Developmental changes of gene expression in heme metabolic enzymes in rat placenta

Naomi Ihara[a], Reiko Akagi[b,*], Kohei Ejiri[a], Takafumi Kudo[a], Kazumichi Furuyama[c], Hiroyoshi Fujita[c]

[a] *Department of Obstetrics and Gynecology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-0914, Japan*
[b] *Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, 111 Kuboki, Soja 719-1197, Japan*
[c] *Departments of Molecular Biology and Biochemistry, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-0872, Japan*

Received 1 September 1998; received in revised form 9 October 1998

Abstract Transcription levels of the non-specific δ-aminolevulinate synthase (ALAS-N) and heme oxygenase-1 (HO-1) in the placenta at the terminal stage of pregnancy were comparable to those in the female adult liver and in the spleen, respectively. Immunohistochemical studies demonstrated that both enzymes were exclusively expressed in the trophoblast. During gestation, transcript of ALAS-N slightly increased, while HO-1 mRNA significantly decreased. Induced acute fetal hypoxia resulted in an increase in ALAS-N mRNA and in a decrease in HO-1 mRNA. These findings indicate that placental heme metabolism is influenced by the oxygen supply.

Key words: Placenta; Trophoblast; Fetus; Heme; δ-Aminolevulinate synthase; Heme oxygenase; Hypoxia

1. Introduction

The placenta is a unique organ which transports essential substances for fetal development to the fetus from the uterine artery to the umbilical vein via trophoblasts. For the rapid growth of the fetus, a huge amount of oxygen needs to be supplied through the placenta. Though hemoproteins are thought to be responsible for oxygen transfer from the mother to the fetus, little is known about heme metabolism and its possible changes in the placenta during fetal development.

Heme content is mainly controlled by its biosynthesis and degradation [1]. The two major organs for heme biosynthesis are the erythroid bone marrow cells and the liver [2]. Erythroid heme synthesis is exclusively dependent on the erythroid-specific isoform of δ-aminolevulinate synthase [3]. In contrast, in the liver, heme content is determined by the level of the non-specific isoform of δ-aminolevulinate synthase (ALAS-N), which is the rate limiting enzyme of heme biosynthesis and regulated by cytosolic free heme in a negative feedback manner [1–5]. It is likely that heme biosynthesis in the placenta may also be dependent on ALAS-N. However, little is known about its tissue-specific heme synthesis in this organ.

The major site of heme degradation in adult animals is the spleen, where a markedly elevated activity of heme oxygenase (HO), the rate-limiting enzyme of heme catabolism, is observed [6]. To date, three HO isozymes have been identified, i.e. inducible HO-1, constitutive HO-2 and a poor heme catalyst, HO-3 [7–10]. HO-1 gene is activated not only by its substrate heme, but also by various kinds of oxidative stress [6] yielding bilirubin IXa, an effective anti-oxidant [11,12]. This information suggests that oxygen concentration may also influence HO-1 gene expression in feto-placental tissues. In fact, HO-1 has been shown to be an oxygen-regulated protein 33 (ORP 33) [13]. Thus, hypoxic environment of fetus [14] may influence the expression of HO-1 in the placenta, resulting in an alteration in heme metabolism.

In this study, we examined developmental changes in mRNAs of key enzymes for heme synthesis and degradation in the rat placenta. Our findings demonstrate that both ALAS-N and HO-1 are expressed in placenta not only in the level of mRNA but also in that of protein. There are also developmental changes in the expression of mRNAs for the enzymes corresponding to heme metabolism, and these changes occur in response to hypoxia in the fetal environment.

2. Materials and methods

2.1. Animals

Female Wistar rats from 15 to 21 days of gestation were used. They were housed in a temperature-controlled (24°C) room with a 12-h light/12-h dark cycle, and were allowed free access to water and food. Animals were sacrificed by decapitation under light anesthesia with ethylether. Placenta, brain and liver from fetus, and brain, liver and spleen from non-pregnant adult female rats were excised. They were frozen immediately in liquid nitrogen and stored at −80°C until total RNA isolation. To induce hypoxia in the fetus, uterine vessels of rats at day 19 of gestation were ligated under light anesthesia with ethylether according to Wigglesworth's method [15]. Animals were sacrificed at 2, 3, 4 and 5 h after the ligation and the placenta was excised as described above.

