Rat Hemopexin. Molecular Cloning, Primary Structural Characterization, and Analysis of Gene Expression†,‡

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ABSTRACT: A full-length hemopexin cDNA was isolated from a rat liver cDNA library and the derived amino acid sequence was obtained. Rat hemopexin shows a 76% amino acid homology with human hemopexin. The amino-terminal domain of rat hemopexin contains two histidine residues that are conserved in the human and rat sequences and are the most likely heme axial ligands. Analogous to human hemopexin, the rat hemopexin consists of 10 internal repeating peptide motifs characteristic of the pexin gene family. A complete conservation of cysteine residues is seen between the human and rat sequences suggesting an identical disulfide bridge structure in both proteins. Our analysis of the primary structure of rat hemopexin reveals characteristics typical for members of the pexin gene family and suggests a conserved evolutionary role for the C-terminal (non-heme-binding) domain of this protein. The full-length rat hemopexin cDNA was used to analyze changes in hemopexin gene expression during development and experimental inflammation. RNA blot analysis showed a single 2.0-kb hemopexin mRNA present in fetal liver at day 14. Hemopexin-specific mRNA was not detected in embryonic or fetal tissues at earlier stages of development and was confined to the liver throughout fetal, newborn, and adult life. The abundance of hemopexin mRNA was found to increase throughout gestation, with a sharp increase in the first postnatal weeks, reaching maximum levels in adult animals. Endotoxin-induced inflammation resulted in a 5-fold increase in hepatic hemopexin mRNA content within 48 h without associated changes in hemopexin transcript size. Adult animals exposed to hyperoxia (95% oxygen) showed a 3-fold increase in hepatic hemopexin mRNA content. Extrahepatic hemopexin gene expression was not detected during inflammation or hyperoxic exposure. The data also indicate that typical of the acute-phase response in rodents the regulation of hemopexin synthesis occurs at a pretranslational level. In contrast to many plasma proteins, hemopexin gene expression is entirely confined to the liver during development, inflammation, and tissue injury.

Hemopexin is a plasma β_2-glycoprotein with the highest known heme affinity of any characterized heme-binding protein (Muller-Eberhard, 1988). The abundance and high-affinity heme binding of hemopexin suggest a functional role for this protein as a heme scavenger essential in reutilization of heme-bound iron (Smith & Morgan, 1981) and/or in prevention of heme-catalyzed oxidative damage (Vincent et al., 1988; Gutteridge & Smith, 1988). The structural determinants of heme binding by hemopexin and the subsequent cellular metabolism of heme remain largely unknown. A high-affinity hemopexin receptor that specifically binds the heme–hemopexin complex has been reported for hepatocyte plasma membranes (Smith & Morgan, 1981), murine hepatoma cells (Smith & Ledford, 1988), placenta (Taketani et al., 1987), and human polymorphonuclear leukocytes (Okazaki et al., 1989). Although this receptor is thought to function specifically in removing hemopexin-bound heme from the serum, in in vitro experiments hemopexin appears to inhibit rather than to promote heme uptake (Sinclair et al., 1988).

Limited proteolytic digestion of hemopexin results in two equally sized homologous domains (Takahashi et al., 1985a). The N-terminal domain of hemopexin has been identified as the heme-binding domain in human (Takahashi et al., 1985a), rabbit (Morgan & Smith, 1984), and pig (Spencer et al., 1990). Protein modification and magnetic circular dichroism studies suggest that the axial heme ligands in hemopexin are histidine residues (Morgan & Muller-Eberhard, 1976; Morgan & Vickery, 1978). Although hemopexin binds heme in a one to one complex with a $K_d < 1 \times 10^{-13}$ (Hrkal et al., 1974), the heme pocket is readily accessible to solvent (Morgan et al., 1976) and one of the heme axial ligands is easily replaced with small diffusible ligands with high ligand field strength (Hrkal et al., 1981; Shaklai et al., 1981). The precise structural features that cause the exceptionally tight binding of heme to hemopexin remain unclear. A conformational change occurs in the hemopexin molecule following heme binding (Muller-Eberhard & Grizzuti, 1971). This change, which involves both the N- and the C-terminal domain, is thought to be required for the receptor-mediated heme transport (Smith et al., 1988) with the C-terminal domain providing the structural elements necessary for the cell association functions of hemopexin.

Hemopexin serum concentrations increase markedly following birth. In most species hemopexin is an acute-phase reactant with serum levels increasing several fold following experimentally induced inflammation (Muller-Eberhard, 1988). The mechanisms that determine the serum concentrations of hemopexin have not been examined. The hemopexin serum concentration in rats increases after administration of interleukin 6 (IL-6)[1] (Markinovic et al., 1989), and, similarly, this cytokine induces hemopexin synthesis in rat hepatoma cells (Baumann & Muller-Eberhard, 1987). A cytokine (IL-6) responsive cis-acting DNA motif has recently been described for several acute-phase plasma protein genes in-

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[1] Abbreviations: IL-6, interleukin 6; SDS, sodium dodecyl sulfate; aa, amino acid(s).

