Developmental Expression of Heme Oxygenase Isozymes in Rat Brain

TWO HO-2 mRNAs ARE DETECTED*

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Blot hybridization of RNA isolated from rat brain revealed the presence of two HO-2 homologous transcripts (1.3 and 1.9 kilobases (kb)) at all stages of development ranging from 1 day before birth to adulthood. The level of both HO-2 messages appeared to be developmentally regulated and a gradual increase was observed from prenatal day 1 to adulthood. The two transcripts were highly homologous as assayed through hybridization studies using probes derived from the 3′ end, middle, and 5′ end of a cloned rat testis HO-2 cDNA. The 1.3-kb mRNA was essentially identical in size to the testis HO-2 cDNA. The message was efficiently translated in the brain, and is believed to encode the HO-2 protein. It seems unlikely that the 1.9-kb species represents a precursor of the 1.3-kb mRNA, as it was also translated in vivo, although less efficiently than the smaller mRNA species. Neither of the two HO-2 mRNA species were induced by bacterial endotoxin. Unlike HO-2, only one HO-1 transcript of ~1.8 kb could be detected. This transcript was of very low abundance and was not developmentally regulated, but could be increased by bacterial endotoxin. The product of this induced message, however, was not detectable by Western immunoblot analysis using antibody raised against liver HO-1. An immunoprecipitate could be detected in brain microsomes by radioimmunoassay using the same antibody. This protein, however, exhibited antigenic properties different from that of the purified liver HO-1 or that of spleen microsomal HO-1. Brain heme oxygenase activity correlated well with the amount of immunoreactive HO-2 protein and both reflect the abundance of the 1.3-kb mRNA message over the course of development.

Oxidative cleavage of the α meso carbon bridge of heme b (Fe-protoporphyrin IX), resulting in the formation of biliverdin IXα, is catalyzed by the microsomal heme oxygenases (1). The concerted activity of NADPH-cytochrome P-450 reductase is essential for this activity. In mammalian species and certain fish, the dual nucleotide-dependent cytosolic enzyme, biliverdin reductase, converts biliverdin to bilirubin (2, 3). In placental animals this conversion is postulated to be essential for the elimination of heme degradation products from the developing fetus (4).

In all systems tested to date, including human, rabbit, and rat, two isozymes of heme oxygenase have been identified (5–8). These isozymes, which have been referred to as HO-1 and HO-2, are encoded by different genes (9) and are related only by their catalytic activities and regions of homologous primary sequence (9, 10). HO-1, which has been recently identified as the 32,000-dalton stress protein (11–13), is the form inducible by a variety of exogenous and endogenous chemicals, including bacterial endotoxins (1, 14–16), and is found ubiquitously in all organs with the exception of the brain (8, 17). HO-1 levels in the adult rat and rabbit brain are below both the detection limit of Western immunoblotting and the resolving capability of routine purification schemes (8, 17). In contrast, HO-2 which is apparently the noninducible isozyme (5), is highly abundant in rat and rabbit brain.

Given the precedence for the developmentally linked repression of gene expression for hemoproteins such as fetal hemoglobin and certain cytochrome P-450s (18–20), the disparate representation of HO-1 and HO-2 in the adult brain could perhaps be attributed to a developmentally related differential regulation of the two genes. Alternatively, the brain HO-1 gene could be transcriptionally and/or translationally inactive throughout development.

We have examined the molecular basis for the very low or negligible level of HO-1 protein in the adult rat brain, and have compared the relative abundance and expression of the mRNAs encoding the two heme oxygenase isoforms over the course of rat brain development. We present evidence for the existence of two HO-2 homologous transcripts and demonstrate that both HO-2 messages are in significantly greater abundance than the single HO-1 message species in rat brain. Furthermore, the levels of both HO-2 mRNAs, but not of HO-1 mRNA, are developmentally regulated. Only one HO-2 message species seems to be efficiently translated to a protein immunohistochemically similar to rat testis HO-2.

MATERIALS AND METHODS

Restriction enzymes were purchased from Boehringer Mannheim. Two oligonucleotide primers were obtained from Research Genetics: E2 (5′-TGCACTCCTGCGAGAGAT-3′), homologous to HO-1 cDNA nucleotides +71 to +90, and E3 (5′-AGGAAACTGAGTGAGGAC-3′), complementary to HO-1 cDNA nucleotides +833 to +814 (21). Sprague-Dawley rats were used as tissue source for all experiments. Rat liver HO-1 and rat testis HO-2 were purified as described before (17) and resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 20% (v/v) glycerol and used for measurement of heme oxygenase activity or Western immunoblotting. Biliverdin reductase was purified as described before (3). For induction experiments, adult rats were given intraperitoneal injections of bacterial endotoxin (Salmonella typhimurium lipopolysaccharide, 1.5 mg/kg, Sigma). The control rat was given saline subcutaneously. Twelve hours post-administration, rats were killed, brains removed, and mRNA prepared as described below.

