J. Biochem. 2009;145(6)733–738
doi:10.1093/jb/mvp030

Post-Transcriptional Regulation of the Expression of Ferrochelatase by Its Variant mRNA

Masayoshi Sakaino[1], Takao Kataoka[1] and Shigeru Taketani[1,2,*]

[1]Department of Biotechnology and [2]Insect Biomedical Center, Kyoto Institute of Technology, Kyoto 606-8585, Japan

Received January 27, 2009; accepted February 17, 2009; published online February 27, 2009

Ferrochelatase (FECH) catalyses the insertion of ferrous ions into protoporphyrin IX to produce haem at the haem-biosynthetic pathway. The present study characterized a variant mRNA of mouse FECH, which was generated by skipping exon II (FECH-v). FECH-v mRNA was expressed in various tissues, including the liver and kidney, of mice. The mRNA was also expressed in mouse and human non-erythroid and erythroid cells to a different extent but could not be translated into functional FECH. The ratio of FECH-v/FECH increased in hemin-treated Balb/3T3 cells, while it decreased after treatment with succinylacetone, an inhibitor of haem biosynthesis, strongly suggesting that FECH expression was decreased by increasing the level of intracellular haem. These results demonstrated the haem-dependent negative feedback regulation of the expression of FECH at a post-transcriptional level.

Key words: ferrochelatase, haem biosynthesis, hemin, mRNA variant, post-transcription.

Abbreviations: bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EPP, erythropoietic protoporphyria; FCS, fetal calf serum; FECH, ferrochelatase; FECH-v, ferrochelatase variant; kDa, kilodalton (s); MEL, mouse erythroleukemia; RT–PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecylsulfate; SA, succinylacetone.

For the last step in the haem-biosynthetic pathway, FECH catalyses the insertion of ferrous ions into protoporphyrin IX to form haem. FECH in erythroid cells is positively regulated at both the transcriptional and translational levels (*1*, *2*). In addition, the enzyme activity was inhibited by hemin and metal ions (*3*, *4*). *N*-alkylporphyrins are also potent inhibitors of FECH (*4*). The mammlian enzyme nuclear encoded is translated, as a precursor form (48 kDa), and translocated into the mitochondrion, where it is proteolytically processed to its mature size of 41–42 kDa (*5*). Genes for mouse, human and rat FECH have been characterized and consist of 11 exons (*1*, *5*, *6*). X-ray and chromatographic analyses demonstrated the three-dimensional structure of the homodimeric enzyme containing an iron–sulphur cluster at the carboxyl terminus (*7*, *8*). In humans, erythropoietic protoporphyria (EPP) is an inherited disease caused by a deficiency of FECH (*5*, *9*). The extent of enzyme deficiency in EPP can be low, as compared with that in asymptomatic gene carriers. Several haplotypes of the low expression alleles of FECH gene have been reported (*10*).

By examination of EST databases, variant mRNAs of mouse and rat FECH were found, which corresponded to the skipping of exon II (designated as FECH-v). However, the regulation of the expression and function of the FECH-v mRNA has not been demonstrated. This study characterized the mouse and human FECH-v mRNAs, examined their expression levels and the elucidated role of FECH-v mRNA in cells. As a result, a new regulation mechanism of the expression of FECH at the post-transcriptional level is proposed.

MATERIALS AND METHODS

Materials—Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Co. (Tokyo, Japan) and Toyobo Co. (Tokyo, Japan). Mesoporphyrin IX was purchased from Porphyrin Products (Logan, UT, USA). Antibodies against bovine ferrochelatase and actin were obtained, as previously described (*11*, *12*). All other chemicals were of analytical grade.

Plasmids—The FECH-v cDNA was isolated by PCR from a mouse liver cDNA library, digested with *Sma*I and ligated into a *Eco*RV-digested pEF-neo vector (*13*). The resulting plasmid, pEF-mouse FECH-v, was introduced into *Escherichia coli* XL1-Blue. The pEF-mouse FECH plasmid carrying the full-length cDNA of mouse FECH was generated as described previously (*13*). Mouse FECH-v cDNA (214–1263) was by PCR using primers (5′-AAGAATTCGATGTTAAACATGGGAGG-3′ and 5′-AAAAGCTTTCACAGCTGTTGGCTGG-3′). The resulting DNA fragment after digestion with *EcoR*I/*Hind*III was inserted into *EcoR*I/*Hind*III-digested pET vector (*14*), and pET-FECH-v (his-tag) was obtained. The pET-FECH-1 (his-tag) was obtained, as described previously (*14*).

