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Erythroid Expression of the Heme-Regulated eIF-2α Kinase

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The role of heme-regulated eIF-2α kinase (HRI) in the regulation of protein synthesis in rabbit reticulocytes is well documented. Inhibitors of protein synthesis with properties similar to those of HRI have been described in some nonerythroid cell types, but it has not yet been determined whether these eIF-2α kinase activities are mediated by HRI or one or more as yet uncharacterized kinases. We have studied the expression of mRNA, polypeptide, and kinase activities of HRI in various tissues from both nonanemic and anemic rabbits. Our results indicate that HRI is expressed in an erythroid cell-specific manner. HRI is present in the bone marrow and peripheral blood of both nonanemic and anemic rabbits but not in any of the other tissues tested. HRI mRNA is present at low levels in uninduced mouse erythroleukemic (MEL) cells and human K562 cells and accumulates to higher levels upon induction. The accumulation of HRI mRNA in differentiating MEL cells is dependent upon the presence of heme. The addition of 3-amino-1,2,4-triazole (AT), an inhibitor of heme biosynthesis, to the induction medium markedly reduced HRI mRNA accumulation. Simultaneous addition of hemin and AT to the dimethyl sulfoxide induction medium largely prevented the inhibition of HRI mRNA induction by AT. These findings indicate that HRI is expressed in an erythroid cell-specific manner and that the major physiologic role of HRI is in adjusting the synthesis of globins to the availability of heme.

Protein synthesis in intact reticulocytes and their lysates is dependent on the availability of heme. In heme deficiency, inhibition of protein synthesis occurs as a result of the activation of the heme-regulated inhibitor HRI (5, 21, 23, 31). HRI is a cyclic AMP-independent protein kinase which specifically phosphorylates the 38-kDa α subunit (eIF-2α) of eukaryotic initiation factor 2 (eIF-2) (18, 26, 28, 46). Phosphorylation of eIF-2α in reticulocyte lysates results in the binding and sequestration of eIF-2B in an eIF2(αP)–eIF-2B complex. The unavailability of eIF-2B, which is required for the exchange of GTP for GDP in the recycling of eIF-2 and the formation of the ternary complex (eIF-2/GTP/Met-tRNA$_f$), results in the cessation of the initiation of protein synthesis (reviewed in references 21, 23, and 31).

HRI is present in an inactive form in hemin-supplemented reticulocyte lysates (33, 34) and becomes active under conditions of heme deficiency (33, 34) or heat shock (4, 15) or upon treatment with sulfhydryl reagents such as *N*-ethylmaleimide or *o*-iodosobenzoate (20), oxidized glutathione (16, 19, 25), and heavy metal ions (22). It has been shown that hemin binds directly to HRI (17) and that this binding promotes intersubunit disulfide bond formation (8). Disulfide bond formation in HRI correlates with the maintenance of protein synthesis, the reversal of inhibition of protein synthesis during heme deficiency, and the inhibition of in situ eIF-2α phosphorylation (55).

Inhibitors of protein synthesis initiation with properties similar to those of HRI have been purified to various extents from human erythroid cells (43) and murine erythroleukemia (MEL) cells (35, 48, 49). Recently, the eIF-2α kinases from both uninduced and induced MEL cells have been purified and shown to be very similar, although not identical, to rabbit reticulocyte HRI (35, 48). This difference is most likely due to species-specific variation. Inhibitors of protein synthesis initiation with properties similar to those of HRI have also been reported in some nonerythroid cell types such as Ehrlich ascites cells (9), rat liver cells (12), and HeLa cells (11). Most recently, Olmsted et al. (39) purified two eIF-2α kinases from Ehrlich ascites cells which they believe to be distinct from HRI and the double-stranded RNA-dependent eIF-2α kinase (PKR). Furthermore, increased eIF-2α phosphorylation has been observed in HeLa and Ehrlich ascites cells under conditions of heat shock and nutrient starvation (11, 13, 14, 39, 50), in GH_3 pituitary cells treated with calcium-mobilizing agents (45), and in vasopressin-treated rat liver (24). It has not yet been determined whether the eIF-2α kinase activities measured in these cells are mediated by HRI or one or more as yet uncharacterized eIF-2α kinases.

Previous studies using a monoclonal antibody raised against rabbit reticulocyte HRI suggest that HRI may be erythroid cell specific (41). Western blot (immunoblot) analysis of several rabbit tissues and cultured cells using an anti-rabbit HRI monoclonal antibody (MAb F) detected HRI only in the peripheral blood and bone marrow of an anemic rabbit. This monoclonal antibody did not detect cross-reacting HRI in rat or human reticulocytes or in mouse or human erythroleukemia (MEL and K562) cells even after these cells were induced to enter into erythroid differentiation. Since the monoclonal antibody recognizes only limited regions of the rabbit reticulocyte HRI, it remains to be determined whether there are HRI-like isozymes in nonerythroid cells.

Our recent cloning of the rabbit reticulocyte HRI cDNA (7) has permitted us to study further the expression of HRI in mammalian cells. Results from Northern (RNA) blot and Western blot analyses, coupled with results from kinase assays using immunoadsorbed HRI from various rabbit tissues and other cell lines, provide evidence of the erythroid cell-specific nature of HRI expression. In addition, we show here that HRI expression is increased in both mouse and human erythroid leukemia (MEL and K562) cells when these cells are induced to enter into erythroid differentiation in culture and that in MEL cells, this increase in HRI mRNA expression is dependent on the presence of heme.

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MATERIALS AND METHODS

Cell culture conditions and rabbit tissue preparation. MEL cells (from Paul-Henri Romeo, INSERM U91, Paris, France) were grown in Dulbecco's modified Eagle medium (DMEM; BioWhittaker)–10% horse serum–25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4)–100 U of penicillin per ml–100 μg of streptomycin per ml at 37°C and 5% CO_2. These cells grew best on Falcon brand Primaria plates. MEL cells were induced to differentiate by the addition of either 1.5% dimethyl sulfoxide (DMSO) or 75 μM hemin or the two combined in DMEM containing 12% horse serum for a period of 3, 4, or 5 days. After the initial experiments, a 4-day induction period became standard.

Human chronic myelogenous leukemia (K562) cells (American Type Culture Collection) were grown in RPMI 1640 (Gibco BRL)–10% fetal calf serum–25 mM HEPES (pH 7.4)–100 U of penicillin per ml–100 μg of streptomycin per ml at 37°C and 5% CO_2. Cells were induced to differentiate by the addition of 75 μM hemin to the culture medium for a period of 4 days.