Probes—We have recently described the isolation and character-
ization of a 1300-bp HO-2 cDNA purified from a rat testis cDNA library (10). In the present study we used the full length cDNA: a 221-bp PurI/EcoRI fragment derived from the 3' end of this HO-2 clone (nucleotides +888 to +1108), a 290-bp EcoRI/SacI fragment derived from the 5' end of the same clone (nucleotides +1 - +104), or a 274-bp cDNA fragment (9) corresponding to a central portion of this clone containing nucleotides +437 to +688, as HO-2 hybridization probes (10).

A cDNA fragment corresponding to HO-1 nucleotides +71 to +833 reported by Shibahara et al. (21) was generated via an adaptation of the polymerase chain reaction technique as described previously (22). Briefly, 6.5 µg of poly(A)* RNA, isolated from adult rat spleen, was used as template for first-strand cDNA synthesis using a Bethesda Research Laboratories (BRL) cDNA synthesis kit according to the manufacturer's specifications. Following phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation, 60 ng of the recovered cDNA products were added as template to a standard PCR reaction mixture (22) using HO-1-specific oligonucleotide probes E2 and E3 as primers. After 20 cycles of amplification, 16% of the primary reaction product was purified from an ethidium bromide-stained 1.2% agarose gel and prepared for use as an HO-1 hybridization probe as described below. The identity of the purified cDNA fragment was confirmed by subjecting a second aliquot to direct sequencing with the "P-labeled E2 oligonucleotide (9) by dideoxynucleotide chain termination according to the method of Higuchi et al. (23). All probes used during the course of this study, including the mouse a-actin cDNA probe (24) were labeled by the random priming method according to the manufacturer's instruction (Random Primers DNA Labeling System, BRL).

**Polysome Extraction, Sucrose Gradient Analysis of Polysome Extracts, and RNA Isolation**—Two grams of frozen rat brain tissue were homogenized in 1.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl2, 50 µM/ml cycloheximide, 200 µg/ml heparin, and 0.2 µl/ml diethyl pyrocarbonate) in a Dounce homogenizer on ice. The homogenate was centrifuged at 5,000 x g for 5 min at 4°C, and the resulting supernatant spun at 25,000 x g for 5 min at 4°C. The post-mitochondrial supernatant was recovered and aliquots corresponding to 20 A260 units each were immediately fractionated by layering onto 11.0 ml 7-47% linear sucrose gradients (containing 50 mM Tris acetate, pH 7.0, 50 mM NaCl, and 12 mM MgCl2), and centrifuging at 27,000 rpm for 4 h at 4°C in an SW-41 rotor. The gradients were then analyzed through an ISCO model 183 gradient fractionator equipped with a model UA4 absorbance monitor. Fifteen µg of carrier yeast tRNA, 100 µl of 3 M sodium acetate, pH 5.2, and 2 volumes of 100% ethanol were added to each 1.0 ml fraction, and the samples were allowed to precipitate at −20°C overnight. The precipitate was collected and resuspended in 0.5 ml of 10 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 3 mM EDTA, 0.1% diethyl pyrocarbonate and 1% SDS. Resuspended fractions were then extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) at 65°C for 3 min and once with an equal volume of chloroform at room temperature. Each sample was then precipitated in 2.5 volumes of ethanol in a dry ice/ethanol bath, collected by centrifugation, dried under vacuum, and resuspended in 10 µl of sterile water. The entire sample of each was subjected to Northern blot hybridization analysis as described below for rat brain total RNA.

**RNA Preparation and Northern Blot Analysis**—Total RNA was purified from rat brain by the guanidine isothiocyanate/cesium chloride method as described by Chirgwin et al. (25). Poly(A)* RNA was isolated by oligo(dT)-cellulose chromatography (26) and was fractionated on a denaturing formaldehyde-agarose (1.2%) gel and transferred to nitrocellulose paper. The filter was baked at 80°C for 2 h, prehybridized for 6-12 hr at 42°C with 10 ml of hybridization solution containing 25 mM KPO4, pH 7.4, 5 x SSC, 5 x Denhardt's solution (26), 50 µg/ml salmon sperm DNA, and 50% deionized formamide, and hybridized for 12-16 hr at 42°C in 5 ml of the above hybridization solution containing 10% dextran sulfate and the appropriate "P-labeled cDNA probe (2-5 x 106 cpm/µg, 1-2 x 107 cpm/ml). The filter was then washed at 47°C for 2 x 15 min with 1 x SSC, 0.1% SDS and 2 x 15 min with 0.25 x SSC, 0.1% SDS, air-dried, and exposed to x-ray film (Kodak) with intensifying screen at −80°C. If the filter was to be reprocessed, the original probe was removed by treating the filter with boiling water for 10 min.

