Cloning and expression of cDNA for rat heme oxygenase
(expression cDNA library/antibody screening/transient expression/enzyme induction/hemin)

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ABSTRACT Two cDNA clones for rat heme oxygenase have been isolated from a rat spleen cDNA library in λgt11 by immunological screening using a specific polyclonal antibody. One of these clones has an insert of 1530 nucleotides that contains the entire protein-coding region. To confirm that the isolated cDNA encodes heme oxygenase, we transfected monkey kidney cells (COS-7) with the cDNA carried in a plasmid, and the primary structure of heme oxygenase was deduced. Heme oxygenase is composed of 289 amino acids and has one hydrophobic segment at its carboxyl terminus, which is probably important for the insertion of heme oxygenase into endoplasmic reticulum. The cloned cDNA was used to analyze the induction of heme oxygenase in rat liver by treatment with CoCl₂ or with hemin. RNA blot analysis showed that both CoCl₂ and hemin increased the amount of hybridizable mRNA, suggesting that these substances may act at the transcriptional level to increase the amount of heme oxygenase.

The microsomal heme oxygenase plays an essential role in physiological heme catabolism (1, 2). Heme oxygenase catalyzes the oxidative degradation of heme to biliverdin (1), which is subsequently converted to bilirubin by biliverdin reductase (3). In the rat, the activity of heme oxygenase is highest in the spleen, where senescent erythrocytes are sequestrated and destroyed (2). Other tissues such as bone marrow and liver also perform this function, especially in hemolytic states and after splenectomy (2, 4). Heme oxygenase is highly inducible by its substrate heme in kidney (2, 5), liver (2, 5), and macrophages (6–8). Heme oxygenase is also induced by various other substances such as metal ions (9, 10), endotoxin (11), and bromobenzene (12).

We are particularly interested in the induction of heme oxygenase by heme, because heme (ferrous protoporphyrin IX) is an essential component of hemoglobin and of other hemoproteins. Furthermore, hemin (ferric chloride protoporphyrin IX) has interesting biological properties such as stimulation of neurite outgrowth (13), promotion of adipocyte differentiation (14), and stimulation of globin mRNA accumulation in erythroleukemic cells (15, 16). Previously, we demonstrated that hemin increased the levels of functional mRNA for heme oxygenase in cultured pig alveolar macrophages (17) and in rat liver (18), suggesting that hemin acts at the transcriptional level to increase the amount of heme oxygenase. To understand the molecular mechanisms of induction of heme oxygenase, it is essential to know the structure of the gene for heme oxygenase.

In this study, we have isolated cDNA clones for rat heme oxygenase by antibody screening, and we have confirmed that our cDNA actually encodes heme oxygenase by expressing cDNA in monkey kidney cells. We determined the nucleotide sequence of the cloned cDNA and deduced the amino acid sequence of heme oxygenase.

MATERIALS AND METHODS

Preparation of RNA. Total RNA was prepared from rat spleen by the method of Chirgwin et al. (19). Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography (20).

Construction of cDNA Expression Library and Antibody Screening. cDNA was synthesized from 10 µg of rat spleen poly(A)⁺ RNA and converted to double-stranded cDNA by using RNase H and DNA polymerase I (21). Double-stranded cDNA was methylated at internal EcoRI sites with EcoRI methylase (22). Addition of EcoRI linkers and ligation to λgt11 DNA (23, 24) was carried out as described by Schwarzbauer et al. (25). After in vitro packaging (Amer sham), the recombinant phage were plated on Escherichia coli strain Y1090 (24, 25) and incubated at 37°C for 6 hr with 2 mM isopropyl β-D-thiogalactopyranoside. Each plate was then overlaid with a nitrocellulose filter and incubated for 12 hr at 37°C. The filters were air dried, washed with 50 mM Tris-HCl, pH 7.5/150 mM NaCl (TBS) and treated with TBS containing 3% bovine serum albumin for 1 hr at room temperature. The following procedures were performed at room temperature unless otherwise indicated. The filters were washed with TBS containing 0.1% Triton X-100 (TBST) and treated with rabbit antirat heme oxygenase IgG (18) for 2 hr in TBST containing 3% bovine serum albumin. For reducing the background, anti-rat heme oxygenase IgG was treated with bacterial extracts at 4°C overnight, and any precipitate was removed by centrifugation. Then, the filters were washed extensively with TBST and treated with 32P-labeled protein A (Amersham) in TBST containing 3% bovine serum albumin for 1 hr. Filters were washed with TBST, air dried, and autoradiographed overnight at −70°C with intensifying screens.