Human epitheloid carcinoma (HeLa S3) cells (from Louane Hann, Massachusetts Institute of Technology, Cambridge) were grown in DMEM–10% fetal calf serum–25 μM gentamicin–25 mM HEPES (pH 7.4) at 37°C and 5% CO_2. NIH 3T3 cells were grown in DMEM–10% calf serum–25 mM HEPES (pH 7.4)–100 U of penicillin per ml–100 μg of streptomycin per ml under the same conditions.

Rabbit tissues (normal and anemic liver, kidney, spleen, lung, heart, brain, pancreas, bone marrow, and peripheral blood) were obtained from four New Zealand White rabbits, two of which were made anemic by the subcutaneous injection of a 1% solution of 1-acetyl-2-phenylhydrazine for 5 consecutive days. After resting 4 days, the animals were anesthetized and then bled by heart puncture. This step proved critical in the removal of contaminating blood from the various organs. Organs were quickly removed and immediately frozen in liquid nitrogen. Before preparation of lysate or isolation of mRNA, tissues were extensively washed with cold phosphate-buffered saline (PBS) to remove traces of peripheral blood.

Cell and tissue lysate preparation. Rabbit tissue (0.5 g) or cultured cells (10^7) were used to prepare the lysates. The tissues were first frozen in liquid nitrogen, ground to a fine powder, and resuspended in cold PBS. Cells were harvested by centrifugation at 1,000 × *g* for 4 min at 4°C and washed five times with cold PBS. Both the ground tissue preparations and the packed cells were resuspended in 2 volumes of cold lysis buffer (20 mM Tris-HCl [pH 7.4] containing 50 mM KCl, 2 mM dithiothreitol, and 1 mM EDTA) and incubated on ice for 15 min. Cell lysis was completed by 20 to 30 strokes in a Dounce homogenizer, using a tight-fitting pestle. Lysates were centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatants were immediately frozen and stored at −80°C. Protein concentrations were determined with a Bio-Rad protein assay kit as specified by the manufacturer (Bio-Rad, Richmond, Calif.).

mRNA isolation and Northern blot analysis. mRNA was isolated from 10^8 cells or 1.0 g of rabbit tissue by oligo(dT) selection (Fast Track mRNA isolation kit; Invitrogen) and analyzed by Northern blotting, using standard techniques. In brief, mRNA sample concentrations were determined spectrophotometrically, and equal amounts of mRNA from each sample were separated through 1.0% agarose formaldehyde denaturing gels, using morpholinepropanesulfonic acid (MOPS)-EDTA buffer (47). Following electrophoresis, mRNA was transferred to supported nitrocellulose (BA-S NC; Schleicher & Schuell), cross-linked to the membrane by UV light (GS Genelinker; Bio-Rad), and hybridized to α-^{32}P-labeled cDNA probes. cDNA inserts were radiolabeled by the random priming reaction (Boehringer Mannheim), using [α-^{32}P]dCTP (>3,000 Ci/mmol; New England Nuclear). Unincorporated nucleotides were removed from the labeled DNA preparations by using Sephadex G-50 DNA-grade NICK columns (Pharmacia). The membranes were prehybridized in a solution containing 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA per ml, and 50% formamide at 42°C for 12 to 16 h. Hybridization took place under identical conditions with the addition of radiolabeled cDNA probe (5×10^8 to 1×10^9 cpm/μg) for 12 to 16 h. The blots were then washed three times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% SDS at room temperature, once in 1× SSC–0.1% SDS at 55°C, and finally twice at higher stringency in 0.2× SSC–0.1% SDS at 55°C. Blots were exposed to Kodak X-Omat AR film at −70°C. Before blots were reprobed, they were first washed with 0.03× SSC–0.1% SDS at 80 to 85°C for at least 1 h to remove the previously hybridized probe. The amount of hybridization was quantitated with a 2202 Ultrascan laser densitometer (LKB) and a 3390A integrator (Hewlett-Packard).

Western blot analysis. Proteins from the various rabbit tissues or from uninduced and induced cells were separated by electrophoresis through SDS–7.5% polyacrylamide gels and transferred to supported nitrocellulose (Schleicher & Schuell), using a Trans-Blot SD semidry transfer apparatus (Bio-Rad) at 15 V for 45 min. The nonspecific binding sites on the protein blots were blocked with either 5% nonfat milk or 2% bovine serum albumin in TBST (10 mM Tris [pH 7.4], 0.9% NaCl [TBS], 0.05% Tween 20) for at least 1 h. Blots were incubated for 3 h at room temperature with a monoclonal anti-rabbit HRI antibody (MAb F) (41). Immunoreactive proteins were visualized by colorimetric reaction, using a 1:7,500 dilution of anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Promega) and the nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) color development system (Promega). For detection of MEL cell HRI, 2 μg of protein from both uninduced and induced MEL cell extracts was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% gel), transferred to nitrocellulose, and incubated with a p56 anti-rabbit HRI peptide antibody (1:500 in TBS–0.5% Tween–0.05% deoxycholate). The p56 antibody was raised in rabbits and directed against the P-56 peptide conjugated to keyhole limpet hemocyanin.

Immunoprecipitation of HRI by the p74 antibody and protein kinase assays. HRI was immunoadsorbed from 75 μg of protein from cultured cells and normal and anemic rabbit tissues by overnight incubation at 4°C with the p74 antibody in the presence of protease inhibitors leupeptin (5 μg/ml; Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The p74 antibody was raised in rabbits and directed against the P-74 peptide conjugated to keyhole limpet hemocyanin. Immunoprecipitates were purified by protein G-Sepharose 4 Fast Flow (Pharmacia) and washed extensively in TBS–0.5% Triton, TBS–0.5% Triton–0.35 M NaCl and 0.5% deoxycholate, and finally TBS alone.

Protein kinase assay mixtures (50 μl) contained 20 mM Tris-HCl (pH 7.4), 40 mM KCl, and 2 mM magnesium acetate. Phosphorylation reactions were done as described previously (8). Proteins were separated by electrophoresis through SDS–10% polyacrylamide gels, stained with Coomassie blue, dried, and exposed to Kodak X-Omat AR film at room temperature.