2.2. cRNA probes

cRNA probes for rat ALAS-N [16] and HO-1 [17] were transcribed from pKRA2cA and pRHO-1, respectively, as described previously [3,18]. Template for rat HO-2 cRNA is rat cDNA corresponding to 443–948 bp [19], which was cloned from the rat fetal brain library using PCR and constructed into pGEM-T vector (Promega, Madison, WI, USA). All probes for Northern blot analysis were biotin-^{14}CTP labeled antisense riboprobes which were prepared according to the manufacturer's instructions using Non-radioactive RNA Labeling System (Life Technologies, Gaithersburg, MD, USA).

2.3. Northern blot analysis

Twenty μg of total RNA, isolated from 3–5 placentas according to the method of Cathara et al. [20], were applied for Northern blot analysis as described previously [3,18]. After blotting onto a sheet of BIODYNE A nylon membrane (Pall BioSupport Division, Pall, Port Washington, NY, USA), samples were hybridized with cRNA probes, treated with RNase A (1 μg/ml), followed by washing under a stringent condition [3,18]. Detection of mRNAs was carried out by using PHOTOGENE Nucleic Acid Detection System (Life Technologies, Gaithersburg, MD, USA). Chemiluminescent signals were visualized by exposing the membrane to X-ray films. Levels of mRNAs were quantitated by densitometry using a BioImage Analyzer (Millipore, Bedford, MA, USA).

*Corresponding author. Fax: (81) (866) 94-2156.
E-mail: akagirei@opufhw.fhw.oka-pu.ac.jp

PII: S0014-5793(98)01324-6

2.4. Immunohistochemistry

Placenta from a rat on day 19 of gestation was fixed by 10% neutralized formalin for 24 h at room temperature, and were embedded in paraffin, then sliced into 4-μm thick sections. After deparaffinization and rehydration, they were subjected to immunohistochemical analysis. Endogenous peroxides were blocked by 3% hydrogen peroxide, followed by incubation with primary antibodies, i.e. rabbit polyclonal anti-rat liver HO-1, rabbit polyclonal anti-rat testis HO-2 antibody (Stress gen Biotechnologies, Victoria), or rabbit polyclonal anti-rat liver ALAS antibody [21], at 37°C for 3 h. The antigen-antibody reaction was detected using an anti-rabbit immunoglobulin and avidin-biotin immunoperoxidase staining kit (DAKO, Carpinteria, CA, USA). The positive reaction was visualized by 3,3′-diaminobenzidine staining. Untreated rabbit serum was used as a control for non-specific staining. Sections were counterstained with Mayer's hematoxylin solution.

3. Results and discussion

3.1. Messenger RNA expression of ALAS-N and HO-1 in various rat organs

To understand the characteristics of gene expression of heme metabolic enzymes in the placenta, levels of mRNAs for ALAS-N and HO-1 in the placenta at day 19 of gestation were compared with those in other organs. Northern blot analysis revealed that the level of transcript for ALAS-N in the placenta was as much as 1.3-times of that in adult female liver, which is one of the major organs for non-erythroid heme biosynthesis in adult animals (Fig. 1). None of the feto-placental organs so far examined contains ALAS-N mRNA higher than the placenta.

