Tissue Distribution and Immunoreactivity of Heme-Regulated eIF-2α Kinase Determined by Monoclonal Antibodies†

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ABSTRACT: A highly purified preparation of heme-regulated inhibitor (HRI), an eIF-2α kinase, from rabbit reticulocyte lysates has been used for generating monoclonal antibodies (mAB). Two hybridoma clones secreting HRI-specific antibodies (mAB A and mAB F) were obtained. Both antibodies immunoprecipitated biosynthetically labeled as well as phosphorylated HRI in reticulocyte lysates and also recognized denatured HRI in a Western blot. In in vitro protein kinase assays, preincubation of HRI with the antibodies significantly diminished both autokinase and eIF-2α kinase activities. HRI from reticulocyte lysates could be quantitatively removed by immunoprecipitation with mAB F, and such HRI-depleted lysates were able to maintain protein synthesis under conditions of heme deficiency. With these monoclonal antibodies, HRI was detected only in the reticulocytes and bone marrow of anemic rabbits, among several rabbit tissues tested. The antibodies did not detect cross-reacting HRI in rat or human reticulocytes or in mouse erythroleukemic cells or human K562 cells even after induction of differentiation, although eIF-2α kinase activity was detected in them. Polyclonal anti-rabbit HRI antibody detected HRI in rat reticulocytes. However, no cross-reacting HRI was detected by polyclonal antibody in human reticulocytes or other cell types tested. These findings suggest that HRI is not ubiquitous, and may be erythroid-specific, and that it is antigenically different in different species.

In heme-deficient reticulocytes and their lysates, inhibition of protein synthesis occurs as a result of activation of the heme-regulated inhibitor [HRI;[1] reviewed in Ochoa (1983) and London et al. (1987)], also called heme-controlled repressor (HCR) (Gross & Rabinovitz, 1973). Activation of HRI, a cAMP-independent protein kinase, is accompanied by its autophosphorylation as well as phosphorylation of the 38-kDa α-subunit of the eukaryotic initiation factor, eIF-2 (Levin et al., 1976; Kramer et al., 1976; Ranu & London, 1976; Farrell et al., 1977). Phosphorylated eIF-2 binds to the reversing factor (RF, also called guanine nucleotide exchange factor and eIF-2B) and forms an RF–eIF-2(αP) complex that sequesters RF. As a consequence of the unavailability of RF that is required for the exchange of GTP for GDP in the recycling of eIF-2, initiation of translation ceases (Amesz et al., 1979; Siekierka et al., 1982, 1984; Matts et al., 1983, 1986; Panniers & Henshaw, 1983; Pain & Clemens, 1983; Matts & London, 1984; Thomas et al., 1984).

Although inhibitors with properties similar to those of rabbit reticulocyte HRI have been reported in nonerythroid cells, rabbit reticulocyte HRI has been the most widely investigated [for review, see London et al. (1987)]. Purified HRI from rabbit reticulocyte lysates sediments as a ~6.6S component in glycerol gradients (Levin et al., 1980) and appears to be a homodimer of a 92-kDa polypeptide (Trachsel et al., 1978; Hunt, 1979). Activation of HRI in reticulocyte lysates is caused by various conditions and reagents: heme deficiency, heat shock, the sulfhydryl reagents *N*-ethylmaleimide (NEM) and iodosobenzoate, oxidized glutathione (GSSG), and heavy-metal ions (Hunt, 1979; London et al., 1987; Hurst et al., 1987). The binding of hemin directly to purified heme-reversible HRI has been demonstrated (Fagard & London, 1981). Recently, we have observed that binding of hemin to HRI promotes intersubunit disulfide bond formation which is a likely mechanism of negative regulation of HRI (Chen et al., 1989).

The activation of HRI by one or more associated proteins has been suggested (Kudlicki et al., 1987; Rose et al., 1987, 1989; Matts & Hurst, 1989). Spectrin-related polypeptides of molecular mass of 80–120 kDa (Kudlicki et al., 1987; Rose et al., 1987) have been copurified with HRI, and a 90-kDa heat shock protein (hsp90) has been found associated with HRI in situ (Rose et al., 1989; Matts & Hurst, 1989). However, the reports on the effect of such HRI–hsp90 association on HRI activation are contradictory (Rose et al., 1989; Matts & Hurst, 1989).

With regard to tissue-type or cell-type specificity or immunological cross-reactivity among species, very little is known. Inhibitors with properties similar to those of rabbit reticulocyte HRI have been described from human erythroid cells (Freedman et al., 1974; Petryshyn et al., 1984), murine erythroleukemia (MEL) cells (Sarre, 1989), nonerythroid cells such as Ehrlich ascites cells (Clemens et al., 1976), rat liver (Delaunay et al., 1977) and HeLa cells (De Benedetti & Baglioni, 1986), and wheat germ extracts (Ranu, 1980). None of these HRI-like preparations with eIF-2α kinase activity, however, has been shown to have cross-reactivity or immunological identity with rabbit reticulocyte HRI.

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[1] Abbreviations: HRI, heme-regulated inhibitor; eIF-2α, eukaryotic initiation factor 2α; mAB, monoclonal antibody; NEM, *N*-ethylmaleimide; GSSG, oxidized glutathione; hsp, heat shock protein.

As a part of our investigation on the structure–function relationships of HRI and the expression of HRI during erythroid cell differentiation, this paper is concerned with the production and characterization of monoclonal antibodies to rabbit reticulocyte HRI and their use in determining its distribution in various tissues and species.

Materials and Methods

Materials. Materials used for HRI purification were obtained from sources described previously (Trachsel et al., 1978). Chemicals for gel electrophoresis were purchased from Bio-Rad Laboratories. Hemin, creatine phosphate, creatine phosphokinase, ATP, *N*-ethylmaleimide (NEM), GSSG, 4-chloro-1-naphthol, Pristane (2,6,10,14-tetramethylpentadecane), and fetal bovine serum (Hybrimax) were purchased from Sigma Chemical Co. Freund's adjuvant and horseradish peroxidase conjugated second antibodies were obtained from ICN Biomedicals. Protein G–Sepharose 4 Fast Flow was purchased from Pharmacia LKB Biotechnology Inc. PEG 1500 and the mouse monoclonal isotyping kit were obtained from Boehringer Mannheim Biochemicals. Radioactive chemicals, [γ-^{32}P]ATP, [^{14}C]leucine, and [^{35}S]methionine, were obtained from Du Pont-New England Nuclear. Tissue culture reagents, Dulbecco's modified minimum essential medium (D-MEM), sodium pyruvate, hypoxanthine, aminopterin, thymidine (HAT), penicillin–streptomycin, HEPES buffer, and azaguanine were purchased from Gibco.