PCR amplification of HRI- and NF-E2-specific sequence from rabbit tissue mRNA. Poly(A)$^+$ RNAs from various anemic and nonanemic rabbit tissues (500 ng) were used in reverse transcription reactions (Invitrogen cDNA cycle kit for reverse transcription PCR)

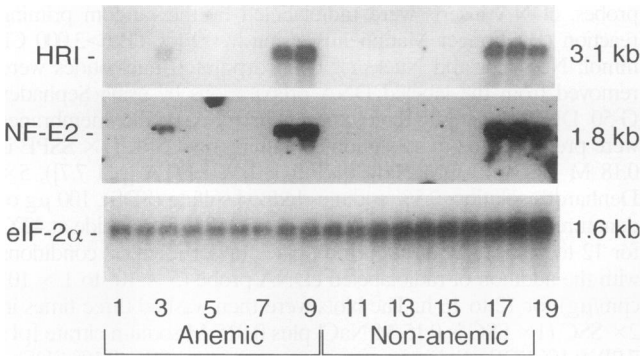

FIG. 1. HRI mRNA expression in anemic and nonanemic rabbit tissues. Five micrograms of poly(A)$^+$ RNA from anemic and nonanemic rabbit liver (lanes 1 and 10), kidney (lanes 2 and 11), spleen (lanes 3 and 12), lung (lanes 4 and 13), heart (lanes 5 and 14), brain (lanes 6 and 15), pancreas (lanes 7 and 16), bone marrow (lanes 8 and 17), and peripheral blood (lanes 9 and 18) was used for this Northern blot analysis. In lane 19, 1 μg of poly(A)$^+$ RNA from an anemic rabbit reticulocyte lysate was loaded as a positive control for HRI mRNA expression. Blots were hybridized to either the full-length rabbit HRI cDNA (top), murine erythroid cell-specific nuclear factor NF-E2 1.2-kb PCR product (middle), or rabbit eIF-2α cDNA as a control (bottom).

as specified by the manufacturer. PCRs were carried out with 100 ng of cDNA in a 50-μl volume containing 1 mM each primers 004 and 007A, which encode nucleotides 1,468 to 1,504 (conserved kinase domain VII) and 1,667 to 1,693 (conserved kinase domain IX) of the rabbit reticulocyte HRI cDNA, respectively, or 1 mM each primers ONF3 and ONF2, which encode nucleotides 301 to 321 and 1095 to 1134 of the mouse NF-E2 cDNA, respectively. These primers define a fragment 226 nucleotides in length for HRI and 834 nucleotides in length for NF-E2. Reactions were carried out for 35 cycles with 2 min at 94°C, 2 min at 47°C, and 3 min at 72°C in the presence or absence of Perfectmatch (Stratagene). Reaction products were separated by electrophoresis through a 1.0% agarose gel and visualized by ethidium bromide staining.

Southern blot analysis of PCR products. PCR products were separated by gel electrophoresis on a 1.2% agarose gel and blotted to a nitrocellulose membrane by using standard methods. After transfer, the membrane was prehybridized in a solution containing 6× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml at 68°C for 12 to 16 h. Hybridization took place under identical conditions with the addition of 0.01 M EDTA and radiolabeled cDNA probe (5×10^8 to 1×10^9 cpm/μg) for 12 to 16 h. The blot was then washed three times in 2× SSC–0.1% SDS for 5 min each at room temperature and then washed with 1× SSC–0.1% SDS for 20 min at room temperature, 1× SSC–0.1% SDS for 20 min at 50°C, and 0.2× SSC–0.1% SDS for 20 min at 50°C. The blot was exposed to Kodak X-Omat AR film at room temperature.

Benzidine staining. The percentage of benzidine-positive cells was determined as described by Orkin et al. (40). In brief, cells were stained in a solution containing 0.4% benzidine base, 2% hydrogen peroxide, and 12% acetic acid (10 μl of staining solution to 50 μl of cell suspension). Cells grown in the presence of hemin were washed twice with normal medium prior to staining.

RESULTS

Expression of HRI mRNA in various rabbit tissues. The mRNAs from various rabbit tissues (liver, kidney, spleen, lung, heart, brain, pancreas, bone marrow, and peripheral blood of normal and anemic rabbits) were isolated and subjected to Northern blot analysis using the full-length HRI cDNA as a probe (Fig. 1). A 3.1-kb mRNA that hybridized to the HRI cDNA was detected in the bone marrow and peripheral blood of both the normal and anemic rabbits (Fig. 1, top, lanes 8, 9, 17, and 18). HRI mRNA levels in the anemic bone marrow and peripheral blood accumulated to levels twofold greater than