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cluding that of human hemopexin (Poli et al., 1989), implying transcriptional mechanisms for the regulation of the acute-phase reaction. A nuclear protein that binds to the IL-6 responsive element of hemopexin, haptoglobin, and C-reactive protein has been described (Poli & Cortese, 1989). Glucocorticoids have also been shown to alter the plasma concentrations of several acute-phase plasma proteins in the rat either by increasing the rate of transcription (Kulkarni et al., 1985) or by altering the mRNA half-life (Carter et al., 1989). Unlike most acute-phase proteins, the serum concentration of hemopexin is only slightly affected by dexamethasone administration in rat hepatocytes and hepatoma cells (Baumann & Muller-Eberhard, 1987). Besides being regulated during inflammation, the serum concentration of hemopexin increases in response to extracorpuscular heme. For example, in rhesus monkeys hemopexin serum concentrations double upon injection of heme, while the levels of other acute-phase proteins remain unchanged (Foidart et al., 1982).

In this study we have cloned a full-length rat hemopexin cDNA and determined the derived amino acid sequence. We report on a comparison of the sequences of rat and human hemopexin. We also examined the primary structure of rat hemopexin to elucidate the determinants of heme binding of the N-terminal domain and the nature of the C-terminal domain. In addition, we have used the isolated hemopexin cDNA to study the molecular mechanisms regulating hemopexin serum concentrations during development and inflammation in the rat. Because of the potential role of hemopexin in providing antioxidant protection via heme scavenging, we also examined the regulation of hemopexin gene expression during oxidative stress (hyperoxia).

Experimental Procedures

Materials. All restriction endonucleases, DNA-modifying enzymes, and cDNA synthesis reagents were purchased from IBI, BRL Pharmacia-PL Biochemicals, Promega Biotech, or Stratagene and were used according to the manufacturers recommendations. ^{32}P- and ^{35}S-labeled nucleotides were purchased from New England Nuclear, and the sequencing kit was from United States Biochemicals. Nylon membranes were purchased from Amersham and nitrocellulose was obtained from Schleicher and Schuell. The primer extension oligonucleotide was custom synthesized by Synthecell.

Animals. Adult Sprague-Dawley rats were purchased from Harlan Sprague-Dawley and were maintained on a normal diet. The rats were allowed to mate overnight and pregnancy was determined by the presence of a vaginal plug. The gestational age of the fetuses was calculated by assuming a mating took place in the previous 12 h. For oxygen exposure experiments rats were maintained in a humidified plexiglass chamber in 95% oxygen/5% air at 1 atm. The animals had free access to food and water and grew normally during the time of exposure.

Construction and Screening of cDNA Libraries. A cDNA libary was constructed from rat liver mRNA 24 h following a single intraperitoneal dose of endotoxin (100 μg). Poly(A+) RNA was isolated on oligo(dT) cellulose and cDNA was synthesized by a modification of previously described methods (Maniatis et al., 1982; Gubler & Hoffman, 1983). A partial cDNA clone for human hemopexin (Metcalfe and Muller-Eberhard, unpublished results) was labeled with [^{32}P]NTP's by nick translation and used to screen 400 000 recombinants. Positive clones were purified by plaque hybridization and analyzed following subcloning into the pUC18 vector (Yanisch-Perron et al., 1985).

Sequence Analysis. The nucleotide sequences were determined by the dideoxy/chain termination method (Sanger et al., 1977). Sequencing was performed for both strands on deletion subclones created by S1 nuclease/exonuclease III treatment (Barnes et al., 1983) followed by double-stranded sequencing of a mini-plasmid preparation (Hattori & Sakari, 1986). Sequence data were analyzed in the Rockefeller University Computing Services Facility. Homology search programs were based on the improved Pearson-Lipman algorithm (Pearson & Lipman, 1988). Secondary structure predictions were based on described prediction rules (Levin et al., 1986; Chou & Fasman, 1978).

Experimental Inflammation and RNA Isolation. Animals received a single intraperitoneal injection of 100 μg of *Salmonella typhimurium* endotoxin (Sigma #L-6511) and were sacrificed at various time points following injection. After sacrifice, the skin was sterilized with 100% ethanol, and the organs were removed and rinsed in phosphate-buffered saline and frozen in liquid nitrogen. After it was dissolved in guanidinium isothiocyanate, RNA was isolated from each tissue by cesium chloride gradient centrifugation (Chirgwin et al., 1979). RNA samples were analyzed for degradation following agarose gel electrophoresis by visualization of intact ribosomal bands with ethidium bromide. All RNA samples used had an $A_{260/280} > 1.8$.

