Figure 5B

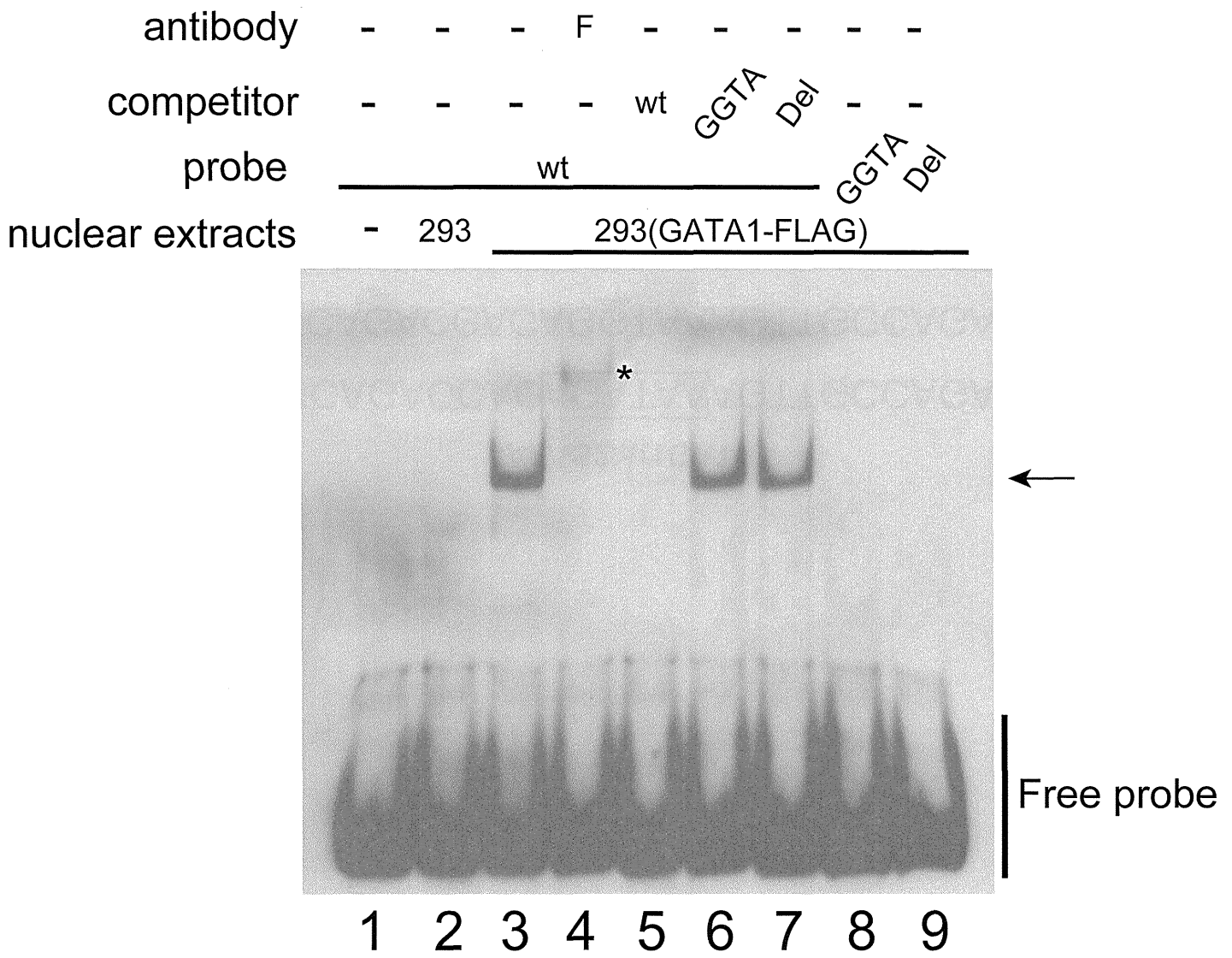

antibody
competitor
probe
nuclear extracts
F
wt
GGTA
Del
293
293(GATA1-FLAG)
*
Free probe
1 2 3 4 5 6 7 8 9