BALB/c mice used for immunization were obtained from Jackson Laboratories. X63. Ag8. 653, a nonsecreting clone of myeloma cells was kindly provided by Dr. J. Lieberman, and K562 and MEL cells were provided by Drs. R. Cavellesco and D. Tuan. Human blood with a 40% reticulocyte count from a patient with pernicious anemia under treatment was generously provided by Dr. Christine Lawrence of the Albert Einstein College of Medicine. Rat reticulocyte lysate was obtained from Dr. R. Eisenstein (MIT).

Initiation factor eIF-2 was purified from the 0.5 M salt wash fraction of reticulocyte ribosomes as described elsewhere (Matts et al., 1983). Anti-hsp90 antibody was a kind gift of Dr. W. J. Welch (UCSF).

Purification of Heme-Reversible HRI. HRI was purified from the postribosomal supernatant of rabbit reticulocyte lysates essentially as described by Trachsel et al. (1978) with some modifications. Postribosomal supernatant was processed through five steps: ammonium sulfate fractionation (to 50% saturation), DEAE-cellulose chromatography, phosphocellulose chromatography, hydroxyapatite chromatography, and glycerol gradient centrifugation (15–50%). Localization and purity of HRI throughout the purification procedures were monitored by heme-inhibitable autophosphorylation of HRI and eIF-2α phosphorylation followed by SDS–PAGE, silver staining and autoradiogaphy. Protein concentration was determined according to Bradford (1976) with Bio-Rad's Protein Assay Reagent.

Protein Kinase Assays, SDS–PAGE, and Autoradiography. Protein kinase assay mixtures (20 μL) contained 20 mM Tris-HCl (pH 7.6), 40–60 mM KCl, 2 mM $Mg(OAc)_2$, and 25–50 μM [γ-^{32}P]ATP (20–30 Ci/mmol). HRI samples were added to the reaction mixtures and incubated for 10 min at 30 °C. Other additions are noted in the figure legends. Reactions were terminated by addition of SDS sample buffer (Laemmli, 1970), and samples were treated at 95 °C for 5 min. Proteins were separated by one-dimensional 10% slab gel SDS–PAGE according to Laemmli (1970). Gels were either Coomassie Blue stained or silver stained, dried, and autoradiographed with Kodak X-OMAT AR film.

Immunization and Hybridoma Production. Immunization of mice was carried out as described elsewhere (Grossi de Sa et al., 1988). Four 2–3 month old female BALB/c mice were immunized with two different HRI preparations, partially purified (preparation A) and highly purified (preparation B), as follows. Each mouse received three subcutaneous injections at 2-week intervals of 5 μg (preparation B) or 50 μg (preparation A) of HRI mixed 1:1 with either Freund's complete adjuvant for the first injection or Freund's incomplete adjuvant for the two subsequent injections. Ten days after the third injection, sera were collected by intraorbital bleeding and were tested for the presence of HRI antibody by an enzyme-linked immunosorbent assay (ELISA) and Western blot. After a period of about 40 days following the third injection, the mice with strong titers of HRI-specific antibody were injected intraperitoneally with either 5 or 50 μg of purified antigen in phosphate-buffered saline (PBS: 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl), after which they were sacrificed on the fourth day for cell fusion.

The spleen of each mouse was aseptically removed and teased apart in serum-free D-MEM. Dispersed spleen cells were then mixed with myeloma cells (X63. Ag8. 653) in a 5:1 ratio and fused by poly(ethylene glycol) (PEG) 1500 as described by Galfre and Milstein (1981). The fused cells were distributed to six 96-well Costar microculture plates (10^5 cells per well) and cultured (37 °C, 6% CO_2) in D-MEM with 20% fetal bovine serum containing HAT. Following ELISA screening of the culture supernatants, positive hybridomas were cultured in medium containing HT. Hybridomas that were subsequently shown to be positive by both ELISA and Western blot were subcloned by limiting dilution as well as soft-agar cloning methods.

Production and Purification of Antibody from Cloned Hybrids. Large-scale production of monoclonal antibodies was achieved by harvesting spent culture media or by ascites production. For the production of ascites, BALB/c mice were Pristane-primed and 2 weeks later were intraperitoneally injected with 5×10^6 to 5×10^7 hybridoma cells. When ascites developed, the mice were tapped twice, and the fluid was collected.

Immunoglobulins (IgGs) were purified from either culture supernatants or ascites fluid by Protein G–Sepharose 4 Fast Flow chromatography as descrbied by the manufacturer (Pharmacia LKB). Purity of the IgG was monitored by SDS–PAGE. The isotypes of the antibodies were determined by both ELISA and Western blot with a mouse IgG isotyping kit (Boehringer Mannheim).

ELISA. Polyvinyl flexible microtiter plates (Falcon) were coated with 50 μL of partially purified HRI (4 μg/mL in PBS) per well overnight at 4 °C. Following saturation with 50 μL of 3% bovine serum albumin (BSA) in PBS (4 h, room temperature), 50-μL culture supernatants were added, and the plates were incubated overnight at 4 °C. Wells were washed with PBS (3×, 10 min each) and incubated (4 h, room temperature) with 50 μL of diluted peroxidase-conjugated rabbit anti-mouse IgG (1:500), in PBS containing 10% rabbit normal serum. The wells were washed (3×, 10 min each) and developed with 50 μL of a solution containing 0.1% of 30% H_2O_2 and 2 mM ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid] in ELISA buffer (0.1 M sodium acetate, 0.05 M NaH_2PO_4). Color development was allowed to proceed for 30 min, and the wells were read in a Titer-tek microplate reader (Dynatech Lab).

Western Blot. Following SDS–PAGE, proteins were electrophoretically transferred to nitrocellulose paper (0.45 μM;

Schleicher & Schuell) according to Towbin et al. (1979). The blots were then processed for immunoreactions as described elsewhere (Pal et al., 1988). In brief, protein blots were saturated with 5% milk in PBS (4 h, room temperature) and incubated with primary antibodies overnight at 4 °C and then with peroxidase-conjugated second antibodies containing 10% normal rabbit serum (4 h, room temperature). Following each antibody incubation, blots were washed in PBS (3×, 10 min). Color development was performed with H_2O_2 and 4-chloro-1-naphthol.