Subcloning and Sequencing of cDNAs. The EcoRI inserts of positive phage clones (λRHO2 and λRHO6) were purified, ligated into EcoRI-cleaved pUC8 plasmid, and used to transform E. coli HB101. To detect the fusion protein in bacterial colonies by antibody, E. coli DH-1 was used for transformation (26). The subcloned DNA fragments were used for further analysis, and nucleotide sequences were determined by the method of Maxam and Gilbert (27).

Cloning of Full-Length cDNA for Heme Oxygenase. A cDNA library was constructed by the method of Okayama and Berg (28) using 2.0 µg of rat spleen poly(A)⁺ RNA and screened with 32P-labeled Dde I(93)/Dde I(240) fragment as a hybridization probe. The numbers in parentheses, shown together with restriction enzymes, indicate the 5' terminal nucleotide generated by cleavage (see Fig. 1). All DNA...
probes used in this study were labeled with [α-32P]dCTP by the method of Feinberg and Vogelstein (29).

Construction of Expression Plasmids Carrying Heme Oxygenase cDNA. The expression vector pKCRH2 (30) was linearized by digestion with HindIII and single-stranded ends were filled in by treatment with DNA polymerase I (Klenow fragment, Boehringer Mannheim). The resulting blunt ends were used for ligation with the following inserts: (i) Xho I(−59)/HindIII(971) fragment isolated from pRH01; (ii) EcoRI(88)/EcoRI(1429) fragment derived from λRH06. Both ends of each fragment were converted to blunt ends before ligation. Recombinant plasmids were cloned in E. coli HB101 and plasmids for each insert with opposite orientation were isolated. The pKCRH021 thus obtained carries the Xho I(−59)/HindIII(971) fragment in the same orientation with respect to simian virus 40 early gene transcription. pKCRH021-anti contains the same insert as pKCRH021 but in the opposite orientation. pKCRH07 has the EcoRI(88)/EcoRI(1429) fragment in the same orientation with respect to simian virus 40 early gene transcription.

Transfection of COS-7 Cells and Assay of Heme Oxygenase. COS-7 cells (31) were maintained in Dulbecco’s minimal essential medium containing 2.5% fetal calf serum and 2.5% newborn calf serum (32). Confluent cells, seeded in 140-mm dishes, were fed with medium 4 hr before addition of plasmid DNA. Plasmid DNA (60 μg per dish) was used to transfect COS-7 cells by the calcium phosphate method (33, 34). After a 48-hr incubation, cells were collected and stored at −70°C until assay for heme oxygenase. Thawed cells were disrupted by sonication and pellets (microsomes) were prepared by centrifugation at 105,000 × g (8). The microsomes were suspended in 50 mM potassium phosphate buffer, pH 7.4/0.1% Triton X-100, and assayed for heme oxygenase (35). One unit of the enzyme was defined as the amount catalyzing the formation of 1 nmol of bilirubin per min. Protein amount was determined by using commercial reagent (Bio-Rad).

RNA Blot Analysis. Total RNA was prepared from untreated rat liver and from liver treated with hemin or with CoCl2. Administration of these reagents to rats was carried out as described (18). Total RNA (10 μg) was denatured (36), electrophoresed on a 1.1% agarose gel containing 1 M formaldehyde, transferred to a nitrocellulose filter, and hybridized with 32P-labeled EcoRI fragment (nucleotide residues −23 to 87) derived from ARH02. The size markers were rRNA and E. coli rRNA (37).

