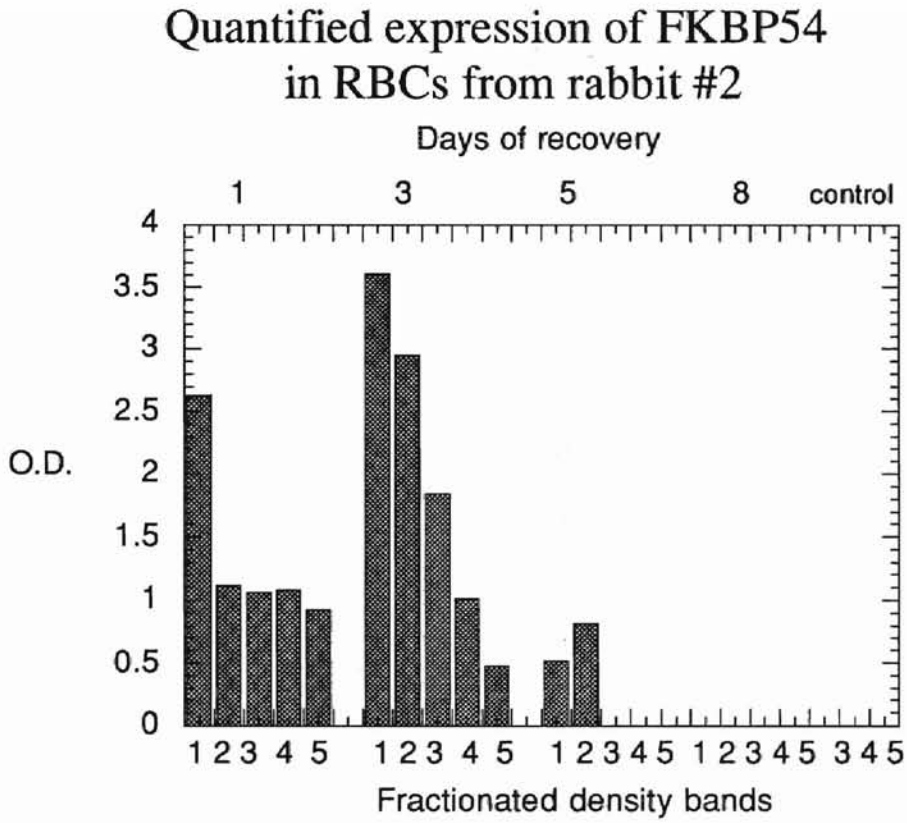

Figure 18. Quantified expression of FKBP54 in red blood cells from rabbit #2. Fractionated density bands 1-5 indicate the density of the fractionated whole cells by histopaque lightest to heaviest. 100 μg of protein from each sample was separated by SDS/PAGE and transferred to PVDF membranes. After probing for p23 with the JJ 5 antibody, western blots were probed for FKBP59 and FKBP54 with the EC1 polyclonal rabbit antibody and the FF1 polyclonal rabbit IgG antibody simultaneously using the p23 blots.. Optical density (O.D.) was determined by scanning densitometry.