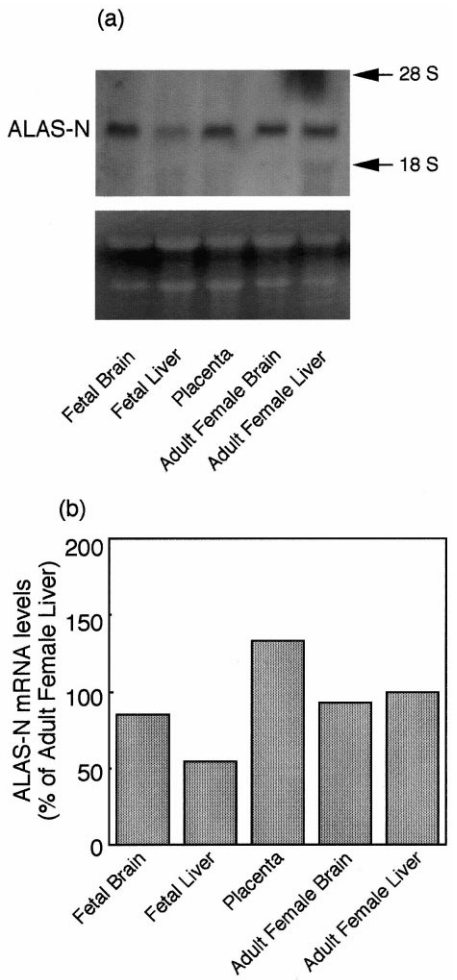

Fig. 1. ALAS-N mRNA expression in various organs. Figure shown is one of three independent experiments. a: ALAS-N mRNA level was estimated by Northern blot analysis as described in Section 2. Each lane contains 20 μg of total RNA from day 19 of gestational fetal brain, fetal liver, placenta, adult female brain and liver. Fractionated RNA stained with ethidium bromide is shown in the lower part of the panel. b: Relative amount of ALAS-N mRNA in each sample was expressed as a percentage of that in adult female liver.

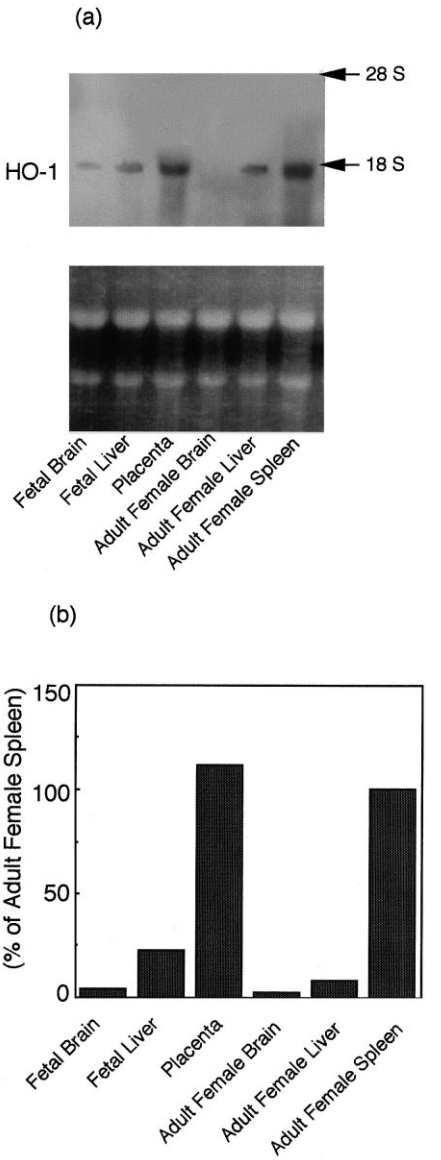

Fig. 2. HO-1 mRNA levels in various organs. Figure shown is one of three independent experiments. a: HO-1 mRNA level was estimated by Northern blot analysis as described in Section 2. Each lane contains 20 μg of total RNA from day 19 of gestational fetal brain, fetal liver, placenta, adult female brain, liver and spleen. Fractionated RNA stained with ethidium bromide is shown in the lower part of the panel. b: Relative amount of HO-1 mRNA in each sample was expressed as a percentage of that in adult female spleen.

Since HO-3 is reported to be a poor heme catalyst among three HO isozymes [8], mRNA levels for HO-1 and HO-2 were examined as measurements of gene expression for the heme degradation. HO-1 mRNA level in the placenta was approximately 14 times of that in the adult liver (Fig. 2). It