*To whom correspondence should be addressed. Tel: +81-75-724-7789, Fax: 81-724-7760, E-mail: taketani@kit.ac.jp

RT–PCR Analysis—Total RNA was prepared from mouse tissues and cultured cells, as described previously (*15*). Single-strand cDNA derived from the RNA was synthesized using an oligo(dT) primer, using Revatra Ace (Toyobo Co.), followed by PCR, using the primers 5′-CAGAGGATCCCTGGCCGT-3′ (forward) and 5′-TGGAAGTCCACATCTTGA-3′ for mouse FECH and 5′-GAGGCTGCCCAGGCAA-3′ (forward) and 5′-CTCCC TGCTTGGAAGTCCAT-3′ for human FECH. The cDNAs obtained were analysed using a 1% agarose gel electrophoresis. The DNA amount in the gel was quantified using Image J software.

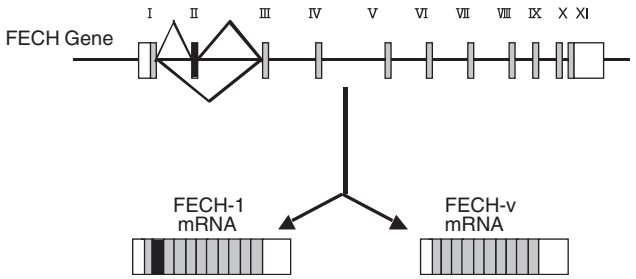

Fig. 1. **Schematic presentation of the production of FECH-1 and FECH-v mRNAs from the mouse FECH gene.**

Cell Cultures—Mouse cell lines (Balb/3T3, L929, MethA, Hepa1-6 and MEL), human cell lines (HeLa, HepG2, PLC/PRF/5 and HEK 293T) and monkey Cos-7 cells were grown in DMEM supplemented with 10% FCS and antibiotics (*13*, *15*). Human erythroleukemia K562 cells were grown in RPMI-1640 medium supplemented with 7% FCS and antibiotics. The cells (5×10^5) in a 3.5-cm-diameter dish were then transfected with pEF-FECH and pEF-FECH-v and cultured for 16 h (*16*). Immunoblotting was carried out using anti-FECH as