**Radioimmunoassay (RIA)—** Purified rat liver HO-1 (20 µg) was

1. The abbreviations used are: bp, base pair(s); SSS, sodium dodecyl sulfate; kb, kilobase(s); RIA, radioimmunoassay.

**RESULTS AND DISCUSSION**

In our previous studies, using chromatographic procedures and Western immunoblotting techniques, we were unable to detect HO-1 protein in rat brain (8, 17). In the present study we have used more sensitive and specific RIA techniques to reexamine the presence of HO-1 protein in brain microsomes. Solubilized microsomes from an HO-1-enriched organ, spleen, were used as a positive control. As shown in Fig. 1, when purified HO-1 protein (reference HO-1) was added to brain microsomes (50 µg), the inhibition curve (O) had the same slope (0.62 ± 0.05 versus 0.59 ± 0.05) as that generated by spleen microsomes (Δ). This parallelism between the two curves indicates that the RIA system is capable of measuring HO-1 protein in a microsomal preparation. When the RIA was used to measure HO-1 in brain microsomes, however, the inhibition curve generated was not parallel to spleen or reference HO-1 and appeared to plateau at about 40% binding (Fig. 1, inset). These data suggest that the factor(s) responsible for immunoreactivity with antisera to HO-1 in the brain microsomes are antigenically dissimilar from those present in spleen or purified HO-1. This antigenic dissimilarity may reflect the existence of a modified form of HO-1 protein sharing limited antigenic epitopes with the previously characterized HO-1 protein (5). Based on our previous RIA data showing that purified testis HO-2 protein is not recognized by HO-1 antisera (31), we suspect that the presently observed binding inhibition does not result from cross-reactivity of brain microsomal HO-2 protein with the HO-1 antibody. At this time, however, we cannot rule out the possibility of nonspecific interactions in the RIA system causing the observed behavior.

To determine whether the apparent low abundance of HO-1 protein in the brain reflects a similar paucity of HO-1 mRNA, or is due to inefficient translation of a relatively abundant message species, we assayed the steady-state level of HO-1 homologous mRNA in adult rat brain and compared it with that of the spleen (Fig. 2a). When hybridized with an...
FIG. 1. Radioimmunoassay analysis of HO-1 in adult rat brain. Brain microsomal fractions obtained from adult rats were solubilized and used for assessment of HO-1 protein concentration by RIA as described under "Materials and Methods." Spleen microsomal fractions, used as the positive control, decreased radioligand (\textsuperscript{125}I-HO-1) binding in a dose-dependent fashion. Shown (\(\Delta\)) is the computer-fitted inhibition curve for doses of spleen microsomes ranging between 1.56 ng (86% binding) and 200 ng (18.9% binding). The slope of this curve was 0.59 ± 0.05. The computer-fitted inhibition curve, slope of 0.62 ± 0.05, for purified HO-1 (reference HO-1, 30–240 ng, 56.9% to 39.5% binding) added to 50 ng of brain microsome protein is shown (○). The inset shows inhibition of \textsuperscript{125}I-HO-1 binding to antibody by solubilized microsomes from adult rat brain (○). Raw data are shown in this case because the inhibition curve generated by brain microsomes was not parallel to that for spleen microsomes or purified HO-1 mixed with brain microsomes, and appeared to plateau at 40–50% binding.

HO-1 cDNA probe prepared by a polymerase chain reaction as described under "Materials and Methods," a blot of poly(A\(^+\)) RNA showed that a discrete message of ~1.8 kb is present in each organ. The high abundance of HO-1 mRNA in spleen (lane 1) reflects the fact that this is the organ known to be highly enriched in HO-1 protein (7). Although detectable, only very little of the ~1.8-kb message was observed in brain poly(A\(^+\)) RNA (lane 2). It is therefore likely that the tissue-specific regulation of HO-1 expression occurs primarily at the level of transcription. At this time, however, the possibility of a role for differential message stability cannot be ruled out. Since no HO-1 homologous mRNA species other than the ~1.8-kb form were detected in the brain poly(A\(^+\)) RNA sample, it seems that the putatively modified form of HO-1 protein, referred to above, could arise through co- or post-translational modification of the "standard" HO-1 protein.