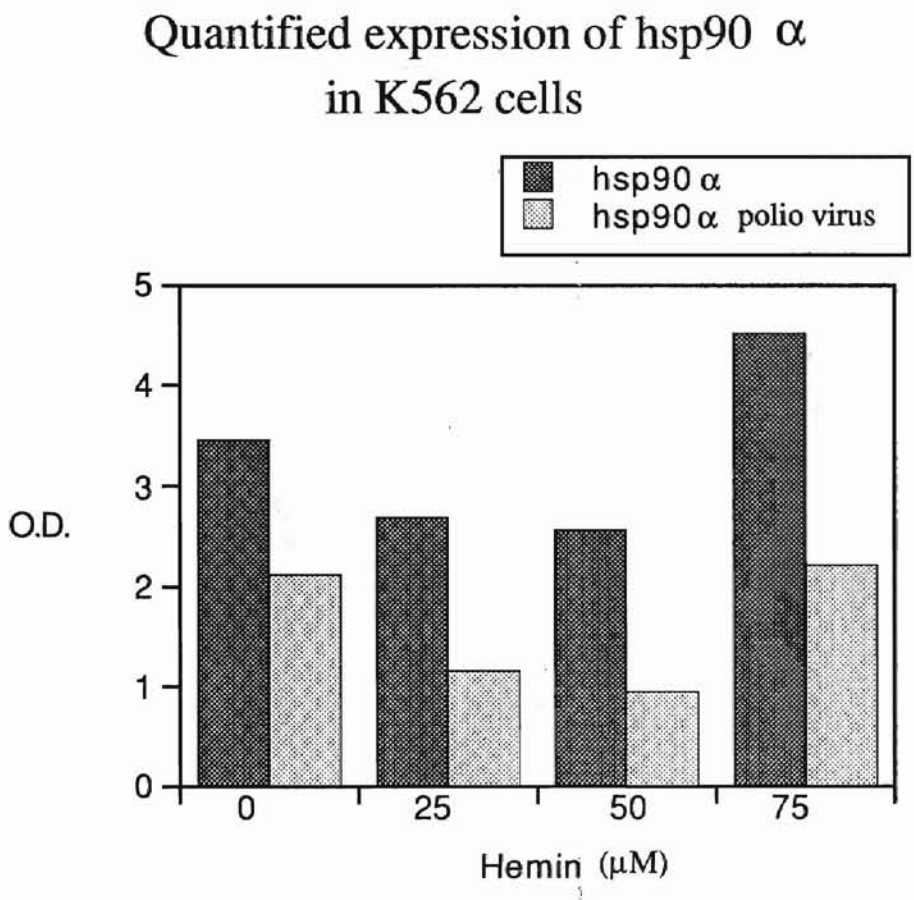

Figure 19. Quantified expression of hsp90α in K562 cells induced for 2 days with hemin. For each sample 3.3 x 10^3 cells were loaded. Proteins separated by SDS/PAGE and transferred to PVDF membrane. Western blots were probed with anti–p84. Optical density (O.D.) was determined by scanning densitometry.