Protein Synthesis in Reticulocyte Lysates. Rabbit reticulocyte lysates were obtained from Green Hectares Custom Laboratory Animal Work (Oregon, WI). Protein synthesis was carried out in 25-μL reaction mixtures (Hunt et al., 1972) under conditions of (i) no hemin added, (ii) 20 μM hemin, (iii) 20 μM hemin plus 5 mM NEM, and (iv) 20 μM hemin plus 1 mM GSSG. Protein synthesis was measured by the incorporation of [^{14}C]leucine into proteins as described in Hunt et al. (1972).

In Situ Phosphorylation of HRI. Protein synthesis was carried out in the presence of unlabeled leucine as described above. The reaction mixtures were pulsed during the first 10 min of incubation with 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) with or without added rabbit eIF-2 (2 μg). The samples were analyzed by means of either pH 5 precipitation or immunoprecipitation by anti-HRI antibodies as described below.

Synthesis of HRI in the Protein Synthesizing Lysate. Protein synthesis was carried out for 1 h under plus hemin conditions as described above but with unlabeled leucine and [^{35}S]methionine. Reaction mixtures were immunoprecipitated by anti-HRI antibodies and were analyzed as described below.

Immunoprecipitation. Protein synthesis reaction mixtures were immunoprecipitated with antibodies either directly or after dilution (1:1) with 2× PBS containing 50 mM NaF and 5 mM EDTA. HRI was immunoprecipitated with anti-HRI polyclonal and monoclonal antibodies, under various conditions as detailed in the legends; nonimmune serum was used as a control. Incubation with the antibodies was done either for 1 h or overnight at 4 °C followed by purification of the immunoprecipitates by incubation (30 min to 1 h, 4 °C) with protein G–Sepharose 4 Fast Flow suspended in PBS containing NaF and EDTA. Immunoprecipitates were washed extensively with PBS containing 50 mM NaF, 5 mM EDTA, and 0.5% Triton X-100 (5×) and finally with the same buffer without Triton X-100. Washed pellets and the supernatants were treated with Laemmli sample buffer and were analyzed by SDS–PAGE. Either the gels were stained, dried, and autoradiographed, or the proteins were transferred to nitrocellulose papers and the papers were autoradiographed and processed further for Western blot analysis.

Distribution of HRI in Various Rabbit Tissues and Other Cell Types. Tissues of rabbits obtained from Green Hectares or the Jackson Laboratory were extracted in 20 mM Tris-HCl (pH 7.4) buffer containing 50 mM KCl, 2 mM DTT, and 1 mM EDTA. These protein extracts and similar extracts from other cells, namely, human and rat reticulocytes, EBV-transformed human B cells, rat hepatoma cells, HeLa cells, noninduced and induced K562 human erythroleukemia cells, and induced MEL cells, were analyzed by Western blot and immunoprecipitation with anti-HRI antibodies. Some of these extracts were also assayed for the presence of eIF-2α kinase activity.

Results

Purification of HRI from Rabbit Reticulocyte Lysates. HRI was purified from about 400 mL of postribosomal supernatant of lysates. At step 2 (DEAE-cellulose column chromatography), two protein peaks, P_1 and P_2, containing HRI activity were eluted at 0.15 and 0.25 M KCl, respectively (data not shown). Although total HRI activity was the same in each peak, the total protein composition appeared more complex in P_2 than in P_1; a greater number of comigrating as well as neighboring protein bands was observed. Hence, HRI in P_1 was further purified as described under Materials and Methods. The HRI preparation after step 4 (hydroxyapatite chromatography) was about 25% pure (preparation A), and after the final step (glycerol gradient centrifugation), it was about 60% pure (preparation B) as demonstrated by a single predominant silver-stained band (Figure 1A, lane 2). Total HRI protein (in preparation B) was calculated to be about 20 μg, with BSA as a standard.

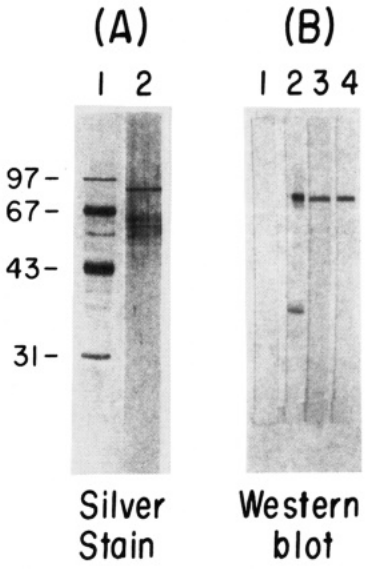

FIGURE 1: Production of monoclonal antibodies to HRI. (A) Silver-stained protein profile of the HRI preparation used for immunization. Proteins in purified preparation of HRI were separated by SDS–PAGE (10%). Lane 1, MW marker proteins; lane 2, HRI preparation. (B) Western blot of partially purified HRI with polyclonal and monoclonal antibodies. Lane 1, nonimmune serum (control); lane 2, polyclonal serum; lanes 3 and 4, culture supernatants containing monoclonal antibodies mAB F and mAB A, respectively.

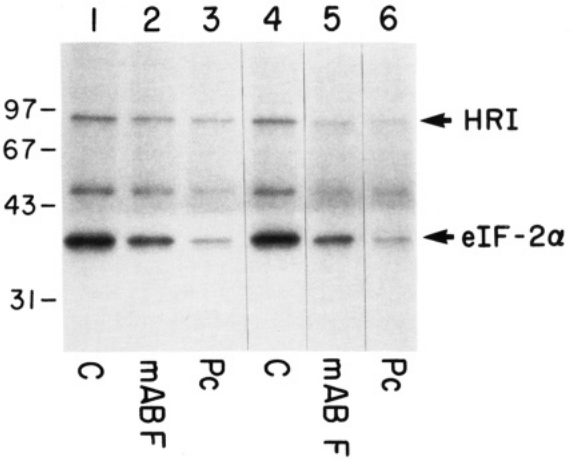

FIGURE 2: Effects of HRI monoclonal antibodies on HRI kinase activities. HRI was preincubated with purified antibodies (IgGs), and a kinase assay was then performed along with added eIF-2 and [γ-^{32}P]ATP as described under Materials and Methods. Preincubation was done with either 0.5 μg (lanes 1–3) or 2.0 μg (lanes 4–6) of IgGs. Lanes 1 and 4, nonimmune serum IgG control (C); lanes 2 and 5, mAB F IgG; lanes 3 and 6, polyclonal IgG (Pc). Phosphorylated HRI and eIF-2α are indicated. The figure is an autoradiogram.