RNA Blot Analysis. RNA blots were analyzed as previously described (Gitlin, 1988). RNA samples were denatured in formaldehyde-containing buffer and electrophoresed in 1% agarose, 2.2 M formaldehyde gels. RNA was transferred to nylon membranes (Hybond-N, Amersham) by capillary blotting and immobilized by UV irradiation. RNA blots were wetted in 5× SSPE and hybridized at 58.5 °C in a solution containing 50% formaldehyde, 5× SSPE, 5× Denhart's solution, 200 μg/mL salmon sperm DNA, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS). Hybridization was performed in the same solution for 12 h at 58.5 °C in the presence of [^{32}P]cRNA antisense transcripts (Melton et al., 1984). After hybridization the filters were washed and analyzed as previously described (Gitlin, 1988). Quantitation of RNA was performed by dot blot hybridization (White and Bancroft, 1982). All dot blots were done in serial dilution from 0.5 to 2 μg of total RNA per sample and in all cases relative hybridization varied directly with the amount of input RNA. Following autoradiography, individual dots were excised and quantitated by liquid scintillation counting. All hybridization experiments for quantitation were repeated twice on RNA samples derived from two separate experiments. There was less than 10% variation between experiments at each time point. The cDNA inserts used in this study were subcloned into bluescript (Stratagene) and included the full-length cDNA's for rat hemopexin, rat α_2-macroglobulin (Gehring et al., 1987), rat β-actin (O'Malley et al., 1987), and rat ceruloplasmin (Fleming & Gitlin, 1990).

Primer Extension Analysis. γ-^{32}P end-labeled oligonucleotide complementary to the coding sequences 87–64-bp downstream from the TATA box (Nagae and Muller-Eberhard, unpublished results; Figure 1) was annealed with 30 μg of total liver RNA from untreated, O_2-treated, and endotoxin-treated animals. The mRNA start sites were determined by using M-MLV reverse transcriptase essentially as described by Leonard et al. (1985).

Results

Cloning and Characterization of Rat Hemopexin cDNA. Following initial screening of the liver cDNA library and analysis of positive clones, a 1425-bp cDNA (pRHx1) was characterized and sequenced. pRHx1 contains eight amino