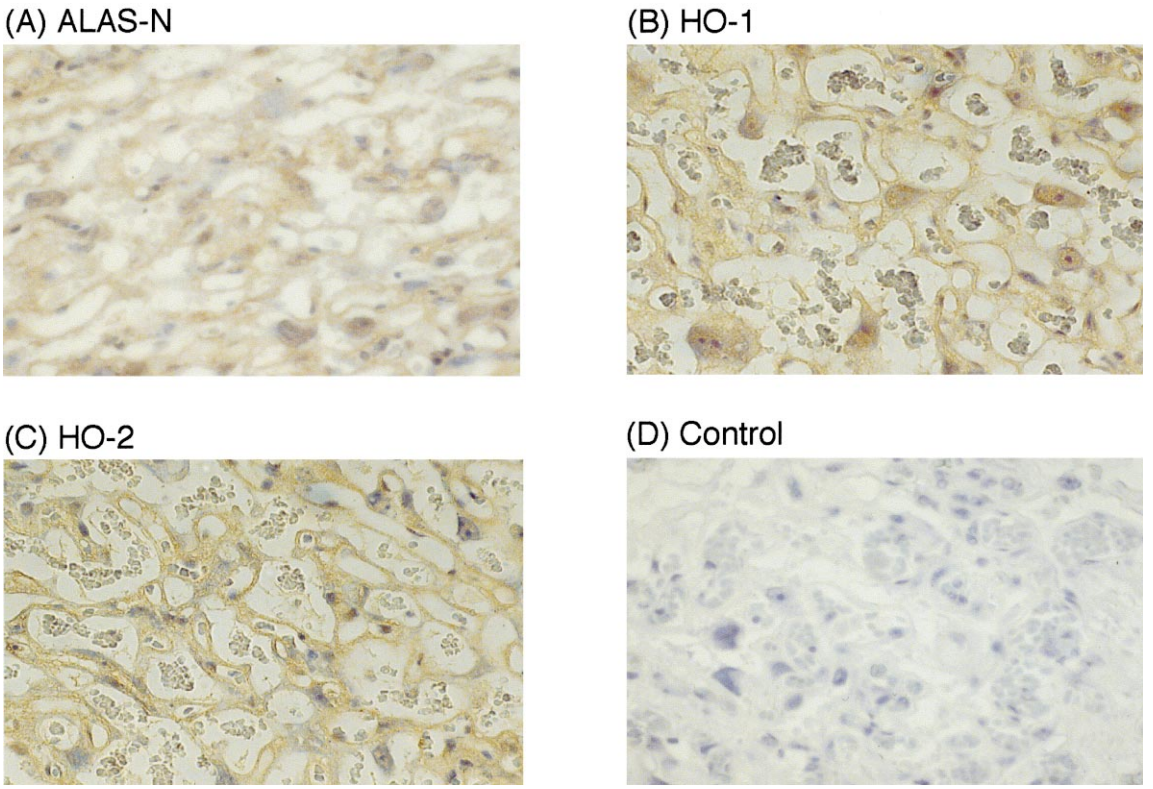

Fig. 3. Immunohistochemistry of ALAS-N and HOs in the placenta. Placental sections from a rat at day 19 of gestation was incubated with (A) rabbit anti-rat ALAS-N antibody, (B) rabbit anti-rat HO-1 antibody, (C) rabbit anti-rat HO-2 antibody, or (D) normal rabbit serum, followed by immunohistochemical staining as described in Section 2 (original magnification ×400).

should be noted that HO-1 mRNA level in the placenta was substantially higher than that in the adult spleen, where the highest activity of HO is observed among adult tissues (Fig. 2). The transcript encoding HO-2 was also expressed in the placenta (Fig. 4a). These findings suggest that the placenta at late gestation is highly active both in heme biosynthesis and in its degradation.

3.2. Distribution of ALAS-N and HO proteins in the placenta

Although Northern blot analysis suggested that the placenta is one of the major organs among feto-placental tissues in the heme biosynthesis and its degradation, it is not clear whether these transcripts are translated into proteins or not. Furthermore, it is also unclear whether the synthesis as well as the degradation of heme in the placenta are carried out in the same cells or not. We therefore carried out immunohistochemical studies of the placenta from a rat at day 19 of gestation. Positive staining was observed for ALAS-N (Fig. 3A), and HO-1 (Fig. 3B) and HO-2 (Fig. 3C), exclusively in trophoblasts, compared with the lack of signals in the section treated with non-immune rabbit serum (Fig. 3D). Positive staining was also observed for HO-1 (Fig. 3B) in erythroid cells, suggesting that there were some hematopoietic progenitor cells in the placenta [22]. Our observations demonstrate that trophoblasts, where the exchange of substance between maternal blood flow and fetal circulation takes place, are responsible for heme metabolism in the placenta. It is thus reasonable to assume that fetal heme is mainly degraded in trophoblasts to excrete the end product bilirubin into the maternal circulation, since fetus expresses little amount of UDP glucuronyl transferase during the early stage of pregnancy [23].