RESULTS AND DISCUSSION

cDNA Cloning. Heme oxygenase represents ∼0.33% of total peptides synthesized on free polysomes isolated from pig spleen (38). Therefore, rat spleen poly(A)+ RNA was used to construct cDNA libraries. From 8 × 10⁶ cDNA clones in a λgt11 expression library, two phase clones, λRH02 and λRH06, were isolated by antibody screening (Fig. 1). EcoRI sites at both ends of λRH06 were produced by the addition of EcoRI linkers. The 3′ EcoRI site was produced in the poly(dA) tract located 5 residues downstream from nucleotide residue 1429 (see Fig. 3). Thus, the clone λRH06 contains 1530 nucleotides, except for linker sequences, as well as 5 residues of poly(dA) tract. For convenience, we indicate the 5′ and 3′ ends of λRH06 as EcoRI(−101) and EcoRI(1429), respectively. Clone λRH02 contains the insert of 110 nucleotides (nucleotide residues −23 to 87) except for a part of linker sequence at its 5′ end. The 5′ end of λRH02 is indicated as EcoRI(−23). Apparently λRH02 is derived from the cDNA in which the internal EcoRI site was not protected by EcoRI methylase against EcoRI digestion. For further analysis, three EcoRI fragments excised from both phage clones were subcloned in pUC8 plasmid.

Because λRH06 is not a full-length cDNA in comparison with the estimated size of mRNA (∼1800 nucleotides) by RNA blot analysis (see Fig. 5), we constructed another cDNA library by the method of Okayama and Berg (28), and

Fig. 1. Restriction map and sequencing strategy of cloned cDNA encoding rat heme oxygenase. Restriction map shows only the relevant sites, identified by numbers indicating the 5′-terminal nucleotide generated by cleavage (see Fig. 3). The only exception to this numbering is in the EcoRI sites produced by the addition of EcoRI linkers, indicated as dotted lines. The poly(dA) tract and poly(dG) tail are not included in the restriction map. The protein-coding region is indicated by an open box, and the putative membrane segment is indicated by a stippled box. The arrows indicate the direction and extent of sequence determinations. The short vertical lines and the slash marks at the end of arrows indicate the site of 5′-end labeling in cDNA and vector DNA, respectively. The short solid and dotted lines on clones indicate the internal EcoRI sites and artificial EcoRI sites, respectively. bp, Base pairs.
we screened the library with the Dde I/93I (Dde I/240) fragment as a hybridization probe. From 3 × 10^7 transformants, we isolated pH01, which contains 1557 nucleotides excluding the dG tail and the poly(dA) tail (~150 residues) (Fig. 1).

Expression of Heme Oxygenase cDNA in COS-7 Cells. As the amino acid sequence of heme oxygenase is not known, we sought to confirm that the isolated cDNA actually encodes heme oxygenase. Consequently, we transfected COS-7 cells with the expression vector pKCRH2 (31) containing our cDNA, and then we measured the activity of heme oxygenase. COS-7 cells have endogenous heme oxygenase activity and this basal activity did not change after transfection with plasmid pKCRH2 (Fig. 2B). Accordingly, we used the activity of heme oxygenase in cells transfected with pKCRH2 as a control.

The structure of the expression plasmid pKCRH02 is indicated in Fig. 2A. The pKCRH07, carrying the EcoRI (88)/EcoRI (1429) fragment, lacks the amino-terminal segment of heme oxygenase (amino acid residues 1–29) (Fig. 3). The cells transfected with pKCRH07 have the same activity of heme oxygenase as the control vector (Fig. 2B). In contrast,

![Fig. 2. Structure of expression plasmid carrying cDNA for heme oxygenase and expression of heme oxygenase in transfected COS-7 cells. (A) Schematic representation of expression plasmid pKCRH02. The origin of the segments is shown on the inner circle. The protein-coding region of heme oxygenase cDNA is indicated by an open box and its 5' and 3' noncoding regions are shown by closed boxes. pKCRH02 is represented the same way as in the original paper (30). Cross-hatched boxes, simian virus 40 (SV40) sequences; stippled boxes, exonic sequences of rabbit β-globin gene; slashed box, its 3' flanking sequence. The segment derived from plasmid pBR327 is indicated by solid line. Transcription occurs clockwise. HindIII sites, marked with closed circles, were converted to blunt ends and used to construct pKCRH02 as well as pKCRH02-anti and pKCRH07. (B) Expression of heme oxygenase in transfected COS-7 cells. After transfection with the indicated plasmid, microsomal fractions were prepared for assay of heme oxygenase. Basal activity was measured in untransfected cells (indicated as none). For comparison, activity of rat spleen was included.