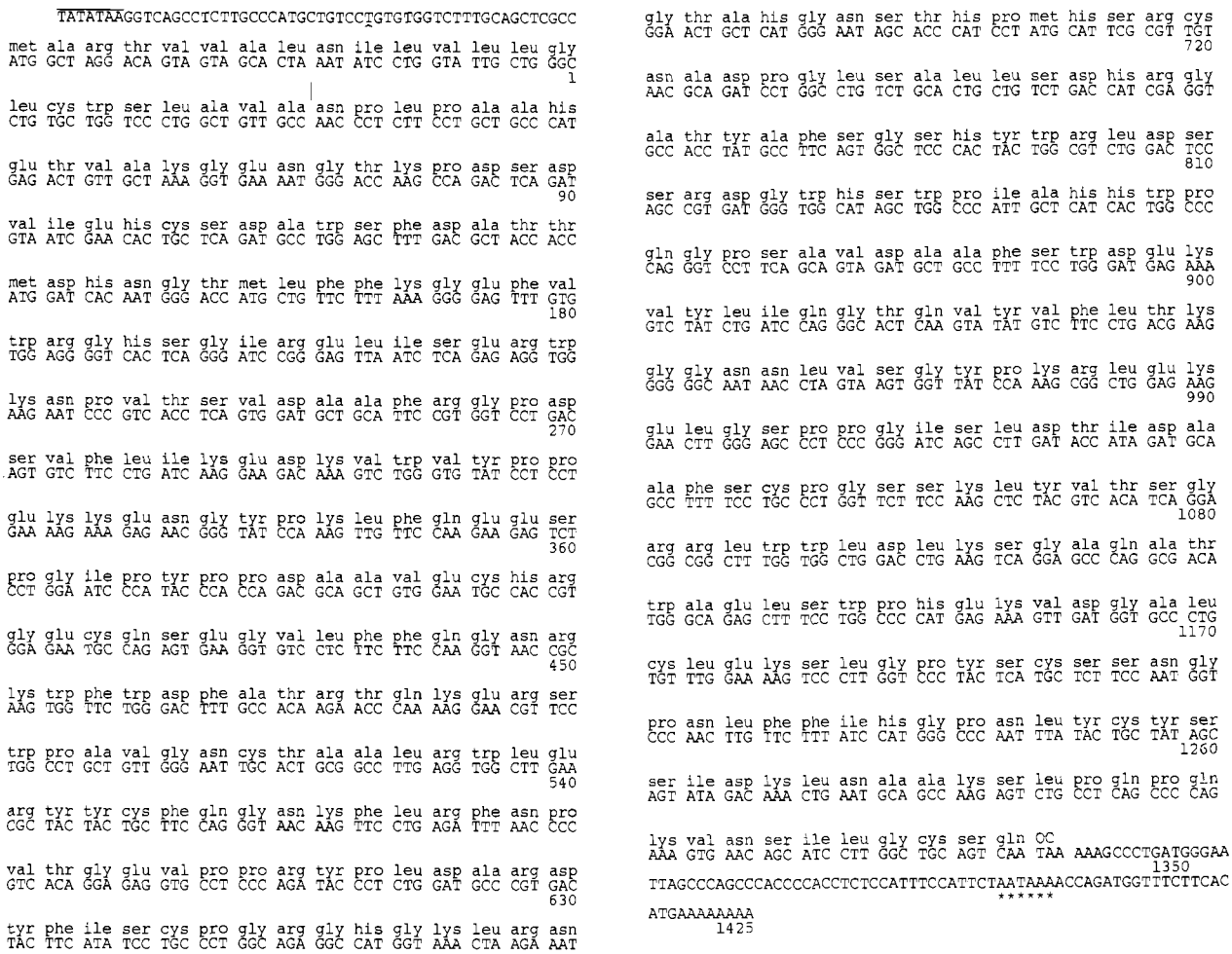

FIGURE 1: The nucleotide sequence of rat hemopexin cDNA. Numbering starts at the first nucleotide of the cDNA clone pRHx1. The 5′-untranslated sequence and the nucleotide corresponding to the first eight amino acids of the rat hemopexin signal sequence were deduced from the genomic clone (Nagae and Muller-Eberhard, unpublished results). The horizontal line shows the position of the TATA box, and the transcription initiation site (deduced from data in Figure 5) is indicated by an arrow. The vertical bar indicates the signal peptidase cleavage site as implicated by the N-terminal amino acid sequence data for rat hemopexin (Wellner et al., 1988). The polyadenylation signal is designated by stars.

acids of the putative signal sequence, the entire rat hemopexin coding region, and 90 bp of the 3′-untranslated sequence including a canonical polyadenylation site and a poly(A) tail (Figure 1). The remaining sequence corresponding to the amino-terminal portion of the signal peptide was deduced from the sequence of the rat hemopexin gene (Nagae and Muller-Eberhard, unpublished results). The Pearson–Lipman algorithm was used to search for sequence homology and revealed an overall 76% amino acid similarity with the human hemopexin sequence (Figure 2). All cysteine residues are conserved in the rat hemopexin, compared to the human hemopexin sequences, indicating that the same arrangement of disulfide bridges is present in the proteins of both species. Recent proton NMR studies (Peyton and Muller-Eberhard, unpublished results) support the earlier spectroscopic results which indicate that the heme axial ligands of hemopexin are histidines. The N-terminal domain of hemopexin contains five conserved histidine residues. Two of these are located in particularly highly conserved domains and most likely represent the heme axial ligands (Figure 2).

A comparison of the N- and C-terminal halves of rat hemopexin reveals a 22% homology, suggesting that a similar gene duplication event has taken place for the rat protein as has been described for the human protein (Altruda et al., 1985). Ten internal repeats characteristic of human hemopexin and other members of the pexin gene family (Jenne & Stanley, 1987) are also present in the primary structure of rat hemopexin (not shown).

Regulation of Rat Hemopexin mRNA Levels. The full-length hemopexin cDNA was used to examine the changes in hepatic hemopexin mRNA size and content during development. As seen in Figure 3 a single 2.0-kb hemopexin transcript was observed by RNA blot analysis of liver tissue at all stages of fetal, neonatal, and adult development, without changes in transcript size. Hepatic hemopexin mRNA was first detected on day 14 of gestation. Quantitation of these developmental changes revealed that at birth hemopexin mRNA is 40% of that found in the adult liver and that the predominant increase in mRNA content occurs during the immediate postnatal period. Hemopexin gene expression was not detected in either yolk sac, placenta, decidua, uterus, or early embryonic tissues (data not shown). In addition, hemopexin mRNA was not detected in extrahepatic tissues including lung, heart, brain, gastrointestinal tract, spleen, or kidney at any stage of fetal development from day 16 to birth (data not shown). Probing of the RNA samples with cRNA probes corresponding to two other liver-specific genes, α_2-macroglobulin and ceruloplasmin, demonstrated a pattern identical with that previously published (Flecher et al., 1988; Fleming et al., 1990), confirming the timing and labeling of samples for each point.

To investigate the molecular basis of the changes in hemopexin serum concentration during inflammation, we ana-

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                10        20        30        40        50      * 60
rat     NPLPAAHETVAKGENGTKPDSDVIEHCSDAWSFDATTMDHNGTMLFFKGEFVWRGHSGIR
        .:::.. .  .  .:..:::::.::.:.:::.:::::::::.:.:::::::::::::..:.  :
human   TPLPPTSAHGNVAEGETKPDPDVTERCSDGWSFDATTLDDNGTMLFFKGEFVWKSHKWDR
                10        20        30        40        50      * 60

                70        80        90       100       110
rat     ELISERWKNPVTSVDAAFR-GPDSVFLIKEDKVWVYPPEKKENGYPKLFQEESPGIPYPP
        :::::::::  ..:::::: :..::::::.:::::::::::.:::::.:.: :::: :
human   ELISERWKNFPSPVDAAFRQGHNSVFLIKGDKVWVYPPEKKEKGYPKLLQDEFPGIPSPL
                70        80        90       100       110       120

      120     130       140       150       160       170
rat     DAAVECHRGECQSEGVLFFQGNRKWFWDFATRTQKERSWPAVGNCTAALRWLERYYCFQG
        :::::::::::::.:::::::.:.::::.:: : ::::::::::..:::::.:::::::
human   DAAVECHRGECQAEGVLFFQGDREWFWDLATGTMKERSWPAVGNCSSALRWLGRYYCFQG
              *130       140       150       160       170       180