3.3. Developmental changes in mRNA of ALAS-N and HOs in the placenta

Present study indicates that the placenta is an active organ in heme metabolic enzyme expression in day 19 feto-placental tissues. Next we investigated the developmental changes of mRNA of ALAS-N and HO-1 in the placenta during pregnancy. Fig. 4 shows changes in transcript encoding ALAS-N from day 15, when the placenta is constructed completely, till the birth of animals. Since the adult tissues which express the maximal level of ALAS-N and HO-1 are liver and spleen, respectively, each mRNA level is expressed as percent of each maximum tissue level. The level of ALAS-N mRNA in the placenta was lower than that in the adult liver until day 18 of gestation, when a significant induction took place, reaching a peak, the adult liver level, at day 19. These findings demonstrate that there is a developmental upregulation of heme biosynthesis in the late gestational placenta.

In contrast to ALAS-N expression, HO-1 transcripts gradually decreased from the maximum level at day 15 of gestation toward the date of birth (Fig. 4). The level of HO-1 mRNA at day 15 of gestation was 3–4 times higher than the level in the adult spleen, then it was decreased to the adult splenic level on the date of birth. A similar change was also recognized in HO-2 mRNA (Fig. 4a). These findings suggest that heme requirement in the placenta increases during the gestation, presumably to supply a greater amount of oxygen to fetal circulation in response to the rapidly growing fetus.

This hypothesis is also consistent with the finding that expression of ALAS-N in the placenta is restricted to trophoblasts, the gate cells of oxygen transport from mother to fetus (Fig. 3A).

3.4. Effect of ligation of uterine vessels on ALAS-N and HO-1 mRNA expression in the placenta

Preceding studies indicate that heme metabolism in the placenta changes from catabolism superior mid gestation stage to biosynthesis superior late gestation stage. The physiological meaning of this change might be attributable to increasing oxygen demand at late gestation stage. Thus, maternal uterine vessels of 19 days of gestation were ligated to produce an acute hypoxic condition for the fetus, and effects on gene expression of heme metabolic enzymes were investigated (Fig. 5). After vessel ligation, ALAS-N mRNA showed a rapid increase, reaching a peak at 3 h, while the HO-1 transcript decreased to approximately 50% after 2 h of ligation. It should be noted that the transcription of HO-1 seems to be essentially nil in the placenta after ligation, since the half-life of HO-1 mRNA is around 2–3 h [24]. A seemingly complete shut-off of the HO-1 gene expression by uterine vessel ligation is in contrast to the known induction of HO-1 in the liver in response to hypoxia [13]. Thus the oxygen regulation of HO-1 in the placenta may represent a unique tissue-specific mechanism.

In summary, our findings in this study demonstrate marked gene expression of the key enzymes in heme catabolism as well as biosynthesis in the placenta, and the possible role of hypoxia in regulating the expression of these genes. Our study is also the first to indicate that fetal heme is primarily catabolized in the placenta, and to define that trophoblasts are the target cells of hypoxia, resulting in major alterations in the placental heme metabolism.