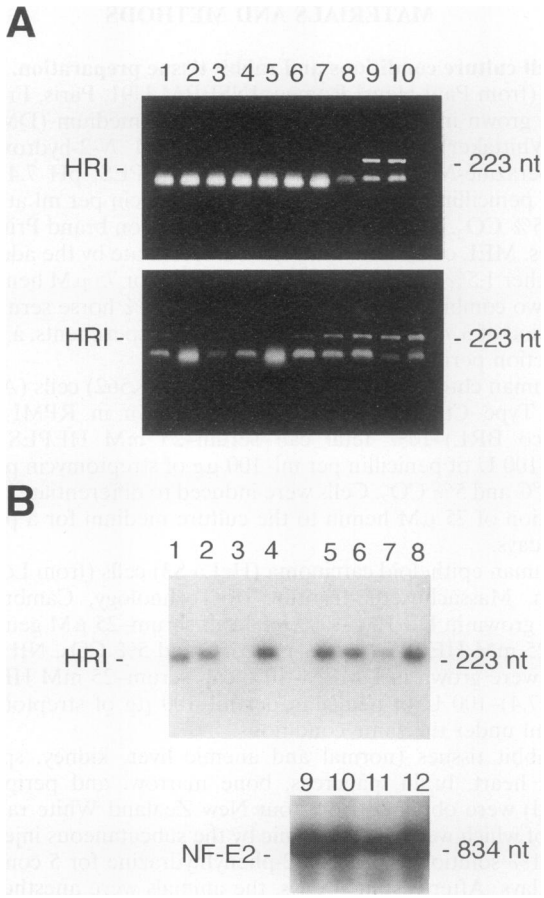

FIG. 2. Expression of HRI mRNA in various rabbit tissues as determined by PCR analysis. (A) Equal amounts of reverse-transcribed cDNA from various anemic and nonanemic rabbit tissues were used in PCRs as described in Materials and Methods. Tissue samples are from liver (lane 1), kidney (lane 2), spleen (lane 3), lung (lane 4), heart (lane 5), brain (lane 6), pancreas (lane 7), bone marrow (lane 8), and peripheral blood (lane 9). HRI cDNA was used as a positive control (lane 10). RNAs from extensively washed tissues recovered from heart-punctured rabbits (top) were compared with those less extensively washed (bottom). The primers used defined PCR fragments of 226 nucleotides (nt) which were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The lower-molecular-weight bands in each of the lanes are primer dimers. (B) RNAs from selected nonanemic rabbit tissues (liver [lanes 1 and 6], spleen [lanes 2 and 7], and brain [lanes 3 and 8]) were used in reverse-transcribed cDNA PCRs with either an HRI-specific (top) or NF-E2-specific (bottom) primer. These reactions were run for 35 cycles in the presence (lanes 1 to 4) or absence (lanes 5 to 8) of Perfectmatch (Stratagene). The products were separated by agarose gel electrophoresis and Southern blotted with the full-length rabbit HRI or mouse NF-E2 cDNA probe (see Materials and Methods). Rabbit reticulocyte mRNA was used as a positive control (lanes 4 and 8).

those in normal samples, as determined by laser densitometry scanning. A small amount of HRI mRNA was detected in the spleen of the anemic rabbit (Fig. 1, lane 3), but no signal was detected in any of the other tissues tested. Limitation of HRI mRNA expression to the bone marrow and, to a lesser extent, spleen in anemic rabbits coincides with the known sites of adult hematopoiesis (3). HRI mRNA expression in these tissues is identical to that of the erythroid cell-specific transcriptional factor NF-E2 (1.6 kb) (1) (Fig. 1, middle). In contrast, eIF-2α (Fig. 1, bottom) and the ε subunit of eIF-2B (32a) were expressed universally in erythroid and nonerythroid rabbit tissues under both anemic and nonanemic conditions.

To examine more closely the erythroid cell-specific nature of HRI mRNA expression, we used PCR to amplify HRI-specific sequences in various nonanemic rabbit tissues (Fig. 2). Initially, such sequences were detected not only in the nonanemic bone marrow and peripheral blood but also in the nonanemic liver, kidney, brain, and pancreas, albeit to a somewhat lesser extent (Fig. 2A, bottom). However, when the tissue samples were washed more rigorously prior to mRNA isolation, these signals were no longer apparent in our PCR analysis (Fig. 2A, top). In these samples, PCR-amplified HRI-specific sequences were detected only in the bone marrow and peripheral blood. To examine the possibility that trace amounts of HRI were present in nonanemic tissues, we blotted the PCR cDNAs and probed for HRI sequence by using our full-length HRI cDNA (Fig. 2B, top). As expected, we observed such sequences in spleen and reticulocyte control samples (Fig. 2B, lanes 2 and 4). Signal was also detected in the liver (Fig. 2B, lane 1) but not in the brain (Fig. 2B, lane 3). When these sequences were PCR amplified in the absence of Perfectmatch (Stratagene), a commercial product that enhances the formation of more homologous template-primer duplexes over those with less homology (Fig. 2B, lanes 5 to 8), we were able to detect a small amount of PCR product in the brain (Fig. 2B, lane 8). The identity of the kinase-related sequences in the liver and brain remains to be determined. It may be due to extremely low levels of HRI in each of these tissues or to trace peripheral blood contamination. In an attempt to distinguish between these two possibilities, we performed PCR experiments using the same tissue mRNA samples but with primer sequences for the erythroid cell-specific transcription factor NF-E2. The NF-E2 PCR product was detected in both the liver and brain in addition to the erythroid samples (Fig. 2B, lanes 9 to 12). These results are consistent with the presence of trace amounts of blood contamination which would result in the amplification of small amounts of the HRI PCR product in nonerythroid cells.

Expression of HRI polypeptide and kinase activities in various rabbit tissues. To determine whether the tissue-specific manner with which HRI mRNA was expressed is reflected in HRI protein expression, we performed Western blot analysis on protein extracts from various tissues of both healthy and anemic rabbits, using the anti-rabbit HRI monoclonal antibody MAb F (Fig. 3). MAb F recognized a single HRI polypeptide of approximately 90 kDa in the bone marrow (Fig. 3, lane 8) and peripheral blood (Fig. 3, lane 9) of both the nonanemic and anemic rabbits. The amount of HRI present in the anemic bone marrow and peripheral blood was significantly greater than that detected in the nonanemic samples. We estimated the amount of HRI protein in the anemic bone marrow to be threefold greater than the amount detected in the nonanemic sample (laser densitometry scan; data not shown). A much more dramatic difference in HRI protein levels was evident in the peripheral blood; the anemic sample contained 12- to 15-fold more HRI protein than the nonanemic sample (laser densitometry scan; data not shown). No HRI protein was detected in any of the other tissues tested, including anemic spleen.

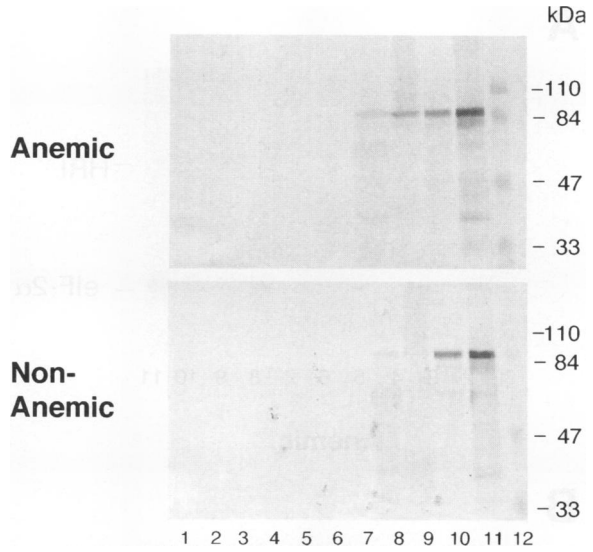

FIG. 3. Expression of HRI polypeptide in various anemic and nonanemic rabbit tissues, determined by Western blot analysis of tissue lysates with anti-rabbit MAb F. Samples are from liver (lane 1), kidney (lane 2), spleen (lane 3), lung (lane 4), heart (lane 5), brain (lane 6), pancreas (lane 7), bone marrow (lane 8), and peripheral blood (lane 9). A previously characterized rabbit reticulocyte preparation known to contain a substantial amount of HRI was loaded (lane 10) along with purified HRI (lane 11) as a positive control; 75 μg of protein from each of the rabbit tissues was loaded per lane. Prestained SDS-PAGE low-range molecular weight marker (lane 12) sizes are noted at the right. Proteins were separated by SDS-PAGE (7.5% gel).