      180       190       200       210 *     220*    *   230
rat     NKFLRFNPVTGEVPPRYPLDARDYFISCPGRGHGKLRNGTAHGNSTH--PMHSRCNADPG
        :.::::.:: :::::::: :.::::.:.:::::::. :::::.:::::::  : . ::...
human   NQFLRFDPVRGEVPPRYPRDVRDYFMPCPGRGHGH-RNGTGHGNSTHHGPEYMRCSPHLV
                190       200       210  *    220*    * 230

        240       250       260       270       280       290
rat     LSALLSDHRGATYAFSGSHYWRLDSSRDGWHSWPIAHHWPQGPSAVDAAFSWDEKVYLIQ
        :::: ::..:::::::.:::::::.:::::::::::::::.:::::::::::::.::.::.:
human   LSALTSDNHGATYAFSGTHYWRLDTSRDGWHSWPIAHQWPQGPSAVDAAFSWEEKLYLVQ
      240       250       260       270       280       290

        300       310       320       330       340       350
rat     GTQVYVFLTKGGNNLVSGYPKRLEKELGSPPGISLDTIDAAFSCPGSSKLYVTSGRRLWW
        :::::::::::: .:::::::::::.:.:.:: ::..:::: :::::.:... .::::::
human   GTQVYVFLTKGGYTLVSGYPKRLEKEVGTPHGIILDSVDAAFICPGSSRLHIMAGRRLWW
      300       310       320       330       340       350

        360       370       380       390       400       410
rat     LDLKSGAQATWAELSWPHEKVDGALCLEKSLGPYSCSSNGPNLFFIHGPNLYCYSSIDKL
        :::::::::::.::.::::::::::.::::: :::.:::.:..:::::::::::..:::
human   LDLKSGAQATWTELPWPHEKVDGALCMEKSLGPNSCSANGPGLYLIHGPNLYCYSDVEKL
      360       370       380       390       400       410

        420       430
rat     NAAKSLPQPQKVNSILGCSQ
        :::::.:::::.:.:.:::..
human   NAAKALPQPQNVTSLLGCTH
      420       430       440
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FIGURE 2: Comparison of rat and human hemopexin amino acid sequences. The numbering starts from the first amino acid of the mature protein; double dot indicates an exact match and a single dot a conservative match. The conserved histidines in the N-terminal and hinge regions are marked with stars. An arrow is drawn underneath arginine-206 to designate the trypsin cleavage site of human hemopexin that divides the protein into two domains. The human Hx amino acid sequence is from Takahashi et al. (1985b).

lyzed hemopexin mRNA size and content in several rat tissues 23 h after a single intraperitoneal dose of saline (C) or endotoxin (I) (Figure 4A).

Endotoxin-induced inflammation resulted in a 4–5-fold increase in hepatic hemopexin mRNA content without changes in hemopexin transcript size. This effect was tissue-specific because no hemopexin mRNA was detected in brain, heart, gastrointestinal tract, lung (Figure 4A), spleen, kidney, adrenals, testes, or skeletal muscle (data not shown) during the time course examined. Reprobing of this blot with a cRNA probe for β-actin confirmed that each lane contained equivalent amounts of RNA (data not shown).

The rat hemopexin cDNA was also used to examine the effects of hyperoxia on hemopexin gene expression in hepatic and extrahepatic tissues of adult rats. As noted above a single hemopexin transcript was detected in the livers of adult rats breathing room air (Figure 4B, control) and the abundance of this transcript was increased 46 h after a single interperitoneal dose of endotoxin (Figure 4B, endotoxin). The abundance of hepatic hemopexin mRNA was similarly increased in rats continuously breathing 95% oxygen for 46 h (Figure 4B, oxygen), and as seen with endotoxin treatment this increase was not associated with changes in transcript size. No hemopexin mRNA was detected in extrahepatic tissues of rats breathing 95% oxygen for up to 75 h, including kidney, lung, heart, spleen, gastro-intestinal tract, and brain (data not shown).

In uninduced, oxygen-treated, and endotoxin-treated rats, hemopexin mRNA transcription is initiated at a single site (Figure 5; compare with Figure 1). No other transcription initiation sites are evident within 300 upstream of this site (partially shown in Figure 5). The transcription initiation site indicated in Figure 5 confirms the result previously obtained by using RNA from IL-6 induced rat hepatoma cells for the primer extension analysis (Nagae and Muller-Eberhard, unpublished results).