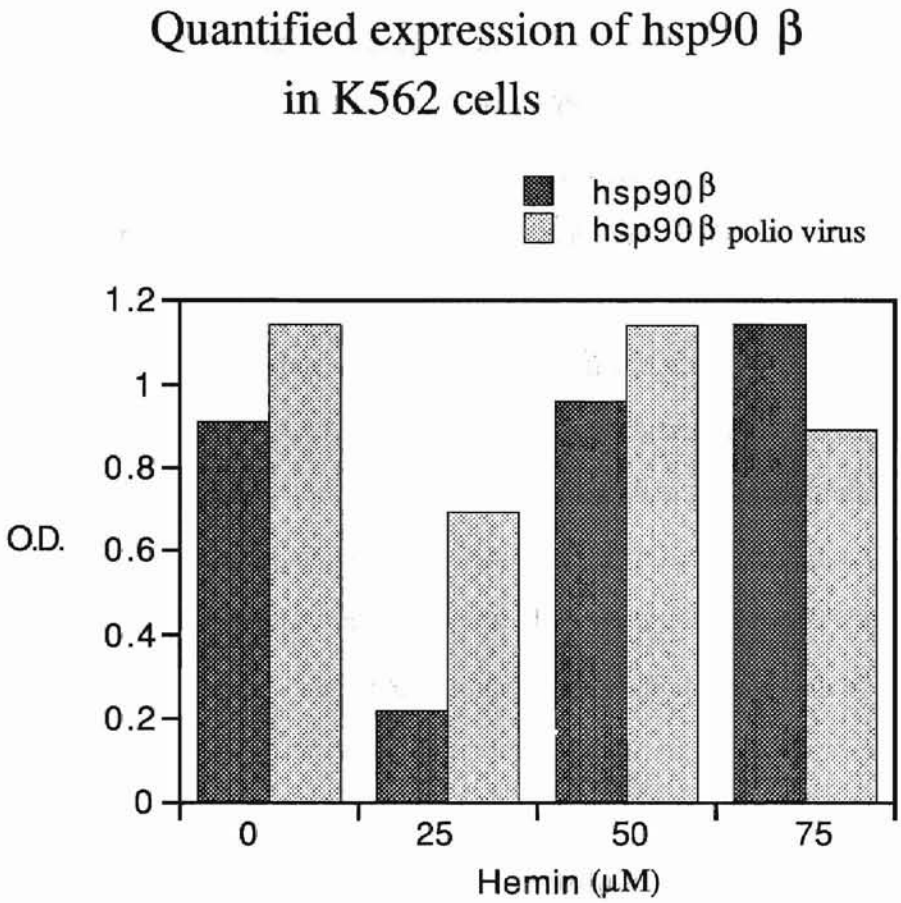

Figure 20. Quantification of expression of hsp90β in K562 cells induced for 2 days with hemin. For each sample 3.3 x 10^3 cells were loaded. Proteins separated by SDS/PAGE and transferred to PVDF membrane. Western blots were probed with anti–p86. Optical density (O.D.) was determined by scanning densitometry.

lowest level of hemin (25 μM) suppressed hsp90β expression (figure 20). The overall expression of hsp90α was decreased in presence of polio virus, while polio virus infection of K562 cells appeared to maintain or increase the expression of hsp90β relative to uninfected cells (figure 20). In general hemin was observed to have the same qualitative effects on hsp90α and hsp90β expression in polio virus infected cells as it did in uninfected cells.

The effect of hemin-induced differentiation and polio virus infection on the expression of hsc72 and hsp73 was also examined (figure 21 and figure 22, respectively). While 75 μM hemin increased the expression of hsc70 in both the uninfected and infected cells, polio virus infection alone, or the induction of cell differentiation by addition of 25 μM and 50 μM hemin appeared to suppress the expression of hsc70 (figure 21). The induction of hsc70 at high hemin levels may be through its induction of the stress response in the cells, since hemin is a strong oxidation reagent. Hsp70 levels were also examined (figure 22). hsp70 expression increased with increasing levels of hemin in a manner similar to hsc70 and infection with polio virus had little effect on hsp70 expression relative to uninfected cells.

Expression of the hsp90/hsp70 cohort, p60, was also analyzed. Little change in p60 expression was observed in K562 cells induced to differentiate with hemin (figure 23) Infection with polio virus decreased the overall expression of p60 regardless of level of hemin present.

Regulation of protein translation rates occurs due to changes in the eIF-2α phosphorylation in response to heat shock (London, 1987; Jackson, 1991). The phosphorylation of eIF-2α has been observed to increase as the protein synthesis rate decreases. Using isoelectric focusing the amount of phosphorylated and unphosphorylated eIF-2α was determined to examine whether hemin-induced differentiation of K562 cells caused a change in the phosphorylated state of eIF-2α. Polio virus infection decreased the