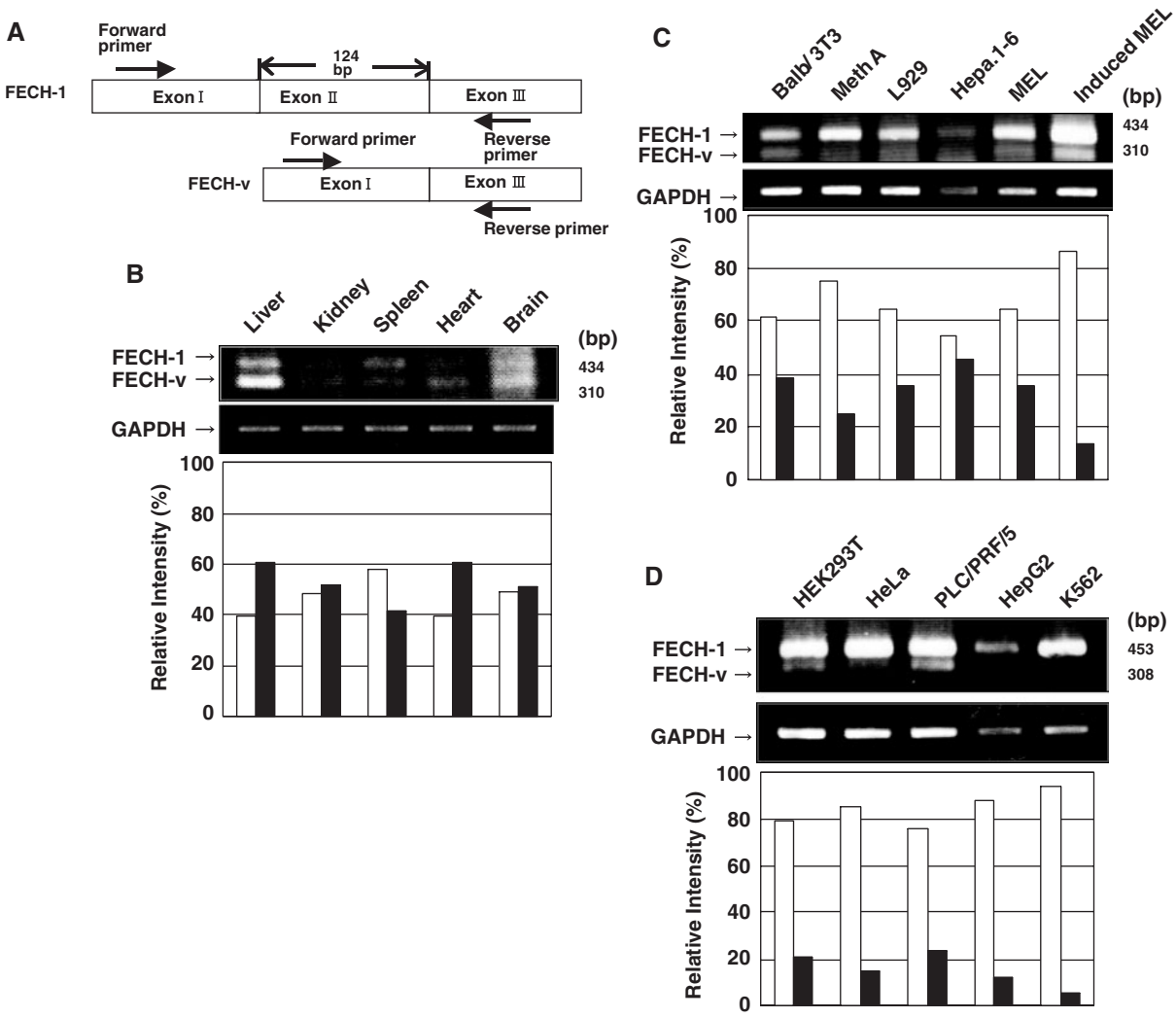

Fig. 2. **RT–PCR analysis of FECH-1 and FECH-v.** (A) The positions of primers for PCR of FECH-1 and FECH-v cDNA. RT–PCR of FECH-1 and FECH-v was carried out, with mRNA from mouse tissues (B), the indicated mouse cell lines (C) and human cell lines (D). A sample of PCR-amplified cDNA product on a 1.2% agarose gel (upper) and the quantified data of all experiments (lower: open bars, FECH-1; solid bars, FECH-v). The sequences of primers used are shown in text.