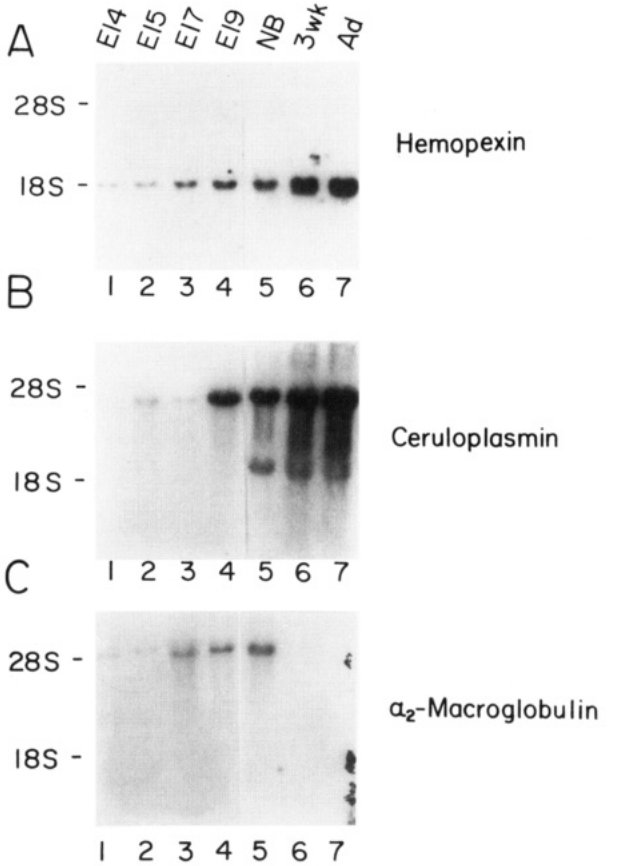

FIGURE 3: Expression of the hemopexin gene in liver during development. RNA blot analysis of hemopexin, ceruloplasmin, and α_2-macroglobulin mRNA in liver from fetal (14, 15, 17, and 19 days), newborn, and adult rats as indicated. Conditions for hybridization are as described in the text. The blot was exposed to XAR film at −70 °C for 5 h.

Because the changes in hepatic hemopexin mRNA content following endotoxin or hyperoxic exposure were not associated with qualitative changes in transcript size, we quantitated these effects using dot blot analysis. As shown in Figure 4C, the abundance of hepatic hemopexin mRNA increased 2-fold within 15 h of endotoxin treatment and this effect continued up to 75 h, reaching a maximum of 5–6-fold. Although the magnitude of the increase in hemopexin mRNA was less during hyperoxic exposure, the kinetics of this process were similar to that seen with endotoxin. A maximum 4-fold increase in hepatic hemopexin mRNA was consistently seen within 46 h of hyperoxia and this did not further increase in animals exposed for periods of up to 85 h (data not shown).

Discussion

The purpose of this study was to isolate a full-length cDNA clone corresponding to rat hemopexin, to characterize and sequence the cDNA, to elucidate the primary structure of rat hemopexin, and to study the molecular mechanisms of hemopexin gene expression. The sequence data reported here agree well with those reported for human hemopexin. The hemopexins of rat and human share a high degree of homology at the amino acid level (76%), and a comparison of the intradomain disulfide bond formation reveals a similarity in their N-terminal and C-terminal domain structure (Takahashi et al., 1985b). The perfect conservation of the cysteine residues of rat and human hemopexin indicates that the same disulfide configuration is present in both proteins.

The internal repeats determined for rat hemopexin are characteristic for those found for other members of the pexin gene family (Altruda et al., 1985; Jenne & Stanley, 1987).