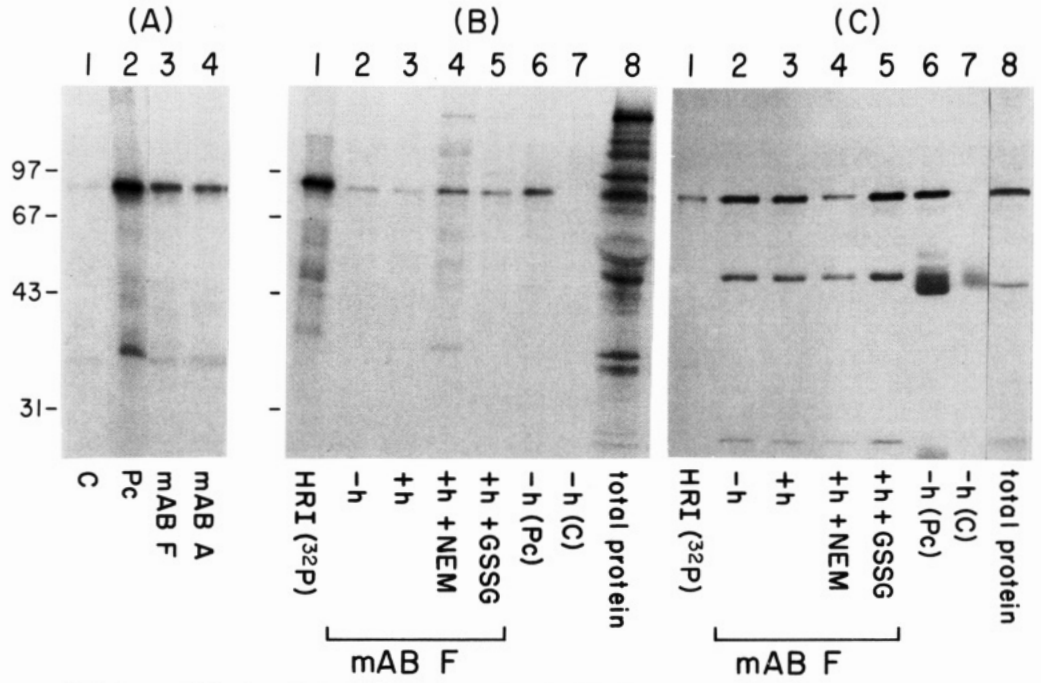

FIGURE 3: Immunoprecipitation and Western blot of HRI by monoclonal antibodies. (A) Immunoprecipitation of partially purified HRI. [γ-^{32}P]ATP-labeled partially purified HRI was immunoprecipitated by nonimmune serum control (C; lane 1), polyclonal serum (Pc; lane 2), and mAB F and mAB A culture supernatants (lanes 3 and 4, respectively). The panel is an autoradiogram. (B) Immunoprecipitation of [γ-^{32}P]ATP-labeled HRI from rabbit reticulocyte lysates. Lane 1, [γ-^{32}P]ATP-labeled purified HRI, HRI(^{32}P); lanes 2–7, immunoprecipitates of mAB F (lanes 2–5), polyclonal serum (Pc; lane 6), and nonimmune serum control (C; lane 7); lane 8, total phosphoproteins of lysates. Immunoprecipitates are from heme-deficient (−h; lanes 2, 6, and 7), hemin-supplemented (+h; lane 3), hemin-supplemented, NEM-treated (+h,+NEM; lane 4), and hemin-supplemented, GSSG-treated (+h,+GSSG; lane 5) lysates. The panel is an autoradiogram. (C) Western blot of the immunoprecipitated HRI as in (B) by mAB F. The description of the lanes is the same as in (B).

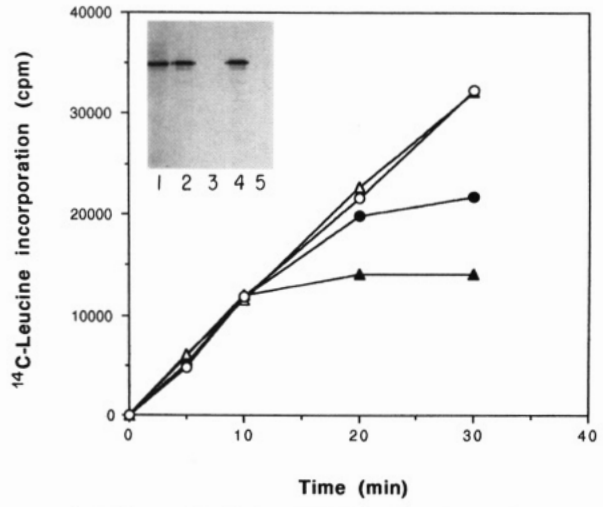

FIGURE 4: Effects of depletion of HRI on protein synthesis in reticulocyte lysates. HRI from reticulocyte lysates was immunoprecipitated by mAB F, and the precipitates (collected by Protein G–Sepharose) and the supernatants were analyzed by Western blot by mAB F (inset). Lane 1 shows the amount of HRI present in the lysate (2.5 μL) used for immunoprecipitation. Lanes 2 and 4 are immunoprecipitates (with 0.5 and 1.0 μg IgG, respectively). Lanes 3 and 5 are supernatants left after precipitation with 0.5 and 1.0 μg of antibodies, respectively. Aliquots of the HRI-depleted lysates were used for protein synthesis as follows: protein synthesis assays (25 μL) were carried out at 30 °C. Aliquots (5 μL) were removed at various time intervals as indicated to determine [^{14}C]leucine incorporation. (△) Hemin-supplemented (20 μM) and (▲) heme-deficient, control lysates. (○) Hemin-supplemented and (●) heme-deficient, HRI-depleted lysates.