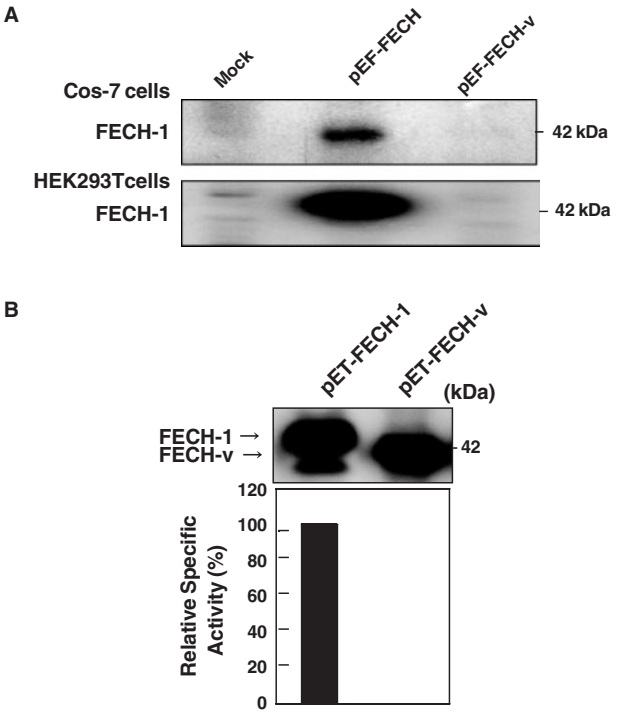

Fig. 3. **(A) Expressions of FECH-1 and FECH-v in Cos-7 and HEK293T cells.** The cells transfected with mock DNA, pEF-FECH and pEF-FECH-v were incubated for 24 h. The cellular proteins were analysed by SDS–polyacrylamide gel electrophoresis, followed by immunoblotting using anti-FECH as the primary antibody. (B) Expressions of FECH-1 and FECH-v in *E. coli*. FECH-1 and FECH-v expressed in cells were purified with Ni^{2+}-beads, and immunoblotting was performed as above. The FECH activity was examined using zinc ions and mesoporphyrin as substrates.

the primary antibody as described previously (*15*, *16*). The FECH activity was measured, as described previously (*14*).

RESULTS

Characterization and Expression of FECH Variant mRNA—A systematic search of the EST databases using the mouse FECH cDNA (*17*) as a query resulted in several partial FECH cDNA sequences from rat and mouse that were dissimilar to the mouse, rat and human FECH mRNAs. This variant mRNA lacked exon II (Fig. 1) and can be translated into a truncated protein by the frame shift of the amino acid sequence but may be translated into a protein using a methionine of a new alternative initiation site corresponding to M72 of the mouse FECH. The complete cDNA of FECH was designated as FECH-1. RT–PCR analysis of the mouse FECH-1 mRNA (exons I–III) revealed that two bands were detected in various tissues including liver, kidney, spleen, brain and heart (Fig. 2A and B). Sequence analysis of two bands confirmed that the upper band carried exons I-III of FECH-1 while the lower band contained exons I and III, but skipped exon II. The proportion of the upper band (FECH-1) to the lower band (FECH-v) varied among tissues, and the density of the lower band from liver, heart and brain was greater than that of the upper band (Fig. 2B). In cultured mouse cells, two bands were also amplified, and the lower band in MEL cells was less abundant than that in non-erythroid Balb/3T3, L929, Hepa1-6 and MethA cells (Fig. 2C). The upper band corresponding to the fragment of FECH-1 cDNA, was increased in DMSO-induced MEL cells. When RT–PCR was carried out using mRNA of human cultured cells, the production of FECH-v mRNA was also observed, but the ratio of FECH-v/FECH-1 in human cells was small and varied among cells, as compared with that in mouse cells (Fig. 2D). These results indicated that two mRNA transcripts, FECH-1 and FECH-v, were produced in various mouse tissues, cell lines and human cells.

The Abnormal Translation Product of FECH-v mRNA—To examine whether the FECH-v mRNA is translated into protein, pEF-FECH and pEF-FECH-v were transfected into Cos-7 cells. After culturing for 24 h, the FECH activity was measured in pEF-FECH or FECH-v-transfected cells. The activity in FECH-v transfected cells was similar to that in mock DNA-transfected cells, while that in FECH-transfected cells was 20-fold higher than the control (data not shown). Data from immunoblotting analysis confirmed the absence of the normal translational product of the FECH-v cDNA (Fig. 3A). Transfection of FECH-v cDNA was performed with HEK293-T cells, but the immunoreactive product was not synthesized in these cells either. A bacterial expression vector (pET-his tag), carrying FECH-1 or FECH-v, was constructed and introduced into *E. coli* (BL21). After purification using the nickel-resin, the FECH activity was measured. No activity was found in *E. coli* expressing FECH-v (Fig. 3B), indicating that exon II was essential for the enzyme activity. These results indicated that FECH-v mRNA does not produce a functional protein.