It has been shown that the anti-rabbit HRI monoclonal antibody efficiently immunoprecipitates HRI from rabbit reticulocyte lysates (41), and while binding of MAb F to HRI does inhibit autokinase and eIF-2α kinase activities somewhat, its activity in both respects is readily observed in protein kinase assays. We used MAb F to immunoadsorb HRI from various cell extracts of both anemic and nonanemic rabbit tissues and assayed the immunoadsorbed pellets for eIF-2α kinase activity. The autokinase and eIF-2α kinase activities were observed only in the immunoadsorbed pellets from peripheral blood and bone marrow (data not shown), results consistent with those obtained from Western blot analysis (41) and Fig. 3.

It is possible that MAb F would be unable to detect HRI-like isozymes if they were present in nonerythroid cells. It was shown previously that MAb F did not cross-react with HRI in rat or human reticulocytes or in mouse or human erythroleukemic cells, even after they had been induced to enter into erythroid differentiation and an eIF-2α kinase activity had been detected in them (41). To gain a broader range of specificity for examining HRI or HRI-like isozymes in different rabbit tissues as well as to study further the regulation and expression of HRI in other cultured cell lines, a polyclonal antibody directed against the synthetic HRI peptide P-74 was prepared. It has been shown that HRI peptide P-74 (amino acids 506 to 525) inhibits eIF-2α phosphorylation by both HRI and PKR eIF-2α kinases (6). There is a very significant homology between the three known eIF-2α kinases (HRI, PKR, and GCN2) around the region of P-74 (7), and it is this region that is believed likely to be involved in eIF-2 binding and eIF-2α phosphorylation (5). We have used the p74 anti-

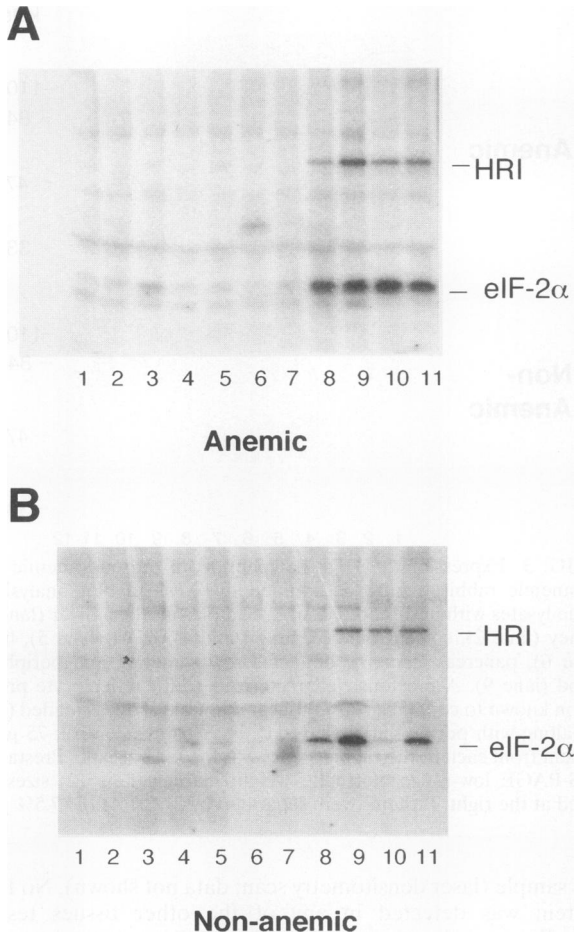

FIG. 4. Autokinase and eIF-2α kinase activities of HRI in various anemic and nonanemic rabbit tissues. Rabbit HRI was immunoadsorbed from various rabbit tissues (75 μg) by using the anti-p74 antibody, and the immunoadsorbed pellets were tested for eIF-2α kinase activity as described in Materials and Methods. The anemic (A) and nonanemic (B) samples include liver (lane 1), kidney (lane 2), spleen (lane 3), lung (lane 4), heart (lane 5), brain (lane 6), pancreas (lane 7), bone marrow (lane 8), and peripheral blood (lane 9), along with rabbit reticulocyte and purified HRI controls (lanes 10 and 11). The reticulocyte control (B, lane 10) contains no eIF-2. Protein kinase assay products were separated by SDS-PAGE (10% gel) and visualized by autoradiography.

body to immunoadsorb HRI from various cell extracts from both anemic and nonanemic rabbit tissues and tested the eIF-2α kinase activity in the immunoadsorbed pellets. The HRI immunoadsorbed from reticulocyte lysates or a purified HRI preparation by the p74 antibody was active both as an autokinase and as an eIF-2α kinase (Fig. 4A, lanes 10 and 11). Preimmune serum did not immunoadsorb significant amounts of HRI. It should be noted that no phosphopeptide in the vicinity of eIF-2α was observed when purified eIF-2 was absent from the protein kinase assay (Fig. 4B, lane 10). A 90-kDa phosphopolypeptide was also observed in the bone marrow and peripheral blood of both the anemic and nonanemic rabbits (Fig. 4A and B, lanes 8 and 9). HRI autophosphorylation was readily detected in the nonanemic peripheral blood, while it was barely detectable in the nonanemic bone marrow. Still, there was sufficient HRI present in the nonanemic bone marrow that eIF-2α phosphorylation was easily detected. Both

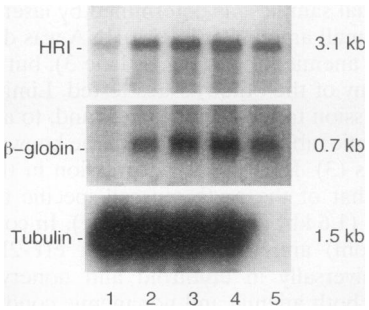

FIG. 5. Expression of HRI mRNA in MEL cells, determined by Northern blot analysis of 5 μg of mRNA isolated from uninduced control (lane 1), hemin-induced (lane 2), DMSO-induced (lane 3), and DMSO-hemin-induced (lane 4) MEL cells. In lane 5, 100 ng of mRNA from anemic rabbit reticulocyte was loaded as a positive control for HRI mRNA expression. The blot was hybridized to the full-length rabbit HRI cDNA (top). The same blot was stripped and reprobed with a human β-globin cDNA (middle) and then again with the rat tubulin cDNA as a loading control (bottom).