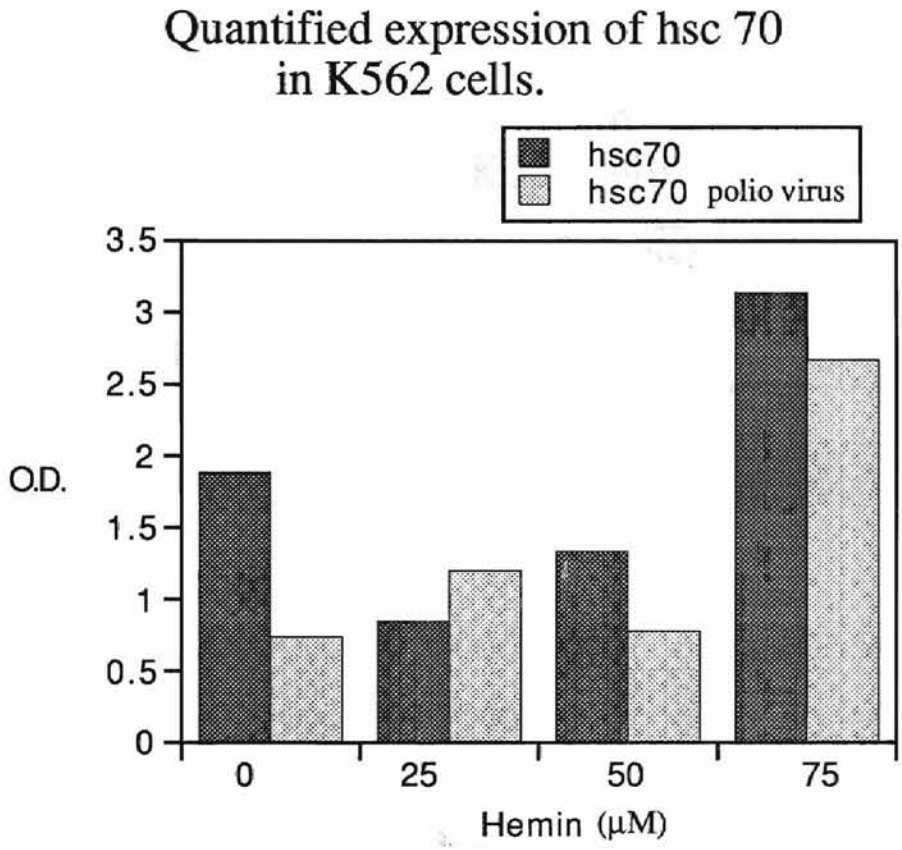

Figure 21 Quantified expression of hsc70 in K562 cells induced for 2 days with hemin. for each sample 1.0 x 10^4 cells were loaded. Proteins separated by SDS/PAGE (13.3 x 25 cm) and transferred to PVDF membrane. Western blots were probed with 4332 antisera. Optical density (O.D.) was determined by scanning densitometry.

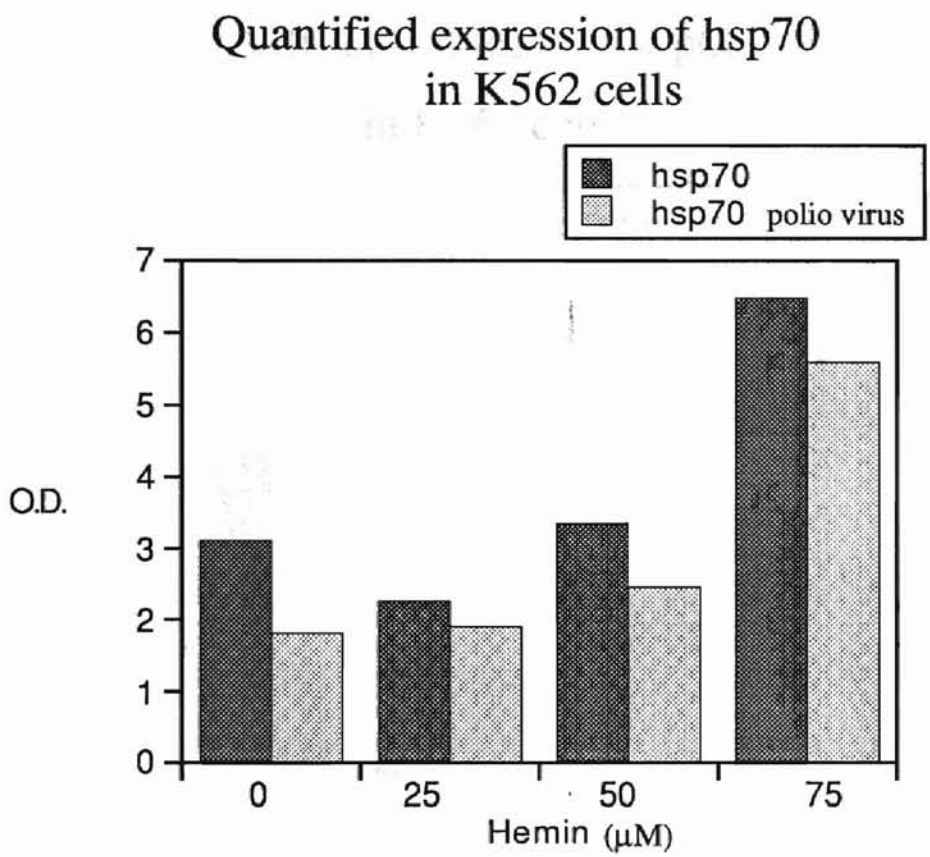

Figure 22 Quantified expression of hsp70 in K562 cells induced for 2 days with hemin. for each sample 1.0 x 10^4 cells were loaded. Proteins separated by SDS/PAGE (13.3 x 25 cm) and transferred to PVDF membrane. Western blots were probed with 4332 antisera. Optical density (O.D.) was determined by scanning densitometry.