An Increase in the Ratio of FECH-v/FECH-1 mRNAs in Hemin-Treated Cells—To obtain information on the role of FECH-v mRNA in cells, the proportion of FECH-v to FECH-1 mRNA under various conditions was examined. RT–PCR of FECH-v/FECH-1 showed that the band of FECH-v in Balb/3T3 cells treated with hemin for 24 h increased in a concentration-dependent manner, while that of FECH-1 decreased (Fig. 4A). When the cells were treated 50 μM hemin for the indicated period, the level of FECH-v mRNA increased gradually with a concomitant decrease of FECH-1 mRNA (Fig. 4B). The treatment of Balb/3T3 cells with SA, an inhibitor of haem biosynthesis, was also carried out. RT–PCR analysis showed that the ratio of FECH-v/FECH-1 was decreased by SA treatment (Fig. 4C). When L929 and K562 cells were treated with hemin, the ratio of FECH-v/FECH-1 also increased (data not shown). The level of total FECH mRNA (FECH-1 + FECH-v) in hemin- or SA-treated cells virtually unchanged. These results indicated that the proportion of the non-functional FECH-v mRNA to FECH-1 mRNA was increased in a manner dependent on the increase in the intracellular level of haem, leading to a decrease of the production in the FECH protein in cells.

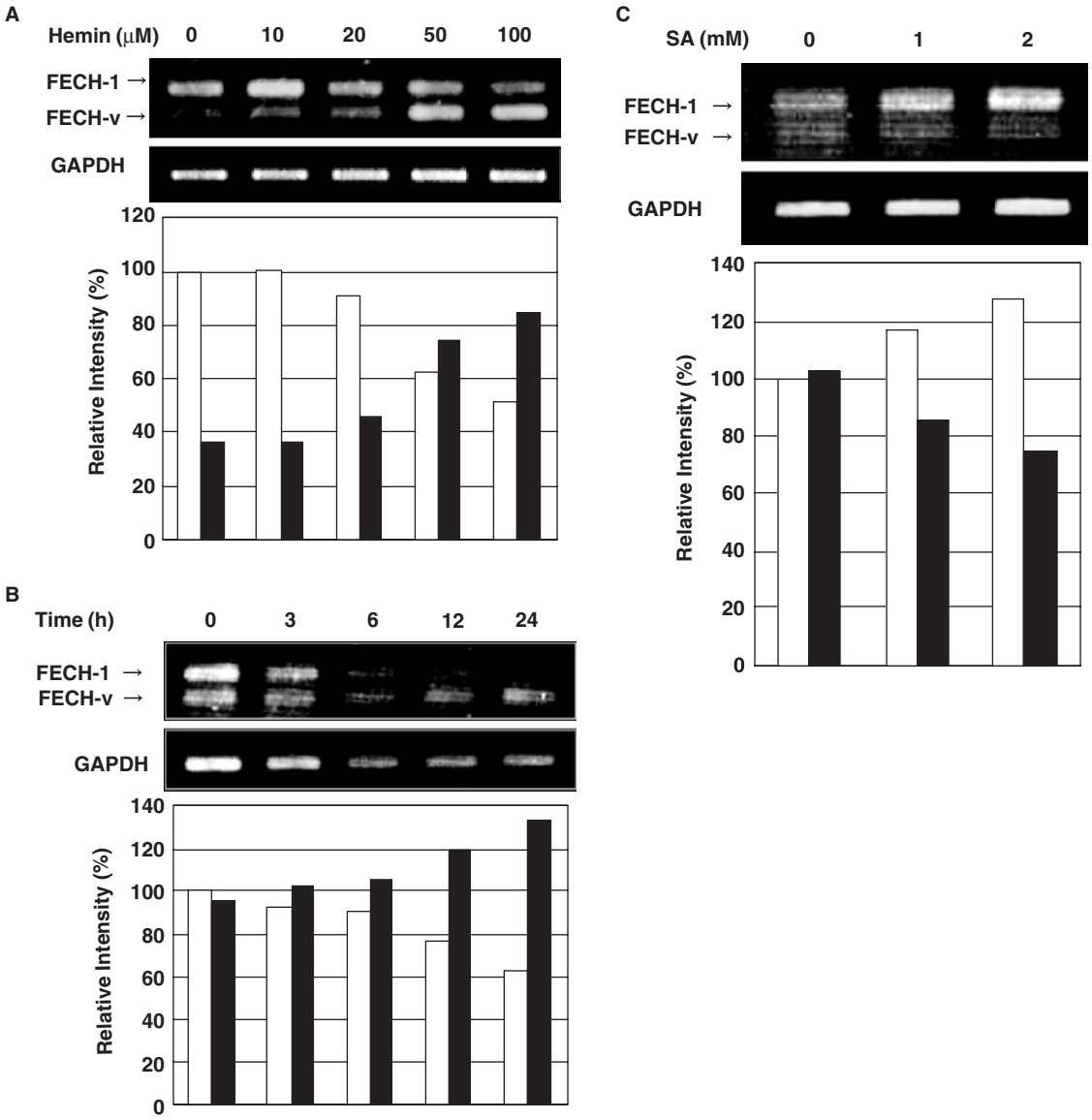

Fig. 4. **Change in the levels of FECH-v and FECH-1 mRNA in hemin- and SA-treated Balb/3T3 cells.** RT–PCR of FECH-1 and FECH-v was carried out with mRNA of Balb/3T3 cells treated with the indicated concentration of hemin for 24 h (A), 50 μM hemin for the indicated period (B) and the indicated concentration of SA for 16 h (C). A sample of PCR-amplified cDNA product on 1.2% agarose gel (upper) and the quantified data of all experiments (lower: open bars, FECH-1; solid bars, FECH-v).

DISCUSSION

In the present study, we report a variant of FECH-1 mRNA ubiquitously expressed in mouse and human. Named FECH-v, it corresponds to the FECH mRNA lacking exon II and has an undefined role. To investigate the regulation of the expression of FECH in various cells, databases were searched with an EST entry showing a variant form of the rat and mouse FECH mRNA and isolated the full length of FECH-v cDNA. The predicted primary structure of the FECH protein showed that the lack of exon II in FECH-1 caused the production of a truncated protein by the frame shift in the amino acid sequence of the mouse, rat and human FECH proteins, but that an alternate initiation methionine residue (M72) in