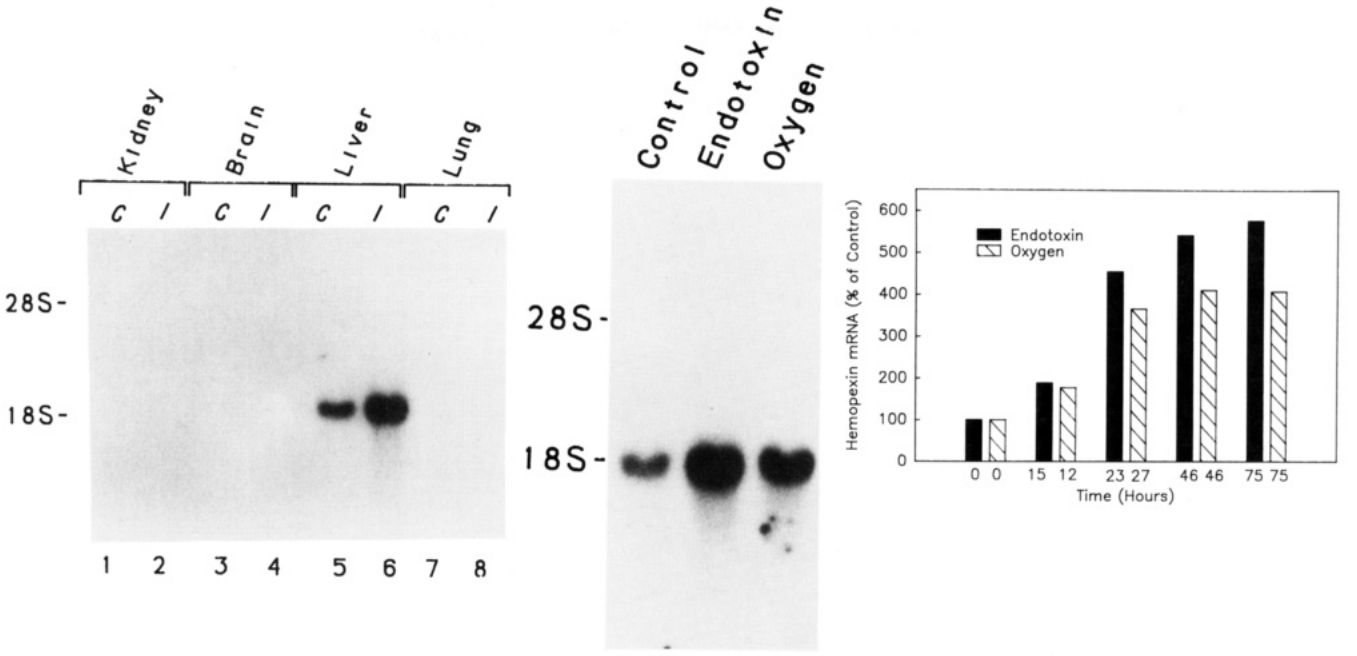

FIGURE 4: Analysis of hemopexin mRNA during endotoxin-induced inflammation and hyperoxia. (A, Left) RNA blot analysis of hemopexin mRNA in the kidney, brain, liver, and lung of an adult male Sprague-Dawley rat 23 h following a single intraperitoneal injection of saline (C) or 100 μg of endotoxin (I). Hybridization and washing of the blot were performed as described in the text. The blot was exposed to XAR film at −70 °C for 4 h. (B, Middle) RNA blot analysis of hemopexin mRNA in the liver of adult Sprague-Dawley rats 46 h following intraperitoneal saline (control) or endotoxin (endotoxin) or following 46 h of continuous exposure to 95% oxygen (oxygen). Blot was exposed for 4 h. (C, Right) Kinetics of hemopexin mRNA changes in liver of adult Sprague-Dawley rats following 100 μg of endotoxin ip (endotoxin) or exposure to 95% oxygen (oxygen) for the indicated tissues. Data were quantitated by dot blot analysis as described in the text. The data shown are expressed relative to the abundance of hemopexin mRNA in control animals (0 time point), which did not change following saline injections throughout the time course shown. The data are from one of three time-course studies and each sample was hybridized in duplicate with less than 12% variation between time points. Each point represents an individual animal and the changes shown were identical in male and female animals.

The conserved glycine residues that are present in the 10 repeats of human hemopexin, human vitronectin (Suzuki et al., 1985), and human collagenase (Goldberg et al., 1988) are not as well conserved in the rat hemopexin sequence. This is also the case when the human proteins are compared to rat collagenase, another member of the rat pexin gene family (Martisian et al., 1985).

The extensive homologies in the sequences of the rat and human hemopexin suggest that it is a physiologically important peptide. The structure of this protein is similar in all vertebrates as judged by immunologic cross reactivity (Cheng et al., 1988) and by the limited protein sequence data available (Wellner et al., 1988). The N-terminal half of the molecule appears to be the heme-binding domain whereas the C-terminus is thought to function in the cellular association of hemopexin (Morgan & Smith, 1984). The highly conserved C-terminal region shared by the rat and the human hemopexin has also been noted in other species by immunological techniques (Cheng et al., 1988), which supports the notion that the C-terminus is essential for hemopexin function or disposal. Heme binding by the N-terminal domain is several orders of magnitude lower as compared to that in the intact molecule, suggesting that the C-terminus provides conformational stability to the folding of the heme-binding domain (Smith et al., 1988). In the N-terminal region five histidine residues are conserved. Three of these are present in the hinge region of the protein (aa 207–231), falling within a less conserved region of the homology pattern (Figure 2). The numerous amino acid deletions or additions between rat and human hemopexin in this region make it unlikely that these histidines serve as axial heme ligands. The most likely heme axial ligands are histidines 56 and 126 in rat hemopexin and 56 and 127 in human hemopexin (Figure 2). These residues are within or near sequences that show a higher than average degree of homology. When a perfect α helix is modeled around rat hemopexin histidine 56 by using an Edmundson wheel, some interesting features of the protein structures in both species become apparent: (1) a tryptophan residue (aa 53) is located close to the heme edge as suggested by previous spectroscopic evidence; (2) an arginine residue (aa 60) is at the mouth of the heme crevice and might interact with one of the heme propionates; (3) a cluster of hydrophobic residues is positioned a short distance toward the interior of the protein. The second putative heme ligand appears to be in the loop region that is stabilized by a disulfide bridge.

During development hemopexin gene expression is entirely confined to the liver. This is in contrast to other acute-phase plasma proteins, such as α_2-macroglobulin and α_1-acid glycoprotein, which are both abundantly expressed in decidua and the yolk sac (Choy et al., 1989; Thomas et al., 1989), and ceruloplasmin, which is equivalently expressed in fetal liver and lung tissue (Fleming & Gitlin, 1990). The temporal pattern of hemopexin gene expression in the liver is consistent with the observed fetal serum levels of hemopexin in human, rabbit, and chicken (Muller-Eberhard et al., 1975; Ross et al., 1971; Grieniger et al., 1986). In addition, previous clinical studies have indicated that serum hemopexin levels increase by 10–100-fold from fetal to adult life (Hanstein & Muller-Eberhard, 1968). This result suggests that the developmental regulation of hemopexin in the rat is similar to that in other species and that this regulation occurs in large part at a pretranslation level.