FIG. 2. Structure of expression plasmid carrying cDNA for heme oxygenase and expression of heme oxygenase in transfected COS-7 cells. (A) Schematic representation of expression plasmid pKCRH02. The origin of the segments is shown on the inner circle. The protein-coding region of heme oxygenase cDNA is indicated by an open box and its 5' and 3' noncoding regions are shown by closed boxes. pKCRH02 is represented the same way as in the original paper (30). Cross-hatched boxes, simian virus 40 (SV40) sequences; stippled boxes, exonic sequences of rabbit β-globin gene; slashed box, its 3' flanking sequence. The segment derived from plasmid pBR327 is indicated by solid line. Transcription occurs clockwise. HindIII sites, marked with closed circles, were converted to blunt ends and used to construct pKCRH02 as well as pKCRH02-anti and pKCRH07. (B) Expression of heme oxygenase in transfected COS-7 cells. After transfection with the indicated plasmid, microsomal fractions were prepared for assay of heme oxygenase. Basal activity was measured in untransfected cells (indicated as none). For comparison, activity of rat spleen was included.

![Fig. 3. Nucleotide sequence of cDNA for rat heme oxygenase and its deduced amino acid sequence. The nucleotide sequence of the message strand is shown. Nucleotides are numbered in the 5' to 3' direction and numbers are shown on the right side of the sequence. Nucleotide residue 1 is the A of the initiating methionine codon ATG, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The deduced amino acid sequence is shown below the nucleotide sequence and are numbered beginning with the initiating methionine. The putative membrane segment and the polyadenylation signal, AATAAA (39), are underlined. The poly(dA) tract (~150 residues) is not included.](image)
the cells transfected with pKCRHO21, carrying the entire protein-coding region in the correct orientation, showed a 6-fold increase in activity of heme oxygenase. The expressed activity is ~2-fold higher than that of rat spleen, which was also measured for comparison (Fig. 2B). Simultaneously, we used pKCRHO21-anti, carrying the same insert as pKCRHO21 but in the opposite orientation, to examine the effect on basal activity of heme oxygenase. Under our conditions, the cells transfected with this plasmid showed similar activity to the control (Fig. 2B).

These results clearly indicate that isolated cDNA encodes heme oxygenase. Furthermore, these results also indicate that the expressed heme oxygenase was actually incorporated into the endoplasmic reticulum of COS-7 cells, as we isolated microsomes for assay of heme oxygenase.

Nucleotide Sequence of cDNA and Deduced Amino Acid Sequence for Heme Oxygenase. The size of cloned cDNA is 1357 nucleotides excluding the 5'3' tail and the poly(dA) tract (=150 residues) (Fig. 3). The 5' untranslated region, composed of 128 nucleotide residues, contains four direct repeats GGAGCC (nucleotide residues -83 to -78, -53 to -48, -46 to -41, and -30 to -25).

It is apparent that both clones ARHO2 and λRHO6 cannot encode immunoreactive fusion proteins because of the presence of the in-frame termination codon TAG (nucleotide residues -15 to -13). This indicates the internal initiation of protein synthesis within the cDNA inserts of both clones as reported in the λgt11 system (23) and another system (40). Furthermore, this also indicates that the initiating methionine is located in the EcoRI(-23)/EcoRI(88) fragment carried by λRHO2. Thus, the translation initiation site is assigned to the ATG at the position 1–3 (Fig. 3).

The open reading frame initiating this methionine codes for a polypeptide of 289 amino acids with a molecular weight of 33,009, which is in good agreement with the reported M of 32,000 of rat liver heme oxygenase (41). The sequence flanking the assigned ATG (nucleotide residues -3 to 4) is consistent with the favored sequence for eukaryotic initiation sites (42). In addition, the subclone, carrying the EcoRI(88)/EcoRI(1429) fragment in proper orientation, produced the protein that reacts with anti-rat heme oxygenase IgG, indicating that our assignment of reading frame is correct. Furthermore, termination codons are found in three different reading frames in the 5' untranslated region (nucleotide residues -40 to -38, -20 to -18, and -15 to -13), and there is no other reading frame that can encode a long polypeptide (>103 amino acid residues). However, there is another potential initiation ATG (nucleotide residues 25–27) in the same frame located 21 nucleotides downstream of the assigned ATG, although the flanking sequence of this alternative ATG (nucleotide residues 22–28) is not favored (42). If this alternative ATG codes for the initiating methionine, heme oxygenase is composed of 281 amino acid residues with a molecular weight of 32,052, which is identical with the reported value (41). The deduced amino acid sequence revealed that heme oxygenase contains no cysteine residues.