exon III can be used. Attempts were made to express the FECH-v protein using mammalian expression vectors. However, FECH-v was not normally produced. Furthermore, there was no activity of FECH-v expressed in *E. coli*, indicating that the function of the amino-acid region of the beginning of exon III is conserved among eukaryotes (*18*, *19*) and prerequisite for FECH activity. Although FECH-v mRNA was not translated into the functional protein, it may play a role in the post-transcriptional regulation of FECH expression.

The level of FECH-v mRNA varied among tissues. In mouse tissues and cells, the ratio of FECH-v/FECH-1mRNA reached to about 50%. In particular, FECH-v mRNA in mouse liver and brain was more abundant than FECH-1mRNA. The ratio of FECH-v/FECH-1

mRNA varied somewhat among samples, suggesting that the level of FECH-v mRNA can be regulated by a specific mechanism. Different culture conditions were examined to determine their effects on the level of FECH-v mRNA, and it was found that the FECH-v/FECH-1 ratio in hemin-treated Balb/3T3, L929 and K562 cells increased while it was decreased by SA treatment. Thus, the intracellular level of haem can be regulated by the FECH-v/FECH-1 ratio although the precise mechanism involved is unclear. These results demonstrated the haem-dependent negative feedback regulation of the expression of FECH at a post-transcriptional level in addition to the inhibition of the FECH activity by hemin (*3*, *4*).

In contrast to a high proportion of FECH-v mRNA to FECH-1 mRNA in mouse cells, the proportion of human FECH-v mRNA was low. The reason for the difference is unclear, but the human FECH-v mRNA may be related to symptoms of EPP. The majority of clinical EPP patients show only 20–30% residual FECH activity rather than the 50% that one would expect from a disease that is dominantly inherited (*20*, *21*). Furthermore, because several patients with EPP exhibit less than 20% of normal activity (*21*) to account for the lowered FECH activity in EPP patients, it was reported previously that asymmetric FECH retains activity, but due to instability, the level of activity is lower (*8*). The generation of FECH-v mRNA in EPP has not been examined, but the present data suggest that the increase in FECH-v mRNA contributes to the lowered enzyme activity in patients with EPP, because an increase in the proportion of FECH-v mRNA to FECH-1 mRNA caused a decrease of the level of FECH.