The present study also demonstrates that the increase in hemopexin serum concentration during inflammation appears to be regulated for the most part at the mRNA level. Endotoxin-induced inflammation results in an increase in the

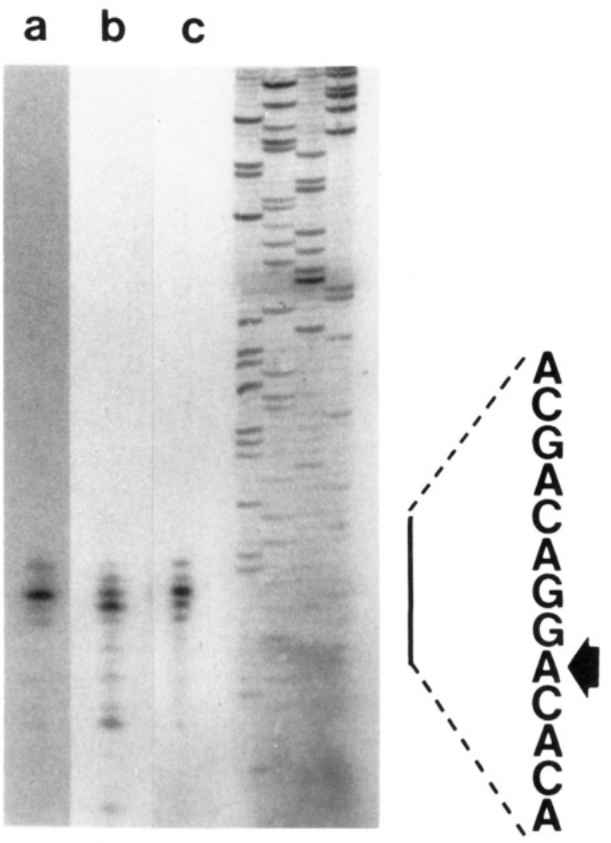

FIGURE 5: Primer extension analysis of hemopexin mRNA from uninduced, O_2-treated, and endotoxin-treated rats. Lanes a, b, and c: Primer extension for 30 μg of total liver RNA from oxygen treated, untreated, and endotoxin treated rats, respectively. Sequence: ^{35}S-sequence ladder (from left to right: G, A, T, C) from the primer extension oligonucleotide primer.

content of hepatic hemopexin-specific mRNA within 15 h following endotoxin administration. This finding is consistent with the observation of an increase of hemopexin serum levels following experimental inflammation in both rats and rabbits (Metcalfe and Muller-Eberhad, unpublished results). In contrast to other acute-phase proteins in the rat including transferrin (Schaeffer et al., 1989), α_2-macroglobulin (Choy et al., 1989), α_1-acid glycoprotein (Thomas et al., 1989), serum amyloid A protein (Meek & Bennett, 1989), and ceruloplasmin (Fleming & Gitlin, 1990), hemopexin gene expression is highly liver specific during the acute-phase response. These results are consistent with the finding that human hemopexin is not expressed in kidney, spleen, or placental cells (Poli et al., 1989).

The effects of hyperoxia on the acute-phase response has not been well-studied. Recent studies in our laboratories indicate that ceruloplasmin gene expression is induced in a tissue-specific fashion in lung tissue by hyperoxic exposure (Fleming et al., 1990). In these same studies, an increase in the hepatic expression of α_2-macroglobulin, a marked acute-phase reactant in the rat, was also observed. In the experiments reported here hyperoxia resulted in an increase in hepatic hemopexin mRNA without expression of hemopexin mRNA in other tissues. The effect of hyperoxia on hemopexin gene expression in liver could be the result of an inflammatory response secondary to hyperoxic tissue injury. However, the failure to see changes in the expression of other acute-phase reactants during oxygen exposure suggests some specificity to this process. The fact that transcription initiates from the same site in uninduced, oxygen-treated, and endotoxin-treated animals excludes the possibility that transcription initiation from an alternate promotor, at a distance not discernible as differences in the mRNA sizes on the Northern blots, would be responsible for this specificity. Changes in local oxygen tension in liver tissue have been shown to affect hepatic gene expression (Jungermann et al., 1989) and hemopexin induction by hyperoxia could involve an oxygen-sensing regulatory factor. Such an oxygen sensor has been shown to function as a trans-activator of erythropoietin gene expression in hypoxic conditions (Goldberg et al., 1988). The relationship of the hyperoxia-induced changes in hemopexin mRNA levels to the role of hemopexin as an antioxidant requires further analysis. The highly tissue-specific pattern of hemopexin gene expression during development, inflammation, and tissue injury suggests that unique cis-acting DNA sequences and/or trans-acting factors may determine hemopexin gene expression in liver. A search for such factors is currently under way in our laboratories, utilizing the rat hemopexin gene.

Registry No. DNA (rat liver clone pRHx hemopexin mRNA complementary), 130405-57-1; hemopexin (rat liver clone pRHx1 precursor reduced), 130405-58-2; hemopexin (rat liver clone pRHx1 hemopexin-specifying messenger), 130405-59-3; RNA (rat liver clone pRHx1 hemopexin-specifying messenger), 130405-60-6.

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