The translation termination codon TAA (nucleotide residues 868–870) is followed by 559 nucleotides of 3' untranslated region. A polyadenylation signal, AATAAA (39), is located 15 nucleotides upstream from the poly(dA) tract.

As heme oxygenase is a microsomal enzyme and is synthesized on free polysomes (17, 18, 38), it must be inserted into endoplasmic reticulum posttranslationally. A hydrophilicity profile (43) revealed one hydrophilic region at the amino terminus (amino acid residues 1–33) and the longest hydrophobic segment at the carboxyl terminus (amino acid residues 268–289) (Fig. 4). This amino-terminal hydrophilic region contains one of the antigenic determinants of heme oxygenase, because clone ARHO2 produced the immunoreactive proteins (see Fig. 1). The presence of hydrophobic segments at carboxyl terminus was reported in other microsomal enzymes such as cytochrome b5 (45) and NADH-cytochrome b5 reductase (46), which are also synthesized on free polysomes (47, 48). These studies suggest that the hydrophobic segments at carboxyl termini are essential to anchor these enzymes to the membrane. Therefore, heme oxygenase is probably inserted into the membrane through the hydrophobic segment at its carboxyl terminus.

The predicted secondary structures (44) for heme oxygenase are also shown in Fig. 4. Overall, α-helix represents 45% of residues; β-sheet, 18%; turn, 12%. The rest of the residues are in random coil. Heme oxygenase seems to have an α/β structure.

We attempted to assign the heme-binding site of heme oxygenase, although further study is required to identify the heme-binding site. There are 10 histidine residues in heme oxygenase and 5 of them are located between amino acid residues 100 and 132. We assume that this region (amino acid residues 100–132) may be important for heme-binding (see Fig. 4). The amino acid sequence of heme oxygenase does not have any significant homology with any known proteins, including globin and myoglobin.

RNA Blot Analysis. To analyze the induction of heme oxygenase in rat liver by treatment with CoCl2 or with hemin, total RNA was subjected to RNA blot analysis using the EcoRI(-23)/EcoRI(88) fragment as a hybridization probe (Fig. 5). The probe hybridized to a discrete mRNA of the same size (~1800 nucleotides). RNA prepared from rat liver was analyzed by computer for hydrophilicity (43) and for secondary structures (44). Hydrophilic regions are plotted above the 0-line and hydrophobic regions are shown below the 0-line. In the secondary structure prediction, residues of α-helix are indicated as open boxes; β-sheet, solid lines; turn, dots; random coil, blank.
Fig. 5. Autoradiogram of RNA blot hybridization. Each lane contained 10 µg of total RNA prepared from rat spleen (lane A), liver treated with CoCl₂ (lane B), liver treated with hemin (lane C), and untreated liver (lane D). The hybridization probe was the 32P-labeled EcoRI(−23)/EcoRI(88) fragment derived from xRHO2. The size markers were rRNA and E. coli rRNA (37). The film was exposed at −70°C for 40 hr.

spleen contains the highest level of hybridizable mRNA among tissues examined, which is consistent with the fact that the spleen has the highest activity of heme oxygenase (2) and that spleen is abundant in functional mRNA for heme oxygenase (38). Both CoCl₂ and hemin treatment apparently increased the levels of hybridizable mRNA in rat liver (lanes B–D). This result is also consistent with the finding that CoCl₂ treatment increased the activity of heme oxygenase in rat liver (9, 10). Furthermore, we previously reported that heme increased the levels of functional mRNA for heme oxygenase in cultured pig alveolar macrophages (17) as well as in rat liver (18). Thus, RNA blot data suggest that both CoCl₂ and hemin act at the transcriptional level to increase the amount of heme oxygenase.

The availability of cDNA clones for heme oxygenase enables us to isolate and characterize the gene for heme oxygenase. Structural and functional analysis of the gene may provide us with the opportunity to study the molecular mechanisms of how heme or CoCl₂ increase the amount of hybridizable mRNA for heme oxygenase.

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