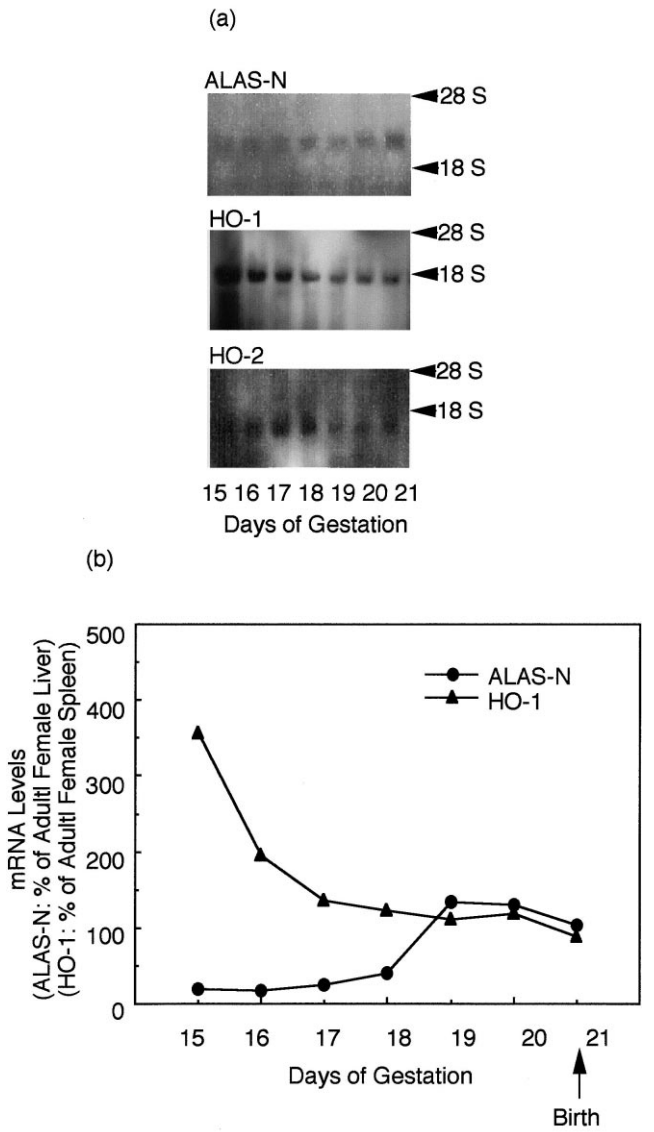

Fig. 4. Developmental changes in mRNA of ALAS-N and HOs in the placenta. Figure shown is one of three independent experiments. a: Levels of mRNA for ALAS-N, HO-1 and HO-2 in the placenta after its complete construction (around day 15 of gestation) were determined by Northern blot analysis. b: Relative amount of ALAS-N (●) and HO- 1 (▲) mRNA in each sample was normalized with the amount detected as 28 S rRNA by ethidium bromide staining, and was expressed as a percentage of that in adult female liver for ALAS-N and in adult female spleen for HO-1, respectively.

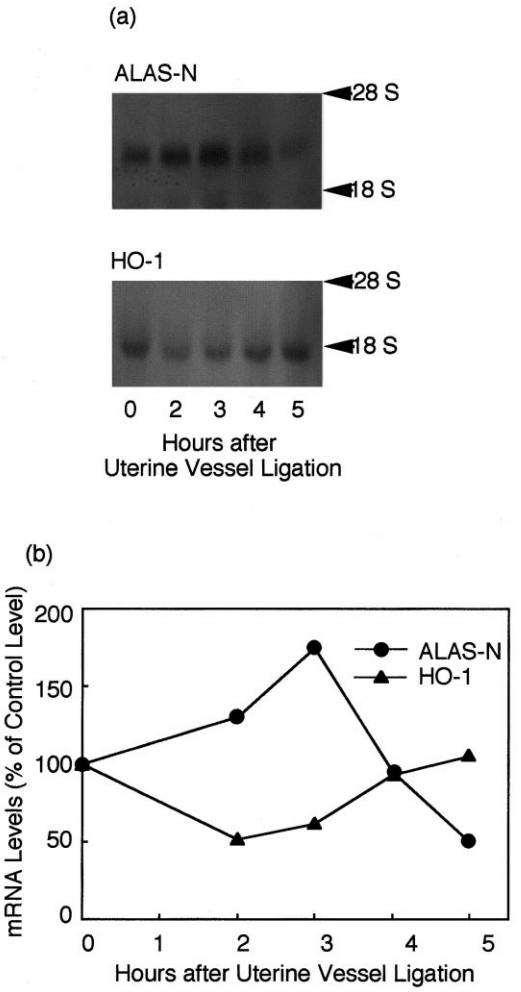

Fig. 5. Effects of hypoxia induced by uterine vessel ligation on ALAS-N and HO-1 mRNA expression in placenta. Uterine vessels of rats at day 19 of gestation were ligated and animals were sacrificed at 2, 3, 4 and 5 h after ligation. ALAS-N (●) and HO-1 (▲) mRNA levels were determined by Northern blot analysis as described in Section 2. Each level was expressed as a percentage of the level of non-ligated control placenta.