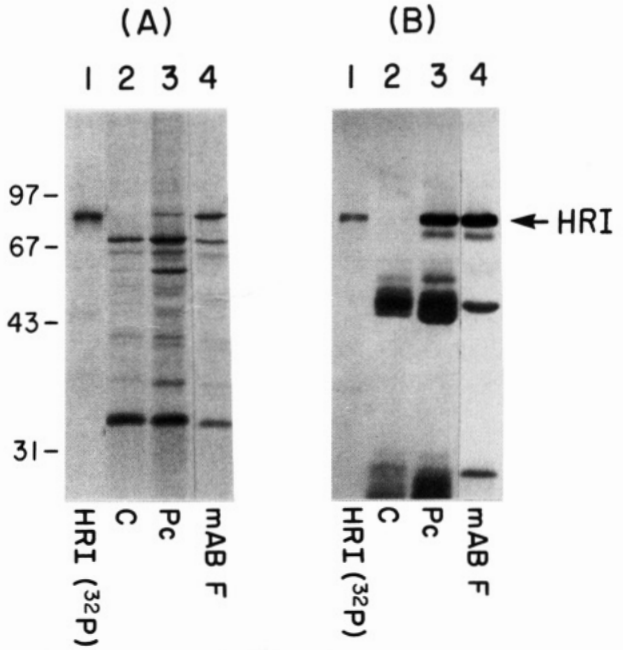

FIGURE 5: De novo synthesis (translation of endogenous mRNA) of HRI in reticulocyte lysates. (A) Immunoprecipitation of [^{35}S]-methionine-labeled HRI. Lane 1, [γ-^{32}P]ATP-labeled purified HRI [HRI(^{32}P)]; lanes 2–4, immunoprecipitates of reticulocyte HRI by nonimmune serum control (C; lane 2), polyclonal serum (Pc; lane 3), and mAB F (lane 4). The panel is a fluorogram. (B) Western blot of immunoprecipitated HRI. Descriptions of the lanes are the same as in (A). Position of HRI in the gel is indicated; other signals in the blot are due to IgG (H and L chains) in the immunoprecipitates.

Immunization, Hybridoma Generation, and Selection. Of the three mice injected (two with HRI preparation A and one with preparation B), all produced antibodies detectable by both ELISA (up to 1:1500 dilution of sera) and Western blot. However, the serum of the preparation B injected mouse recognized only one protein band other than HRI by Western blot (Figure 1B, lane 2) as compared to four to six bands recognized by the sera of the other two mice (data not shown).

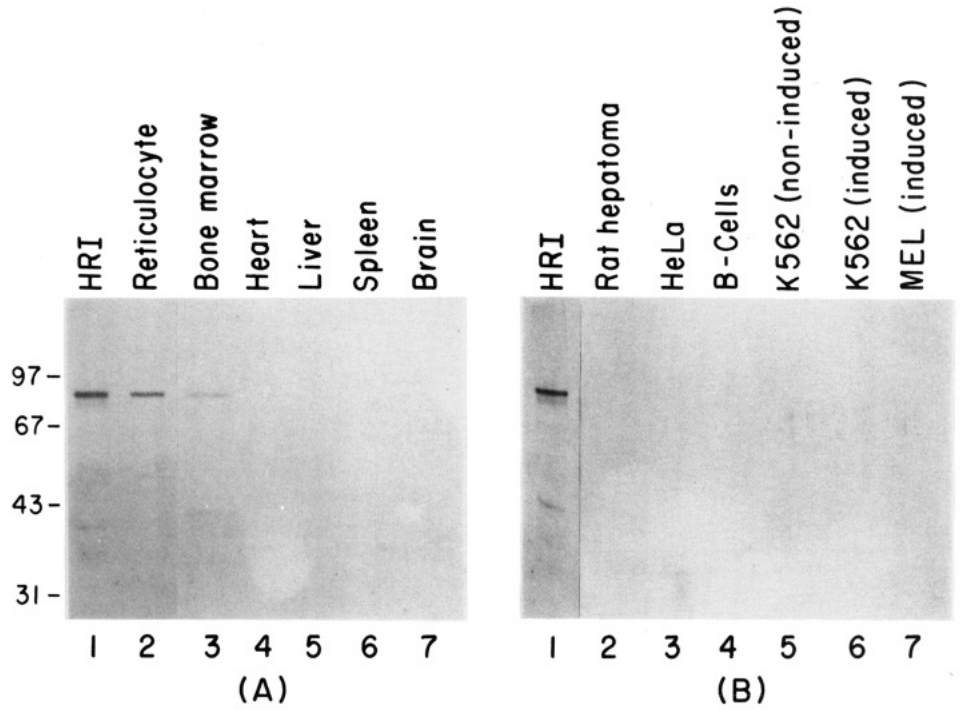

FIGURE 6: Western blot analysis of the cross-reacting HRI in various tissues and cell types. (A) Proteins extracted from various tissues of the rabbit were Western blotted by mAB F. Lane 1, purified rabbit reticulocyte HRI (HRI); lanes 2–7, lysates of different tissues (100 μg of total protein) as indicated. (B) Protein extracts of various tissue and cell types were immunoblotted by mAB F. Lane 1, purified rabbit reticulocyte HRI (HRI); lanes 2–7, extracts of various cell types (100 μg of total protein) as indicated.

Among three cell fusion experiments using spleens of three mice, we obtained HRI-specific hybridomas in only one with the preparation B injected mouse.

Among 576 wells plated with the fused cells, 80 contained hybridomas and were screened by ELISA. Although a positive ELISA reaction was obtained for 15 hybridomas, only three clones were HRI positive as detected by Western blot. Of the three clones secreting HRI-specific antibodies, two stabilized in culture and were expanded, subcloned, and frozen in liquid nitrogen. We designated these monoclonal antibodies mAB F and mAB A (Figure 1B, lanes 3 and 4, respectively). The isotypes of mAB A and mAB F were IgG_{2a} and IgG_1, respectively; both had κ light chains.

Characterization of Anti-HRI Monoclonal Antibodies. Specificity of the monoclonal antibodies to HRI was determined by the following criteria: (1) recognition of purified HRI in a Western blot, (2) inhibition of HRI kinase activities in vitro, and (3) immunoprecipitation of [γ-^{32}P]ATP-labeled purified HRI and HRI in reticulocyte lysates.

It is to be noted that, in various experiments, polyclonal antibody has been used only as a reference during characterization of the monoclonal antibodies. This polyclonal antibody was obtained from the preparation B injected mouse and is not a monospecific anti-HRI antibody.