The variation of IVS3-48 C/T in the FECH gene also explains the difference in the residual enzymatic activities in asymptomatic and symptomatic mutant carriers. The presence of a C at IVS3-48 in the human FECH gene causes reduced FECH expression levels because of partially aberrant splicing of the pre-mRNA, which is responsible for the clinical manifestations of EPP (*10*, *22*). In this regard, it is known that IVS1-23C/T in the human FECH gene is one of the low-expression allele for EPP (*23*, *24*). Variations of IVS1-23C/T also affect the mRNA level and IVS1-23C may interfere with the splicing of the FECH transcript (*25*). Thus, IVS1-23C/T transition under certain conditions may contribute to the exon II skipping in humans, resulting in an increase in the generation of the human FECH-v mRNA. These mechanisms are related to the production of FECH-v mRNA in rat and mouse.

ACKNOWLEDGEMENT

We thank S. Gotoh, T. Ohta and Y. Kohno for excellent technical assistance.

FUNDING

Ministry of Education, Science, Sports and Culture of Japan.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Taketani, S. (1994) Molecular and genetic characterization of ferrochelatase in *Regulation of Heme Protein Synthesis* (Fujita, H., ed.) pp. 41–54, Alpha Med Press, Dayton, OH
2. Ferreira, G.C. (1999) Ferrochelatase. *Int. J. Biochem. Cell Biol.* **31**, 995–1000
3. Rossi, E., Attwood, P.V., Garcia-Webb, P., and Costin, K.A. (1990) Inhibition of human lymphocyte ferrochelatase activity by hemin. *Biochim. Biophys. Acta* **1038**, 375–381
4. Dailey, H.A. and Fleming, J.E. (1983) Bovine ferrochelatase. Kinetic analysis of inhibition by N-methylprotoporphyrin, manganese, and heme. *J. Biol. Chem.* **258**, 11453–11459
5. Taketani, S. and Fujita, H. (1995) The ferrochelatase gene structure and molecular defects associated with erythropoietic protoporphyria. *J. Bioenerg. Biomembr.* **27**, 231–238
6. Dailey, H.A. (2002) Terminal steps of haem biosynthesis. *Biochem. Soc. Trans.* **30**, 590–595
7. Dailey, H.A, Dailey, T.A., Wu, C.K., Medlock, A.E., Wang, K.F., Rose, J.P., and Wang, B.C. (2000) Ferrochelatase at the millennium: structures, mechanisms and [2Fe-2S] clusters. *Cell Mol. Life Sci.* **57**, 1909–1926
8. Ohgari, Y., Sawamoto, M., Yamamoto, M., Kohno, H., and Taketani, S. (2005) Ferrochelatase consisting of wild-type and mutated subunits from patients with a dominant-inherited disease, erythropoietic protoporphyria, is an active but unstable dimer. *Hum. Mol. Genet.* **14**, 327–334
9. Bloomer, J., Wang, Y., Singhal, A., and Risheg, H. (2005) Molecular studies of liver disease in erythropoietic protoporphyria. *J. Clin. Gastroenterol.* **39**, S167–S175
10. Gouya, L., Puy, H., Robreau, A.M., Bourgeois, M., Lamoril, J., Da Silva, V., Grandchamp, B., and Deybach, J.C. (2002) The penetrance of dominant erythropoietic protoporphyria is modulated by expression of wildtype FECH. *Nat. Genet.* **30**, 27–28
11. Kohno, H., Okuda, M., Furukawa, T., Tokunaga, R., and Taketani, S. (1994) Site-directed mutagenesis of human ferrochelatase: identification of histidine-263 as a binding site for metal ions. *Biochim. Biophys. Acta* **1209**, 95–100
12. Taketani, S., Kakimoto, K., Ueta, H., Masaki, R., and Furukawa, T. (2003) Involvement of ABC7 in the biosynthesis of heme in erythroid cells: interaction of ABC7 with ferrochelatase. *Blood* **101**, 3274–3280
13. Ohgari, Y., Nakayasu, Y., Kitajima, S., Sawamoto, M., Mori, H., Shimokawa, O., Matsui, H., and Taketani, S. (2005) Mechanisms involved in delta-aminolevulinic acid (ALA)-induced photosensitivity of tumor cells: relation of ferrochelatase and uptake of ALA to the accumulation of protoporphyrin. *Biochem. Pharmacol.* **71**, 42–49
14. Taketani, S., Ishigaki, M., Mizutani, A., Uebayashi, M., Numata, M., Ohgari, Y., and Kitajima, S. (2007) Heme synthase (ferrochelatase) catalyzes the removal of iron from heme and demetalation of metalloporphyrins. *Biochemistry* **46**, 15054–15061
15. Tahara, T., Sun, J., Nakanishi, K., Yamamoto, M., Mori, H., Saito, T., Fujita, H., Igarashi, K., and Taketani, S. (2004) Heme positively regulates the expression of β-globin at the locus control region via the transcriptional factor Bach1 in erythroid cells. *J. Biol. Chem.* **279**, 5480–5487
16. Yamamoto, F., Ohgari, Y., Yamaki, N., Kitajima, S., Shimokawa, O., Matsui, H., and Taketani, S. (2007) The role of nitric oxide in δ-aminolevulinic acid (ALA)-induced photosensitivity of cancerous cells. *Biochem. Biophys. Res. Commun.* **353**, 541–546
17. Taketani, S., Nakahashi, Y., Osumi, T., and Tokunaga, R. (1990) Molecular cloning, sequencing, and expression of mouse ferrochelatase. *J. Biol. Chem.* **265**, 19377–19380
18. Nakahashi, Y., Taketani, S., Okuda, M., Inoue, K., and Tokunaga, R. (1990) Molecular cloning and sequence