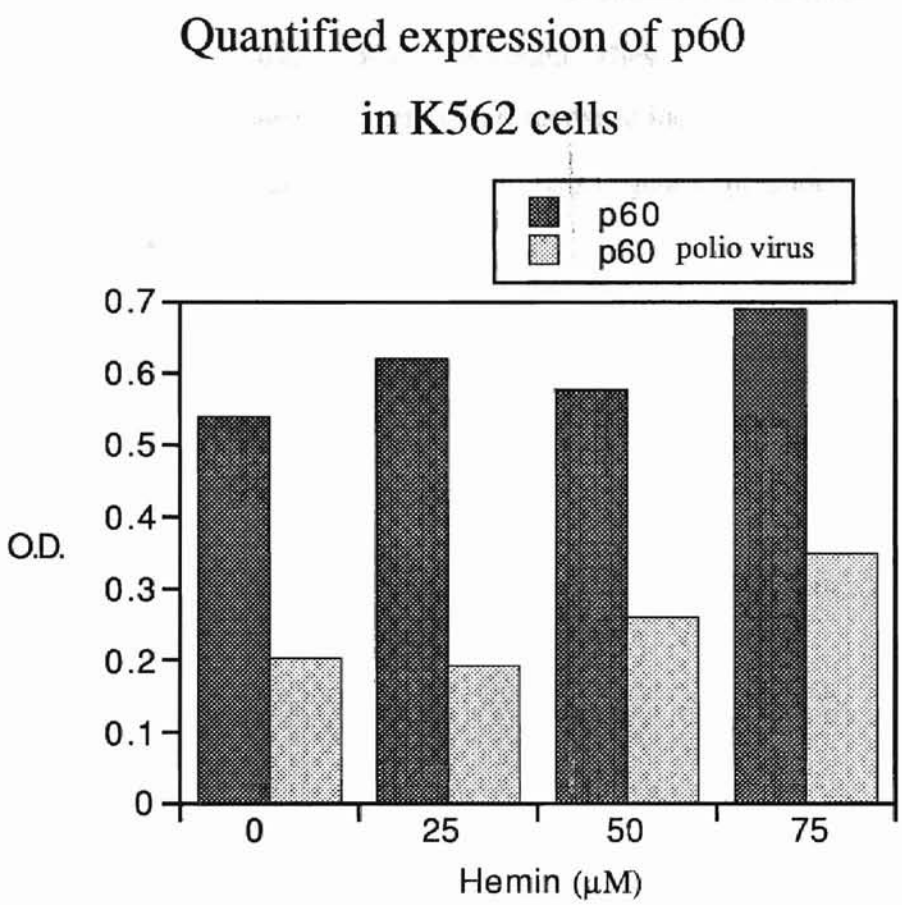

Figure 23 Quantified expression of p60 in K562 cells induced for 2 days with hemin. For each sample 1.3 x 10^5 cells were loaded. Proteins separated by SDS/PAGE and transferred to PVDF membrane. Western blots were probed with F5 antibody. Optical density (O.D.) was determined by scanning densitometry.

amount of eIF-2α in K562 cells, and increased the proportion of phosphorylated eIF-2α to unphosphorylated eIF-2α (figure 24). A similar change was observed upon induction of differentiation in the absence of virus infection. These results suggest that changes in eIF-2α phosphorylation may be occurring in response to induction of differentiation. These changes may be important in regulating changes in gene expression during processes at the translational level.