Western Blot Analysis. Both monoclonal antibodies, mAB F and mAB A, recognized a single polypeptide band of 92 kDa in a Western blot of partially purified HRI (Figure 1B, lanes 3 and 4, respectively); the authenticity of this protein as HRI was further confirmed by aligning it with [γ-^{32}P]ATP-labeled HRI (data not shown). On the other hand, polyclonal serum recognized some additional bands of molecular mass of 38 and 90 kDa (Figure 1B, lane 2). In some HRI preparations as well as in reticulocyte lysates, the 90-kDa polypeptide was also recognized, albeit poorly, by mAB F. The nature of this polypeptide is unknown; it is likely to be either a breakdown product of HRI or differentially phosphorylated HRI. The latter possibility appears more likely because HRI is relatively stable in the lysate.

Effect of Antibodies on HRI Kinase Activity. To determine the effect of anti-HRI antibodies on the autophosphorylation of HRI and the phosphorylation of eIF-2α, purified HRI was preincubated with affinity-purified IgGs for 1 h in ice, and protein kinase assays were performed in the presence of eIF-2 (0.5 μg) and [γ-^{32}P]ATP. As seen in the autoradiogram (Figure 2), compared to the nonimmune serum IgG control (lanes 1 and 4), the monoclonal mAB F IgG (lanes 2 and 5) and the polyclonal IgG (lanes 3 and 6) inhibited HRI autophosphorylation and eIF-2α phosphorylation significantly and in a concentration-dependent fashion. The other monoclonal antibody (mAB A) had similar effects (results not shown). The HRI and eIF-2α bands were excised from the gels, and radioactivity (^{32}P) was measured. The extent of inhibition of eIF-2α kinase activity, however, was more marked with the polyclonal antibody (77%) than with mAB F (58%). None of the monoclonal or polyclonal antibodies had any effect on dsRNA-dependent eIF-2α kinase activity (data not shown).

Immunoprecipitation of [γ-^{32}P]ATP-Labeled HRI. A partially purified HRI preparation containing about 25 ng of HRI was phosphorylated in vitro (see Materials and Methods) and then was immunoprecipitated by the antibodies. Both polyclonal and monoclonal antibodies immunoprecipitated phosphorylated HRI (Figure 3A).

In order to ascertain further the specificity of these antibodies, HRI was immunoprecipitated in situ in reticulocyte lysates under various conditions. As seen in Figure 3B, a substantial amount of phosphorylated HRI was precipitated by both polyclonal (lane 6) and mAB F (lanes 2, 4, and 5) antibodies from heme-deficient lysates (lanes 2 and 6) as well as NEM- (lane 4) and GSSG-treated lysates (lane 5). Very litte ^{32}P-labeled HRI was precipitated in hemin-supplemented lysates (lane 3); nonimmune serum (control) did not precipitate any ^{32}P-labeled HRI (lane 7). These results are consistent with the earlier observations on the extent of the phosphorylation of HRI activated under these conditions in lysates [see London et al. (1987)]. The immunoprecipitated 92-kDa protein which comigrates with purified ^{32}P-labeled HRI (lane

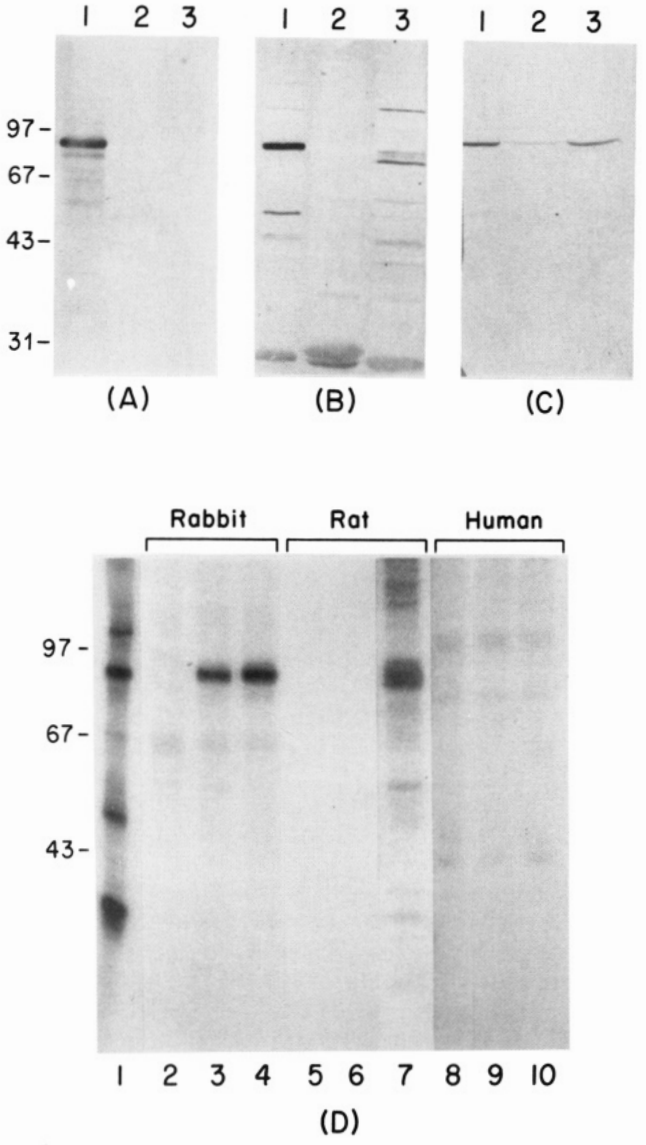

FIGURE 7: Immunoreactivity of HRI from the reticulocyte lysates of rabbit, human, and rat to anti-rabbit HRI antibodies determined by Western blot (A–C) and immunoprecipitation (D). Western blots were done by HRI monoclonal (A), HRI polyclonal (B), and anti-HeLa hsp90 (C) antibodies. In the Western blots, reticulocyte lysates (100 μg of total protein) are of rabbit (1), human (2), and rat (3). (D) [γ-^{32}P]ATP-labeled lysates were immunoprecipitated by control serum (lanes 2, 5, and 8), mAB F (lanes 3, 6, and 9), and polyclonal anti-HRI serum (lanes 4, 7, and 10). Lane 1, phosphorylated partially purified HRI and eIF-2α (as standards). Reticulocyte lysates are of rabbit (lanes 2–4), rat (lanes 5–7), and human (lanes 8–10).