analysis of cDNA encoding human ferrochelatase. *Biochem. Biophys. Res. Commun.* **173**, 748–755
19. Sato, S. and Wilson, R.J. (2003) Proteobacteria-like ferrochelatase in the malaria parasite. *Curr. Genet.* **42**, 292–300
20. Schneider-Yin, X., Gouya, L., Meier-Weinand, A., Deybach, J.C., and Minder, E.I. (2000) New insights into the pathogenesis of erythropoietic protoporphyria and their impact on patient care. *Eur. J. Pediatr.* **159**, 719–725
21. Gouya, L., Puy, H., Lamoril, J., Da Silva, V., Grandchamp, B., Nordmann, Y., and Deybach, J.C. (1999) Inheritance in erythropoietic protoporphyria: a common wild-type ferrochelatase allelic variant with low expression accounts for clinical manifestation. *Blood* **93**, 2105–2110
22. Nakano, H., Nakano, A., Toyomaki, Y., Ohashi, S., Harada, K., Moritsugu, R., Takeda, H., Kawada, A., Mitsuhashi, Y., and Hanada, K. (2006) Novel ferrochelatase mutations in Japanese patients with erythropoietic protoporphyria: high frequency of the splice site modulator IVS3-48C polymorphism in the Japanese population. *J. Invest. Dermatol.* **126**, 2717–2719
23. Yasui, Y., Muranaka, S., Tahara, T., Shimizu, R., Watanabe, S., Horie, Y., Nanba, E., Uezato, H., Takamiyagi, A., Taketani, S., and Akagi, R. (2002) A new ferrochelatase mutation combined with low expression alleles in a Japanese patient with erythropoietic protoporphyria. *Clin. Sci.* **102**, 501–506
24. Schneider-Yin, X., Gouya, L., Meier-Weinand, A., Deybach, J.C., and Minder, E.I. (2000) New insights into the pathogenesis of erythropoietic protoporphyria and their impact on patient care. *Eur. J. Pediatr.* **159**, 719–725
25. Nakahashi, Y., Fujita, H., Taketani, S., Ishida, N., Kappas, A., and Sassa, S. (1992) The molecular defect of ferrochelatase in a patient with erythropoietic protoporphyria. *Proc. Natl Acad. Sci. USA* **89**, 281–285