Transcriptional regulation of this low abundance HO-1 message was tested in vivo by exposure to bacterial endotoxin. The amount of poly(A\(^+\)) RNA assayed was doubled to achieve better visualization of the HO-1 message in the control sample. As shown in Fig. 2b (top panel), when compared with the control (lane 1) administration of endotoxin (lane 2) increased brain HO-1 mRNA abundance by ~3-fold after normalization with \(\alpha\)-actin (bottom panel). This observation indicates that, brain HO-1 transcription can be regulated by endotoxin, an inflammatory agent which causes fever, supporting the possibility of brain HO-1 being a heat-shock protein (32). We note, however, that HO-1 protein remained undetectable in 250 \(\mu\)g of microsomal samples from the brains of treated animals, as assayed by Western blot analysis. This observation is consistent with the above suggestion regarding the possible existence of a post-translationally modified HO-1 protein.

It is well known that rat liver heme oxygenase activity is developmentally regulated and that the peak of activity occurs between days 4 and 7, and then gradually decreases to the adult level by day 21 (33, 34). The abundance of HO-1 mRNA in the liver closely reflects this developmental pattern (35). Accordingly, the possibility was examined that HO-1 protein and mRNA are similarly more abundant in the early postnatal brain and that our observations for adult brain reflect the developmentally dictated repression of HO-1 expression. Western blot analyses of 200-\(\mu\)g samples of brain microsomal proteins obtained from rats at various stages of development are presented in Fig. 3. As shown in the top panel, HO-1 protein could not be detected in rat brain microsomes at any stage of development. In contrast, HO-2 protein (middle panel) displayed a gradual increase in abundance from 1 day before birth to adulthood. Identical samples were run on a Coomassie-stained gel to demonstrate their mass equivalency (bottom panel). As noted in Fig. 4, quantitative data obtained through densitometric analysis of this and similar Western blots indicate that the amount of immunoreactive HO-2 protein increases approximately 2-fold between 1 day before birth and adulthood. This is in good agreement with heme oxygenase-
Fig. 2. Relative distribution of HO-1 mRNA in rat spleen and brain and induction of brain HO-1 mRNA. Poly(A') RNA was isolated from adult rat spleen, brain, or the brain of adult rats treated with endotoxin (1.5 mg/kg, intraperitoneally) and killed 12 h later. Northern analysis was performed as detailed under "Materials and Methods." a, lane 1, spleen; lane 2, brain. Two micrograms of poly(A') RNA were loaded. An HO-1 cDNA fragment was used as the hybridization probe. The film was exposed at -80 °C for 28 h with intensifying screen. b, four µg of poly(A') RNA was loaded in each lane. The blot was probed with a 32P-labeled HO-1 cDNA fragment, then exposed at -80 °C for 44 h with intensifying screen (top panel). The same blot was subsequently reprobed with α-actin (bottom panel). Lanes 1 and 2 contained samples from control and endotoxin-treated rats, respectively.

To determine whether brain HO-1 transcript abundance is subject to developmental regulation, we carried out gel blot hybridization analysis of poly(A') RNAs from rats at various stages of maturation. As shown in Fig. 5a, an HO-1 mRNA of very low abundance was detected at all ages tested (top panel), but no developmental regulation was observed as the -1.8-kb transcript was of nearly equal abundance at all time points after normalization with α-actin (bottom panel). This observation was in good agreement with protein radioimmunoassay data that yielded binding-inhibition curves similar to that presented in the inset of Fig. 1 when solubilized brain microsomes from young rats (1 day and 14 days) were tested. The interesting aspect of these findings is that unlike the liver HO-1 gene, which is developmentally regulated at the transcriptional level (35), the brain HO-1 gene exhibits no developmentally linked regulation, yet retains the ability to respond to inducing agents such as bacterial endotoxin.