1) aligned with the Western blot signals obtained by mAB F (Figure 3C). Immunoprecipitates of the NEM-treated lysates (Figure 3B, lane 4) contained some nonspecific additional phosphoprotein bands, which were also observed in the immunoprecipitates of control serum from NEM-treated lysates (data not shown).

It should be noted that NEM treatment resulted in a decreased Western blot signal as judged by peroxidase staining of HRI in the lysates. While the NEM-activated ^{32}P signal of HRI in the autoradiogram was about 5-fold greater than that in the hemin-supplemented lysate (Figure 3B, lanes 4 and 3, respectively), the peroxidase signal in the Western blot measuring total HRI protein was about one-third as great in the NEM-treated lysate (Figure 3C, lane 4) as in the hemin-supplemented (lane 3), heme-deficient (lane 2), or GSSG-treated (lane 5) lysates. This anomalous effect is, perhaps, the result of diminished recognition by the antibody of an HRI antigen alkylated by NEM.

Protein Synthesis in HRI-Depleted Lysates. To provide further information on the role of HRI in the regulation of protein synthesis during heme deficiency, HRI was completely removed from the lysate by immunoprecipitation with mAB F, and this HRI-depleted lysate was then used for protein synthesis. As seen in Figure 4 (inset), the amount of HRI (Western blot signal) present in 2.5 μL of lysate (lane 1) used for immunoprecipitation was all recovered in the immunoprecipitates of either 0.5 μg (lane 2) or 1.0 μg (lane 4) of IgG; no HRI was detected in the supernatants (lanes 3 and 5); 0.5 μg of IgG was found to be optimal for removing the HRI present in 2.5 μL of lysates. Such a HRI-depleted lysate was able to maintain almost an identical rate of protein synthesis for 20 min during heme deficiency as in hemin-supplemented lysates (Figure 4). The lower rate of protein synthesis after 20 min during heme deficiency could be due to the activation of an undetectable amount of residual HRI.

Synthesis of HRI in Reticulocyte Lysates. Although HRI is present and can be very active in the reticulocytes, little is known about its de novo synthesis in these cells. In order to study the synthesis of HRI, newly translated HRI (using the endogenous mRNA) was immunoprecipitated from [^{35}S]-methionine-labeled reticulocyte lysates. Immunoprecipitates were analyzed by SDS–PAGE and transferred to a nitrocellulose membrane. The nitrocellulose membrane was then autoradiographed (Figure 5A) and then Western blotted with mAB F (Figure 5B). Both polyclonal antibody (Figure 5A, lane 3) and mAB F (lane 4) immunoprecipitated a 92-kDa polypeptide which comigrated with the ^{32}P-labeled HRI marker (lane 1). Other [^{35}S]methionine-labeled polypeptides appeared to be nonspecific since they were also present in the nonimmune (control) serum precipitate (lane 2). In contrast, HRI polypeptide was not detected by the control serum (lane 2).

The identity of the 92-kDa polypeptide as HRI was further demonstrated by Western blotting with HRI mAB F (Figure 5B). The [^{35}S]methionine-labeled 92-kDa polypeptide is superimposed with the Western blot signal of HRI. As expected, HRI was present in all the immunoprecipitates (Figure 5B, lanes 3 and 4) except in that of the nonimmune serum (lane 2). The other bands in lanes 2–4 (Figure 5B) are due to the H and L chains of IgG reacting to anti-mouse IgG–peroxidase.

Tissue- and Cell-Type Specificity of HRI. Inhibitors of initiation of protein synthesis with properties similar to those of HRI have been isolated and purified to various extents from some nonerythroid cell types. It remains to be determined whether the eIF-2α kinase activity in nonerythroid cells is mediated by HRI or by one or more as yet uncharacterized eIF-2α kinases. We have examined this question using our monoclonal and polyclonal HRI antibodies. Total proteins extracted from various rabbit tissues were subjected to Western blot analysis (Figure 6A) with mAB F. HRI was detected only in reticulocytes (Figure 6A, lane 2) and anemic bone marrow (lane 3). No HRI signal was detected in any of the other tissues even when 10 times more total protein was used. Similar results were obtained with both polyclonal and mAB A antibodies to HRI (data not shown).

Western blot analysis of various other (nonrabbit) cell types tested (Figure 6B) revealed no cross-reacting HRI in the following cells: rat hepatoma (lane 2), HeLa (lane 3), EBV-transformed human B (lane 4), noninduced and induced K562 (lanes 5 and 6, respectively), and induced MEL (lane 7). Similar results were obtained with anti-rabbit HRI polyclonal antibody (data not shown). The inability of both polyclonal

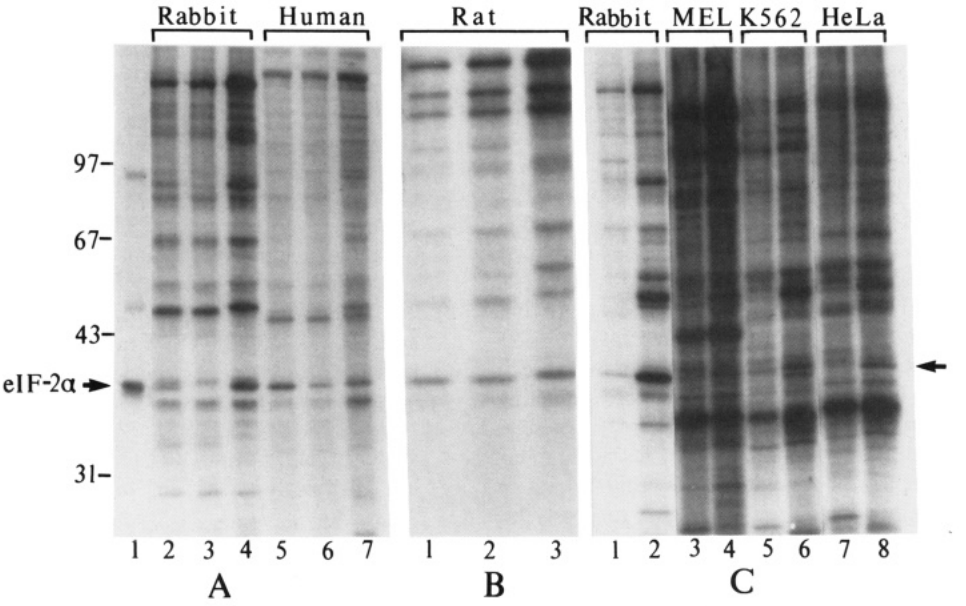

FIGURE 8: Presence of eIF-2α kinase activity in the reticulocytes of human (A) and rat (B) and MEL, K562, and HeLa cells (C). Extracts from these cell types were assayed in vitro for the presence of eIF-2α kinase activity with exogenously added rabbit eIF-2 (0.5 μg). Assays were performed in the absence of hemin [lanes 2 and 5 in (A); lane 1 in (B)], in the presence of 20 μM hemin [lanes 3 and 6 in (A); lane 2 in (B); lanes 1, 3, 5, and 7 in (C)], and in the presence of hemin and 5 mM NEM [lanes 4 and 7 in (A); lane 3 in (B); lanes 2, 4, 6, and 8 in (C)]. The figure is an autoradiogram.