HRI autophosphorylation and eIF-2α phosphorylation were significantly increased in the anemic samples compared with the nonanemic samples. Upon prolonged exposure of the film, a slight increase in eIF-2α phosphorylation was observed in the anemic spleen (data not shown). The spleen retains the ability to reinitiate broad hematopoietic functions under anemic conditions, and therefore HRI-mediated eIF-2α kinase activity in the anemic spleen is not unexpected. However, phosphorylation of neither HRI nor eIF-2α was detected in any of the other tissues tested by this method. Collectively, our results indicated that the expression of HRI mRNA, protein, and kinase activities is erythroid cell specific.

Expression of HRI mRNA in erythroleukemic cells. Cultured erythroleukemic cells from Friend virus-infected mice (MEL cells) undergo considerable erythroid maturation in vitro when grown in the presence of DMSO and become irreversibly committed to terminal differentiation after 12 to 24 h. MEL cells grown in the presence of hemin (75 μM) undergo some of the changes associated with normal erythroid differentiation, including a dramatic increase in cytoplasmic globin mRNA levels, without causing commitment to a loss of indefinite proliferative capacity. Human K562 cells also undergo partial and reversible erythroid differentiation when grown in the presence of 50 to 75 μM hemin.

The extent of erythroid differentiation in MEL cells was determined by benzidine staining. Less than 0.5% of the uninduced MEL cells were benzidine positive. After 4 days of induction, we observed that 11% of the cells treated with hemin alone exhibited a differentiated phenotype, compared with 52% of the DMSO-treated cells. The presence of 3-amino-1,2,4-triazole (AT), an inhibitor of heme biosynthesis, in the DMSO induction medium resulted in a decrease in the number of benzidine-positive cells to 34%. It is important to note that while this is a modest decrease in positively scored cells, the ratio of very lightly stained cells to cells that are more darkly stained was significantly increased (1.2:1 for DMSO-treated cells; 5.4:1 for DMSO-AT-treated cells). These data indicate that the amount of hemoglobin per cell was greatly reduced in AT-treated cells. The number of benzidine-positive cells was restored to 58% upon simultaneous addition of hemin and AT.

To study the relationship of HRI to erythroid differentiation, we examined the expression of HRI in DMSO and/or hemin-

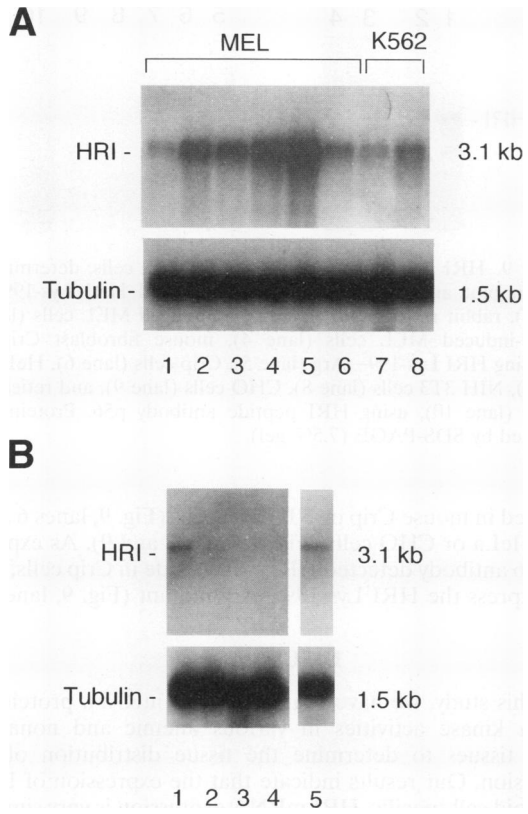

FIG. 6. Effect of DMSO and hemin on the expression of HRI mRNA in MEL and K562 cells. (A) Northern blot analysis of HRI mRNA from uninduced control MEL cells (lane 1), MEL cells treated with DMSO for 3 days (lane 2), 4 days (lane 3), and 5 days (lane 4), MEL cells treated 4 days with hemin plus DMSO (lane 5) and hemin alone (lane 6), uninduced control K562 cells (lane 7), and 4-day hemin-treated K562 cells (lane 8). (B) Northern blot analysis of HRI expression in rabbit reticulocyte control (lane 1), uninduced MEL cells (lane 2), NIH 3T3 cells (lane 3), CHO cells (lane 4), and HeLa cells (lane 5). Each blot was stripped and rehybridized to the rat tubulin cDNA as a loading control (bottom panels).

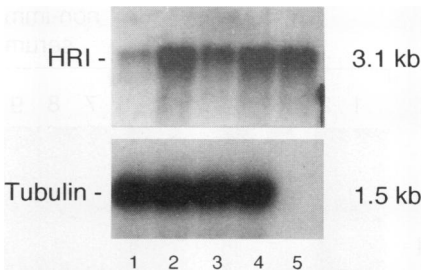

FIG. 7. The induction of HRI mRNA in MEL cells requires heme. Shown is a Northern blot analysis of HRI mRNA from uninduced control MEL cells (lane 1) and MEL cells treated with DMSO (lane 2), DMSO plus AT (lane 3), and DMSO, AT, and hemin (lane 4) for 4 days; 100 ng of rabbit reticulocyte mRNA was loaded as a positive control for HRI mRNA expression (lane 5). The same blot was stripped and rehybridized to the rat tubulin cDNA as a loading control (bottom).

treated MEL cells and hemin-treated K562 cells. Initially, cells were cultured for 3 days in the presence of either 75 μM hemin, 1.5% DMSO, or a combination of the two. After 72 h, the cells were harvested and the mRNA was isolated and analyzed by Northern blot analysis. The full-length rabbit HRI cDNA probe hybridized to a 3.1-kb mRNA in uninduced as well as induced MEL cells (Fig. 5, top). HRI mRNA is present in extremely low abundance, and 5 μg of poly(A)$^+$ RNA was required to detect it by Northern blot analysis. By comparison with the low levels of expression observed in uninduced cells, the levels of HRI mRNA were approximately threefold greater in hemin-treated cells, fivefold greater in DMSO-treated cells, and eightfold greater in cells treated with both hemin and DMSO, as determined by laser densitometry scanning. To ensure that our culture conditions were adequate to induce MEL cells to enter into erythroid differentiation, the same Northern blot was stripped and hybridized with a human β-globin cDNA probe (Fig. 5, middle). Consistent with previous reports (10), little or no detectable globin mRNA was present in uninduced MEL cells, but high levels were observed in response to both hemin and DMSO treatment. The same blot was hybridized to a rat tubulin cDNA as a loading control (Fig. 5, bottom).