Recently, we have described (10) the isolation and sequencing of an essentially full length HO-2 cDNA corresponding to a 1.3-kb mRNA species detected in rat testis poly(A') RNA. Presently, we have also detected a second HO-2 homologous message of ~1.9 kb in the brain. As shown in Fig. 5b, top panel, and in Table I, both HO-2 homologous mRNAs are present at essentially equal relative abundance throughout development and exhibit increases in absolute abundance with age. Densitometric quantitation of this autoradiogram indicates that when normalized to the signal obtained with an α-actin cDNA probe (Fig. 5b, bottom panel), each HO-2 mRNA species increased about 3-fold in abundance during development (Table I). The transcriptional regulation of HO-
Northern blot analysis of developmental changes in both HO-1 and HO-2 mRNA levels. Poly(A)+ RNA was isolated from brains of rats at various stages of maturation and subjected to blot hybridization analysis as described under "Materials and Methods." a, the blot was probed with a 32P-labeled HO-1 cDNA fragment (top panel), and subsequently with an α-actin cDNA probe (bottom panel). b, the blot was hybridized with a 32P-labeled full length HO-2 cDNA probe (top panel), and subsequently with an α-actin cDNA probe (bottom panel). Lanes 1–7 contained 2 µg of poly(A)+ RNA from rat brains aged 1 day before birth, 1, 4, 7, 14, and 21 days after birth, and adult, respectively.

2 gene expression in endotoxin-treated animals was also investigated. Unlike the HO-1 message, transcription of the 1.3- and 1.9-kb HO-2 mRNA species was not induced by endotoxin. Furthermore, the ratio of these mRNA species remained unchanged.

We further tested the degree of sequence homology between the two HO-2 mRNAs by probing rat brain poly(A)+ RNA for each, using fragments from three distinct regions of the intact HO-2 cDNA as hybridization probes. These included an EcoRI/SacI fragment (5' end, nucleotides -186 to +104); a 274-bp HO-2 cDNA fragment described previously (9), middle region of HO-2 cDNA (matching nucleotides +437 to +688), and a PstI/EcoRI fragment (3' end, nucleotides +888 to +1108). All hybridized with approximately equal efficiency to each of the two messages (data not shown), indicating that the two species are highly homologous.

Two lines of evidence indicate that the 1.9-kb HO-2 homologous RNA species is unrelated to the ~1.8-kb HO-1 message species. First, the 1.9-kb mRNA is readily detected in the brain, whereas the HO-1 message is not. Second, the 1.9-kb species can be detected using the 221-base pair probe derived from the 3' end of our HO-2 cDNA (PstI to EcoRI, nucleotides +888 to +1108) described above. The sequence of this region of HO-2 (10) is highly diverged from that of the HO-1 cDNA described by Shibahara et al. (21) and would not hybridize to the HO-1 mRNA under the stringent conditions described under "Materials and Methods."

As we have expressed the 1.3-kb species in Escherichia coli and have demonstrated that it encodes heme oxygenase enzymatic activity (10), we can be certain of this mRNA's function. We further examined the relationship between the two HO-2 mRNA species and used a novel approach to address the question of whether the 1.9-kb RNA species corresponds to a precursor of the 1.3-kb species. If the 1.9-kb RNA is a nuclear precursor, it should not be found associated with mono- or polyribosomes. For these investigations, adult rat brain post-mitochondrial supernatants were fractionated on 7–47% linear sucrose gradients. The absorbance profile of one such gradient is presented in Fig. 6a. Fractions 1–3
contain free RNAs; fractions 4 and 5 harbor free or bound ribosomal subunits and associated mRNAs, and fractions 6-11 contain messages associated with mono- and polyribosomes. Northern blot hybridization analysis of these RNAs extracted from each gradient fraction, using the intact HO-2 cDNA as a probe, is shown in Fig. 6b. Both the 1.3- and 1.9-kb RNA species were found in the ribosome-containing fractions of the gradient. The smaller species seems to be translated far more efficiently than the larger, as the former is associated only with the polyribosomal fractions (fractions 9-11), whereas the latter seems to be distributed amongst the subunit, monoribosomal, and polyribosomal fractions (fractions 4-11). Nevertheless, the observation that the 1.9-kb species is associated with ribosomes at all, indicates that this species is not an immature nuclear precursor RNA. The possibility remains that the 1.9-kb mRNA species represents a second mature message species transcribed from the same HO-2 gene. Alternatively it may represent a product of an as of yet unidentified HO gene.

The biological reason for the existence of two abundant HO-2 mRNAs, at least one of which is translated into an active heme metabolizing protein, in an organ which is by comparison with other organs such as the liver nearly devoid of the microsomal hemoproteins (36) is perplexing. Indeed, the specific activity of brain heme oxygenase is nearly equivalent to that observed in the spleen (16); however, as we know, unlike the spleen, brain has no major role in disposing of circulating hemoglobin. It would be of interest to study the possibility of heme oxygenase having function(s) in the brain aside from that of heme degradation. It may also be of interest, from an evolutionary perspective, to gain an understanding of the exquisitely distinct patterns of expression and regulation of HO-1 and HO-2 in the brain.

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