and monoclonal antibodies to immunoprecipitate [^{32}P]HRI from the ^{32}P-labeled extracts of those cell types (data not shown) further supported the results obtained by the Western blot analysis.

Monoclonal antibodies did not detect cross-reacting HRI in the reticulocyte lysates of two other species, human and rat (Figure 7A, lanes 2 and 3, respectiely). Polyclonal antibody, on the other hand, detected a group of three bands in the region of HRI in rat reticulocytes (Figure 7B, lane 3) but not in human reticulocytes (lane 2). These three bands in rat reticulocytes were also seen in the immunoprecipitates of the polyclonal antibody (see Figure 7D). A ubiquitous protein, hsp90, however, was detected in the lysates of all three species (Figure 7C). It is to be noted that, in 10% Laemmli gel, hsp90 migrates more slowly than HRI (panels C and B of Figure 7) and these anti-HRI antibodies do not cross-react with hsp90. These results were further supported by immunoprecipitation analysis of the [γ-^{32}P]ATP-labeled lysates (Figure 7D). mAB F immunoprecipitated HRI only in rabbit reticulocytes (Figure 7D, lane 3). Polyclonal antibody, on the other hand, immunoprecipitated HRI in both rabbit (lane 4) and rat (lane 7) but not in human (lane 10) reticulocyte lysates.

Presence of eIF-2α Kinase Activity in the Reticulocytes of Rat and Human and in MEL, K562, and HeLa Cells. In spite of the reported heme-dependent eIF-2α kinase activity in human reticulocytes and MEL and HeLa cells, we were unable to detect cross-reacting HRI in them by anti-rabbit HRI antibodies. Therefore, we analyzed the extracts of these cells and K562 cells for the presence of eIF-2α kinase activity. The results presented in Figure 8 clearly demonstrate the presence of eIF-2α kinase activity in the reticulocytes of human (Figure 8A, lanes 5–7) and of rat (Figure 8B, lanes 1–3), although the NEM activation of the kinase in these reticulocytes was significantly lower compared to that in rabbit reticulocytes (compare lane 4 in Figure 8A with lane 7 in Figure 8A and with lane 3 in Figure 8B). The eIF-2α kinase activity in DMSO-induced MEL (Figure 8C, lane 4), K562 (lane 6), and HeLa (lane 8) cells, although detectable, was marginal and far lower than that in the rabbit reticulocytes (Figure 8C, lane 2).

Discussion

Polyclonal antibodies against rabbit reticulocyte HRI have been obtained by several investigators (Kramer et al., 1976; Petryshyn et al., 1979; Gross & Redman, 1987) using either a partially purified native HRI or a SDS-denatured HRI polypeptide excised from SDS–polyacrylamide gels. However, because of the difficulty in obtaining pure homogeneous HRI, such antibody preparations were often contaminated with antibodies to comigrating proteins [see Kudlicki et al. (1987)]. We have prepared monoclonal antibodies to HRI to obtain homogeneous HRI-specific antibodies in high titer.

In the course of three cell fusion experiments, it was evident that a highly purified preparation of HRI was necessary for obtaining HRI-specific hybridomas. Out of three experiments, we obtained two clones secreting two different monoclonal antibodies, mAB A and mAB F, of IgG_{2a} and IgG_1 subclasses, respectively. The specificity of these antibodies for HRI was evidenced by their ability to inhibit HRI kinase activities in vitro (Figure 2) and by their immunoprecipitation of HRI from reticulocyte lysates activated by various means (Figure 3B,C). The efficiency of immunoprecipitation of HRI by the antibodies, mAB F in particular, was especially noteworthy; about 5.0 μg of antibody (IgG) removed HRI completely from 25 μL of lysate.

We took advantage of the ability of mAB F to remove HRI from lysates completely by immunoprecipitation and measured the protein synthesis in such lysates under conditions of heme deficiency. The HRI-depleted lysates were able to maintain a similar rate of protein synthesis in heme-deficient lysates as in control hemin-supplemented lysates for 20 min (Figure 4). This result indicates that HRI is principally responsible for the inhibition of protein synthesis during heme deficiency.

Both monoclonal antibodies recognized SDS-denatured HRI (92 kDa) in the Western blot. They differed in other respects, however; mAB F quantitatively immunoprecipitated HRI from lysates, whereas mAB A had a low affinity for HRI and did not precipitate HRI from lysates as efficiently as mAB F. In addition, mAB A recognized an HRI–hsp90 complex while mAB F recognized only free HRI (details will be published elsewhere).

Erythroid cell specificity of HRI was apparent from our Western blot and immunoprecipitation results (Figures 6 and 7). Although an HRI-like inhibitor has been isolated from perfused rat liver (Delaunay et al., 1977), our antibodies did not detect HRI in rabbit liver, rat hepatoma cells, and several other tissues tested. Furthermore, no cross-reacting HRI was detected in human reticulocytes (Figure 7) or in K562, MEL, and HeLa cells by both polyclonal and monoclonal antibodies (Figure 6B), although low levels of eIF-2α kinase activity were observed in these cells (Figure 8). Polyclonal anti-rabbit HRI antibody detected an HRI-like protein in rat reticulocytes but not in human reticulocytes (Figure 7D). Since as little as 25 ng of ^{32}P-labeled HRI can be detected in the immunoprecipitation experiments (Figure 2A), the amount of cross-reacting HRI, in the nonerythroid tissues and cell lines tested, if present, is less than 25 ng/100 μg of total proteins in the extracts. More conclusive evidence concerning the erythroid specificity of HRI may be obtained in further studies on the presence of HRI mRNA in various cells and tissues.

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