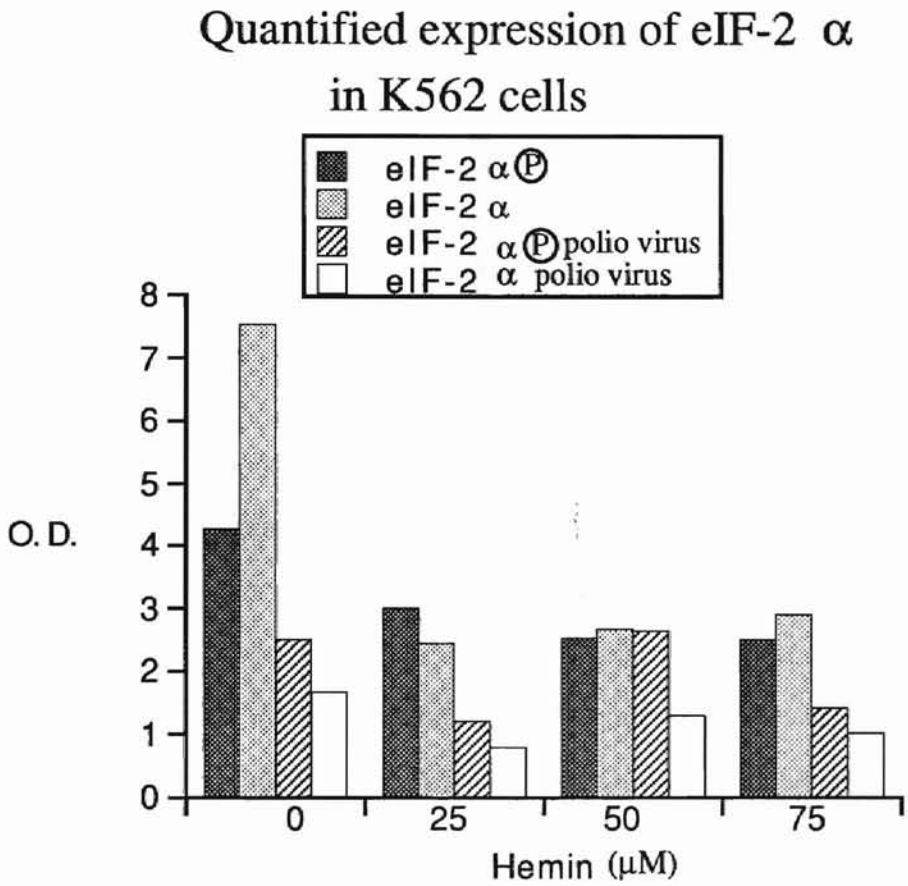

Figure 24. Quantified expression of eIF-2α and the phosphorylated eIf-2α. For each sample 4.0 x 10^5 cells were loaded on an isoelectric focusing gel (pH 4-6). Circled P indicates the phosphorylated eIF-2α. The IEF gels were transferred to PVDF membrane. Western blots were probed with anti-eIF-2α monoclonal antibody. Optical density (O.D.) was determined by scanning densitometry.

CHAPTER IV

DISCUSSION

Heat Shock Response

Almost all types of cells respond to stressful conditions, such as, heat shock, nutritional, and oxidative stress, by synthesizing large quantities of proteins, typically referred to as heat shock proteins (hsp) (Welch, 1990). The heat shock response is tightly regulated, and is required to prevent irreversible cell damage and ultimately cell death (Morimoto, 1992). The induction of hsp mRNA transcription occurs due to the binding of heat shock transcription factor (HSF) to an upstream promoter element (HSE) (Ang, 1991; Sorger, 1991; Craig, 1991; Mosser, 1990). In both human and mouse cells at least two HSF genes, HSF1 and HSF2, have been found and cloned (Scharf, 1990; Schuetz, 1991; Rabindran, 1991; Sarge, 1991). HSF1 has been shown to activate the tanscription of heat shock genes under conditions of elevated temperature, exposure to toxic heavy metals, and amino acid analogs, while HSF2 on the other hand does not (Hunt, 1985). HSF2 DNA binding activity and hsp70 gene transcription are induced during hemin-induced differentiation of the K562 cell line (Theodorakis, 1989; Sistonen, 1992). HSF2 may function to activate heat shock gene expression in the absence of physiological stress such as during differentiation of cells and embryonic development (Morimoto, 1992).