Acknowledgements: We are grateful to Dr. S. Shibahara (Tohoku University, Sendai, Japan) and Dr. M. Yamamoto (Tsukuba University, Tsukuba, Japan) for providing us with pRHO-1 and pKRA2cA and anti-rat liver ALAS antibody, respectively. We are also grateful to Dr. S. Sassa (The Rockefeller University, New York, USA) for his helpful suggestions and manuscript reviewing. This study was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan. (to R.A., T.K. and H.F.).

References

[1] Fujita, H. (1997) Tohoku J. Exp. Med. 183, 83–99.
[2] Kappas, A., Sassa, S., Galbraith, R.A. and Nordmann, Y. (1995) in: The Metabolic Basis of Inherited Desease (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds.) pp. 2103–2159, McGraw-Hill, New York, NY.
[3] Fujita, H., Yamamoto, M., Yamagami, T., Hayashi, N. and Sassa, S. (1991) J. Biol. Chem. 266, 17494–17502.
[4] Riddle, R.D., Yamamoto, M. and Engel, J.D. (1989) Proc. Natl. Acad. Sci. USA 86, 792–796.
[5] Watanabe, N., Hayashi, N. and Kikuchi, G. (1983) Biochem. Biophys. Res. Commun. 113, 377–383.
[6] Shibahra, S. (1994) in: Regulation of Heme Protein Synthesis (Fujita, H., Ed.) pp. 103–116, AlphaMed Press, Dayton, OH.
[7] Maines, M.D., Trakshel, G.M. and Kutty, R.K. (1986) J. Biol. Chem. 261, 411–419.
[8] McCoubrey Jr., W.K., Huang, T.J. and Maines, M.D. (1997) Eur. J. Biochem. 247, 725–732.
[9] Trakshel, G.M., Kutty, R.K. and Maines, M.D. (1986) J. Biol. Chem. 261, 11131–11137.
[10] Trakshel, G.M. and Maines, M.D. (1989) J. Biol. Chem. 264, 1323–1328.
[11] Stocker, R., Glazer, A.N. and Ames, B.N. (1987) Proc. Natl. Acad. Sci. USA 84, 5918–5922.
[12] Stocker, R., Yamamoto, M., McDonagh, A.F., Glazer, A.N. and Ames, B.N. (1987) Science (Wash. DC) 235, 1043–1046.
[13] Murphy, B.J., Laderoute, K.R., Short, S.M. and Sutherland, R.M. (1991) Br. J. Cancer 64, 69–73.
[14] Longo, L.D. (1972) Obstet. Gynecol. Ann. 1, 103–138.
[15] Wigglesworth, J.S. (1964) J. Pathol. Bacteriol. 88, 1–13.
[16] Yamamoto, M., Kure, S., Engel, J.D. and Hiraga, K. (1988) J. Biol. Chem. 263, 15973–15979.
[17] Shibahara, S., Muller, R., Taguchi, H. and Yoshida, T. (1985) Proc. Natl. Acad. Sci. USA 82, 7865–7869.
[18] Fujita, H. and Sassa, S. (1989) Br. J. Haematol. 73, 557–560.
[19] Rotenberg, M.O. and Maines, M.D. (1990) J. Biol. Chem. 265, 7501–7506.
[20] Cathala, G., Savouret, J.-F., Mendez, B., West, B.L., Karin, M., Martial, J.A. and Baxter, J.D. (1983) DNA 2, 329–335.
[21] Yamamoto, M., Fujita, H., Watanabe, N., Hayashi, N. and Kikuchi, G. (1986) Arch. Biochem. Biophys. 245, 76–83.
[22] Sassa, S. (1983) in: The Regulation of Heme Biosynthesis (Goldwasser, E., Ed.) pp. 359–383, Elsevier/North Holland, Harvard, MA.
[23] Balistreri, W.F. (1987) in: Nelson Textbook of Pediatrics, 13th Edn. (Behrman, R.E. and Vaughan, V.C., Eds.) pp. 821–824, Saunders, Philadelphia, PA.
[24] Takahashi, K., Hara, E., Ogawa, K., Kimura, D., Fujita, H. and Shibahara, S. (1997) J. Biochem. 121, 1162–1168.