A time course of HRI mRNA induction in DMSO-treated MEL cells revealed that HRI mRNA levels increased dramatically from 0 to 3 days of induction and continued to increase through 5 days of induction (Fig. 6A, lanes 1 to 4). HRI mRNA levels in 5-day DMSO-treated cells increased approximately 6-fold (Fig. 6A, lane 4), and HRI mRNA levels were observed to increase approximately 10-fold in 4-day DMSO-heme-treated cells (Fig. 6A, lane 5). A twofold increase in HRI mRNA accumulation was observed in MEL cells treated with hemin only (Fig. 6A, lane 6). HRI mRNA was also detected in both uninduced and hemin-induced K562 cells (Fig. 6A, lanes 7 and 8). Uninduced K562 cells express HRI mRNA at low levels. Four-day incubation of K562 cells in the presence of 75 μM hemin induced HRI mRNA to accumulate to levels approximately twofold those of uninduced cells. Both the mouse and human HRI mRNAs comigrated with rabbit HRI mRNA as a 3.1-kb mRNA on 1.0% agarose formaldehyde denaturing gels. For these and all of our Northern blot analyses, equal amounts of poly(A)$^+$ RNA were loaded per lane. It should be noted that many more induced cells were required to recover quantities of mRNA comparable to those of uninduced cells. Therefore, it is most accurate to describe the observed accumulation of HRI mRNA in these cells as an increase in abundance of HRI mRNA relative to total quantities of cellular mRNA. Under the same conditions, a small amount of HRI mRNA was detected in HeLa cells (Fig. 6B, lane 5) but not in NIH 3T3 cells (lane 3) or Chinese hamster ovary (CHO) cells (lane 4).

AT has been shown to inhibit heme biosynthesis selectively in mice (53) and rats (2). We have shown above that HRI mRNA accumulates rapidly in MEL cells treated with 1.5% DMSO (Fig. 5 and 6). In contrast, treatment with DMSO plus 25 mM AT results in a markedly reduced amount of HRI mRNA (Fig. 7, top, lane 3), although levels were still above those of the uninduced cells. The simultaneous addition of hemin to the culture medium was sufficient to prevent the negative effect of AT on HRI mRNA accumulation in DMSO-treated MEL cells (Fig. 7, top, lane 4). This effect was not complete, however, and HRI mRNA levels in the DMSO-AT-hemin-treated cells were not fully restored to those measured in cells treated with DMSO alone. These results indicate a requirement for heme to achieve the increase in HRI mRNA levels in induced MEL cells.

Expression of eIF-2α kinase activity and HRI polypeptide in MEL cells. We used the p74 antibody in an attempt to immunoadsorb cross-reacting HRI from MEL cells and to

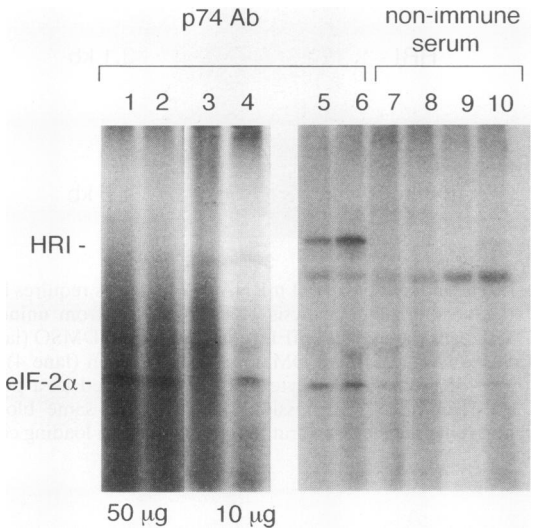

FIG. 8. eIF-2α kinase activities of HRI in uninduced and induced MEL cells. The anti-p74 antibody (Ab) was used to immunoprecipitate MEL cell HRI from 50 μg of uninduced (lane 1), 50 μg of DMSO-induced (lane 2), 10 μg of uninduced (lane 3), or 10 μg of DMSO-induced (lane 4) MEL cell protein extract and from rabbit reticulocyte and purified HRI controls (lanes 5 and 6, respectively); 50 μg of uninduced (lane 7) and induced (lane 8) MEL protein extract and both rabbit reticulocyte (lane 9) and purified HRI (lane 10) controls were used in immunoprecipitation reactions using rabbit nonimmune serum. Kinase reaction products were separated by SDS-PAGE (10% gel).

assay its protein kinase activity. The p74 antibody was able to immunoadsorb an eIF-2α kinase activity from both uninduced and induced MEL cells (Fig. 8, lanes 1 and 2). This activity was totally abolished by preincubation of the immunoprecipitate with 5 μM hemin (data not shown). When immunoadsorption reactions were carried out in a quantitative manner by using smaller amounts of lysate, an increase in eIF-2α kinase activity was observed upon DMSO induction of MEL cells (Fig. 8, lanes 3 and 4). No HRI autophosphorylation was observed in any of the MEL cell kinase reactions. This observation is consistent with earlier reports that phosphorylated HRI is not detected in purified preparations of MEL cell HRI (48). Recently, MEL cell HRI has been shown to undergo autophosphorylation when a more extensively purified and concentrated HRI preparation was used (35). Thus, it is most likely that too little HRI is present in the immunoadsorbed pellets to detect the autophosphorylation of HRI. Rabbit normal serum was unable to immunoprecipitate eIF-2α kinase activity from MEL cell lysates, reticulocyte lysate controls, or purified HRI preparations (Fig. 8, lanes 7 to 10).