Three broad categories of conditions which are known to induce hsp synthesis in whole organisms: include environmental stress; pathophysiological states such as viral infections, hypoxia, and oxidative stress encountered upon reperfusion of ischemic tissue; and non-stressful conditions which include cellular development and differentiation (Morimoto, 1992). Many heat shock proteins were later found to be constitutively

expressed and to function as chaperones facilitating protein folding and assembly *in vivo* (Gething, 1992; Hendrick, (1993). In addition hsps help disaggregate and refold denatured proteins (Cheng, 1989). Members of the major families of hsps, hsp90, hsp70, and hsp 60 are required for proper folding of wide range of proteins (review Gething, 1992). Increased expressions of hsps may indicate their role in folding of new nascent proteins whose transcription must be regulated during the cell cycle or during development. Such proteins would be found during differentiation or other specialized times of cell growth. A reason for the increase in heat shock gene transcription during differentiation may be due to increased requirements for hsps in chaperoning the folding of the new proteins being expressed in large amounts. The hsps may also play a role in protecting cells of cytotoxic effects of stress at critical times during development

Rabbit Reticulocyte Maturation

The effects of many different types of stress on protein synthesis rates have been studied using the rabbit reticulocyte lysate model system. It has been observed in our lab that different preparations of reticulocyte lysate behave differently in the stress-induced inhibition of protein synthesis. The degree of stress-induced protein synthesis inhibition in reticulocyte lysate depends upon the quantity of hsps present, correlating best with hsc73 and FKBP59 expression (Matts (2), 1992). Most studies into differentiation only use cells prior the enucleation stage. The question arises what happens to hsp levels when the nucleus is no longer present or is not functional. The heat shock gene transcripts and those coding for other proteins must be already made. Therefore, regulation of expression is only possible at the levels of translation and degradation. It would be expected that the higher the expression of hsps in the immature reticulocytes, the greater their ability to synthesize, transport, fold, and assemble functional proteins, while maintaining HRI in an inactive state. Therefore, hsp expression should influence the quality of RBC produced, both at the level of hemoglobin accumulation and its ability to carry O_2. Hsp expression

should also influence the ability of a RBC to repair damaged proteins and withstand the oxidative stress incumbent to its function as an O_2 transporter. A correlation between high expression of hsps in erythrocytes and a long half life would be expected. The final steps in maturation of RBC are degradation of the mitochondria and the breakdown of the protein synthesis machinery. In the absence of a need to chaperone the folding or transport of nascent proteins, it is of interest which hsps remain to function in the mature RBC and which ones are degraded. The hsps remaining in the mature cell may influence its ability to withstand the stress incumbent to its function as an O_2 carrier and the half-life of the cell in the circulatory system. In addition, remaining hsps may function to regulate some aspect of erythrocyte function.

When these experiments were initiated, it was expected that HRI expression would be maintained as long as the protein synthesis machinery was present (indicated by brilliant cresyl blue staining of the RNA of the ribosomes). As the ribosomal machinery breaks down a decrease in HRI expression was expected. Figures 3-5 show that in fact HRI expression does decrease and that it was not normally found in peripheral blood at the same detection levels. Scanning error was determined to be $\pm$ 0.40 O.D. It has been reported that HRI is found in peripheral blood using a much higher concentration of whole blood (Crosby, 1994). Since reticulocytes are in the peripheral blood for one day before maturing it is expected that these are the primary cells in which HRI is still found. HRI expression was maintained in RBC's at detectable levels much longer if the rabbit was made anemic through bleeding (figure 5) than in N-acetyl phenylhydrazine. This is probably due to the iron levels of the bleed induced rabbit being much lower and taking longer for the rabbit to recover than when N-acetyl phenylhydrazine was used. When animals are induced to an anemic state with N-acetyl phenylhydrazine the RBC's are destroyed and the iron from the hemoglobin is metabolized and recycled by the spleen. Macroreticulocytes are produced uniquely during the early phases of chemically induced hemolytic anemias. Macroreticulocytes turnover very rapidly and are depleted from the

peripheral blood. The increase HRI expression in whole blood observed on day four (figure 3) may reflect the release of more RBCs in the form of normal reticulocytes in response to the turnover of the macroreticulocytes.

Super complexes involving hsp90, hsp70, FK506 binding proteins, and p23 have been recently found to chaperone the folding of steroid receptors (Johnson (2), 1994). This same type of machinery may also be used in chaperoning other cellular proteins (Schumaker, 1994). As with HRI regulation of protein translation, once the translational machinery is degraded there would not be a need for folding nascent polypeptides. Therefore, a decrease in the proteins making the super complexes might be found during maturation of reticulocytes as the need to chaperone folding of nascent peptides decreases. However, there would be a continual need for hsps in the reversal of proteins damaged oxidatively, as oxidative damage has been demonstrated to markedly lower the stability of proteins. The expression of hsp90, p23, immunophillins (FKBP59 and FKBP54) decreased to undetectable amount upon maturation of reticulocytes to erythrocytes (figures 6-11 and 15-18), suggesting there is little need for the function of these proteins in the absence of the production of nascent polypeptide chains.

In order to study reticulocyte maturation in the absence of N-acetyl phenylhydrazine, peripheral blood was drawn from an untreated rabbit in quantities sufficient to induce an anemia. The blood from this rabbit was fractionated by density of the cells to determine levels of hsps during maturation from the last day reticulocyte to erythrocyte. Hsc70 and p60 were found to decrease to a certain minimal but detectable amounts in the mature red blood cell. What role these proteins have in the mature RBC is not known. While it has been suggested that p60 is a cohort for hsp90 function, this data suggest it may function as a cohort of hsc70. These proteins may be important in protecting aging cells from stress, primarily oxidative. In addition they may function in senescence of red blood cells, as the mechanism of detection and subsequent removal of damaged red blood cells is not totally understood. As the cell matures damaged proteins

accumulate and degrade. Hsc70 may bind to these damaged, degraded or unfolded proteins. As the level of denaturation increases the hsc70 would become sequestered and further denaturation would result in aggregation of proteins. Denaturation of hemoglobin may cause clustering of the band 3 on red blood cells' cytoskeleton, and these clusters would be seen as non-self or foreign by macrophages and removed from circulation in the spleen (Low, 1991). Hsc70 and possibly p60 may be required to keep damaged hemoglobin or other proteins from aggregating, prolonging the half-life of the erythrocyte in the circulation system. Thus hsc70 and p60 may play a role in mechanism by which the recognition of damaged red blood cells by macrophages is regulated.

Future work will be directed at examining the expression of hsps in differentiating reticulocytes and cells in culture, which will hopefully give us more insight into the regulation of expression and the function of hsps *in vivo*.

Polio Virus Infected Cells

To determine the expression of hsps during initial stages of erythroid differentiation, changes in expression of heat shock proteins were evaluated in K562 cell line. The K562 cell line is a model for CFU-E (colony forming units-erythrocyte) found in the bone marrow. The K562 cells were induced to differentiate by addition of increasing molarity of hemin. To test the K562 cells as a possible model for studying post-polio syndrome, cells were infected with polio virus. In uninduced K562 cells the polio virus does not replicate. Induction of K562 cell differentiation is accompanied by the induction of replication of the polio virus. The main goal of this study was to determine if there was a relationship between changes in hsp expression and the induction of replication of the polio virus. The observations made in this study (figure 19 and figure 20) indicate that polio virus infection in general had the same qualitative effect on expression of hsp90α, hsp90β, hsc70, hsp70 and p60 in differentiating cells as it did in undifferentiating cells. The changes in expression of the hsps observed were primarily due to the effect of hemin

level present and not virus infection. No correlation between altered hsp expression and induction of viral replication was observed.

Protein synthesis rates depend in part upon the level eIF-2α phosphorylation present in a cell (Jackson, 1991). Using isoelectric focusing (pH 4-6), the ratio of unphosphorylated to phosphorylated eIF-2α in K562 cells was determined(figure 24). Induction of K562 differentiation by hemin lowered eIF-2α expression and increased the proportion of phosphorylated to unphosphorylated eIF-2α that was present. Infection with polio virus further increased the proportion of phosphorylated to unphosphorylated eIF-2α. However, no inhibition of host cell translation was observed under these conditions (P. Benton per. com.). Of interest then is the mechanism by which protein synthesis is maintained at uninhibited levels in polio virus infected cells in the presence of elevated phosphorylation of eIF2-α, and whether the mechanism underlining the maintenance of protein synthesis may be involved in the induction of polio virus into a lytic cycle.

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