To determine whether HRI protein is increased upon erythroid differentiation, a polyclonal antibody directed against the synthetic HRI peptide P-56 was prepared. The P-56 peptide corresponds to amino acids 166 to 178 of the rabbit HRI polypeptide and is unique to HRI (7). The HRI peptide p56 antibody recognizes the 90-kDa HRI polypeptide in *Spodoptera frugiperda* Sf9 cells which overexpress an HRI Lys-199→Arg mutant, reticulocyte lysate, and MEL cells both before and after induction (Fig. 9, lanes 1 to 4). More HRI polypeptide is present in the induced MEL cells (Fig. 9, lane 4) than in the uninduced cells (Fig. 9, lane 3). This result is consistent with the observed increases in eIF-2α kinase activity (Fig. 8) and HRI mRNA (Fig. 5). HRI polypeptide was not

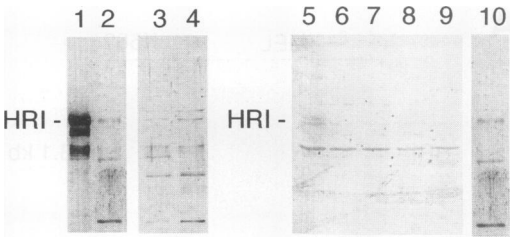

FIG. 9. HRI polypeptide expression in MEL cells, determined by Western blot analysis of baculovirus-expressed HRI Lys-199→Arg (lane 1), rabbit reticulocyte (lane 2), uninduced MEL cells (lane 3), DMSO-induced MEL cells (lane 4), mouse fibroblast Crip cells expressing HRI Lys-199→Arg (lane 5), Crip cells (lane 6), HeLa cells (lane 7), NIH 3T3 cells (lane 8), CHO cells (lane 9), and reticulocyte control (lane 10), using HRI peptide antibody p56. Proteins were separated by SDS-PAGE (7.5% gel).

detected in mouse Crip or NIH 3T3 cells (Fig. 9, lanes 6 and 7) or in HeLa or CHO cells (Fig. 9, lanes 8 and 9). As expected, the p56 antibody detected HRI polypeptide in Crip cells, which overexpress the HRI Lys-199→Arg mutant (Fig. 9, lane 5).

DISCUSSION

In this study, we have examined HRI mRNA, protein, and eIF-2α kinase activities in various anemic and nonanemic rabbit tissues to determine the tissue distribution of HRI expression. Our results indicate that the expression of HRI is erythroid cell specific. HRI mRNA expression is very similar to that of the erythroid cell-specific nuclear transcriptional factor NF-E2. In addition to erythroid cells, NF-E2 has been shown to be expressed in hematopoietic progenitor, megakaryocytic, and mast cell lines but not in macrophage, lymphoid, or fibroblast lines (1). The possible expression of HRI in hematopoietic progenitor and megakaryocytic cells is currently under investigation.

It is important to note that we are able to detect HRI mRNA by Northern blot analysis of mouse and human erythroleukemic cells under the same conditions in which we are unable to detect any such mRNA in nonerythroid rabbit tissues with the full-length HRI cDNA probe. When lower-stringency wash conditions (1× SSC–0.5% SDS, 50°C) were used, some signals were detected in the erythroid and some nonerythroid tissues (data not shown). Most of these low-stringency-hybridizing mRNAs were different from HRI mRNA in size, and often multiple bands were observed (4.2, 3.1, and 2.5 kb in reticulocyte, bone marrow, spleen, liver, and pancreas and 2.2 kb in pancreas). The identities of these different-size messages are yet to be determined. They may represent other eIF-2α kinases. The 2.5-kb message is consistent with the reported size of the PKR mRNA (36).

Similarly, eIF-2α kinase activity is detected in MEL cells with the p74 antibody against rabbit HRI but not in any of the nonerythroid rabbit tissues. In addition, anti-HRI MAB F, which is an extremely high-titered antibody, does not detect any HRI protein in nonerythroid rabbit tissues (Fig. 3) (41). Therefore, the eIF-2α kinase activities present in nonerythroid cells are likely to be different from HRI. Recently, partial purification and characterization of eIF-2α kinases from Ehrlich ascites cells suggest that these eIF-2α kinases are different from both HRI and PKR (39). It is especially important to note that these eIF-2α kinases are not responsive to hemin (42), in marked contrast to HRI from rabbit reticulocytes (5) and MEL cells (35).

HRI mRNA was found to be present in K562 and MEL cells and was increased after induction of erythroid differentiation. The accumulation of HRI mRNA in differentiating MEL cells appears to be dependent upon the presence of heme (Fig. 7), since HRI mRNA was reduced 60% when heme biosynthesis was inhibited by AT. It has been shown previously that AT reduces the level of globin mRNA in DMSO-treated cells but not completely to the low levels present in uninduced cells (32). When hemin is added to cultures treated with DMSO plus AT, globin mRNA levels are restored to those measured in fully induced, DMSO-treated cells. In contrast, while AT significantly reduced erythrocyte membrane antigen (EMA) levels in MEL cells, hemin failed to restore EMA expression, indicating a factor other than the intracellular level of heme as responsible for EMA expression (32). The effects of AT and AT plus hemin on HRI mRNA levels in DMSO-induced MEL cells are similar to their effects on globin mRNA, suggesting a direct relationship between intracellular heme levels and HRI accumulation in MEL cells. It has been demonstrated that erythroid expression of human β-like globin genes is regulated by an upstream 20-bp heme-responsive enhancer which contains the NF-E2 binding site (1, 37, 51). It will be interesting to determine whether a similar mechanism is operative for the erythroid and heme-regulated expression of HRI mRNA.

Our previous studies indicate that hemin inhibits the activity of HRI by bringing sulfhydryl groups, which are essential for ATP binding, in closed proximity and consequently promoting intersubunit disulfide bond formation (8, 55). Formation of a disulfide-linked dimer is not unique to HRI and has been reported for two receptor kinases (reviewed in reference 54), the platelet-derived growth factor receptor (29) and colony-stimulating factor 1 receptor (30). It is interesting that HRI and these two receptor kinases possess kinase insertion sequences which separate the conserved kinase domains into two halves (7, 54). A conserved heme-regulatory motif (HRM) has been identified recently in the erythroid δ-aminolevulinate synthetase precursor (27) and is also found in other heme-binding proteins such as HAP1 (a yeast transcriptional factor for cytochrome genes) (44), hemopexin (38), and catalase (52). HRM contains R/K/N-C-P in which Cys and Pro are invariant. There are two such HRMs in the HRI sequence. They are Cys-406, Pro-407 and Cys-548, Pro-549. The importance of the invariant Cys residue in HRM is consistent with our earlier findings that hemin regulates HRI by direct binding and by promoting intersubunit disulfide bond formation in HRI. These two Cys and Pro residues in HRI are absent in GCN2 and PKR, the other two well-characterized eIF-2α kinases which are not regulated by heme. In fact, there is no Cys-Pro sequence in the entire coding sequence of either GCN2 or PKR.

The data presented here indicate that HRI is expressed in an erythroid cell-specific manner, that HRI mRNA levels are significantly increased during erythroid differentiation, and that this accumulation is dependent on the presence of heme. These results indicate that HRI is tightly regulated by heme in erythroid cells and suggest the main physiological role of HRI may be to regulate a balanced synthesis of heme and globin chains.

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