Figure 6A

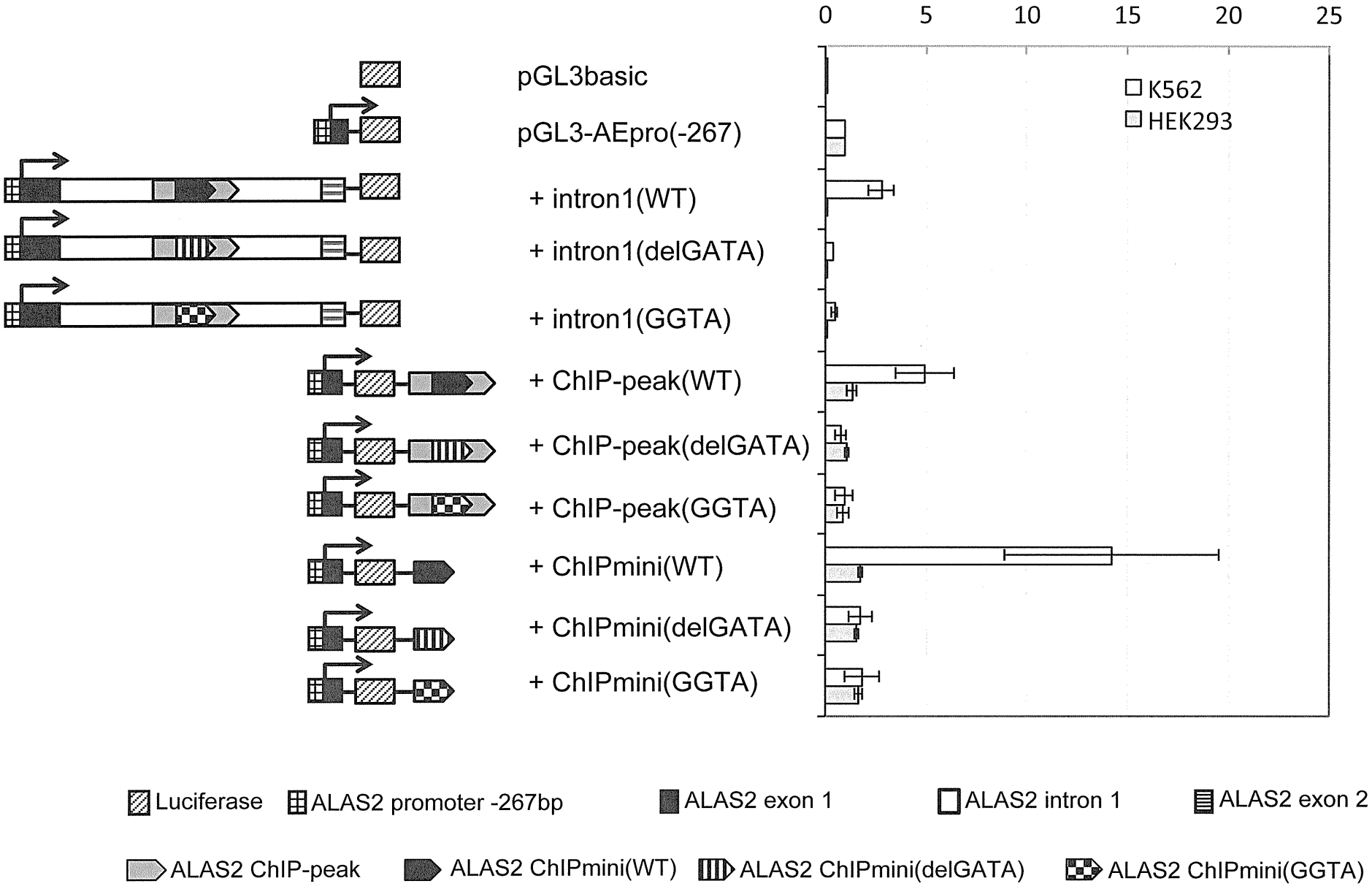

0
5
10
15
20
25
K562
HEK293
pGL3basic
pGL3-AEpro(-267)
+ intron1(WT)
+ intron1(delGATA)
+ intron1(GGTA)
+ ChIP-peak(WT)
+ ChIP-peak(delGATA)
+ ChIP-peak(GGTA)
+ ChIPmini(WT)
+ ChIPmini(delGATA)
+ ChIPmini(GGTA)
Luciferase
ALAS2 promoter -267bp
ALAS2 exon 1
ALAS2 intron 1
ALAS2 exon 2
ALAS2 ChIP-peak
ALAS2 ChIPmini(WT)
ALAS2 ChIPmini(delGATA)
ALAS2 ChIPmini(GGTA)

Figure 6B

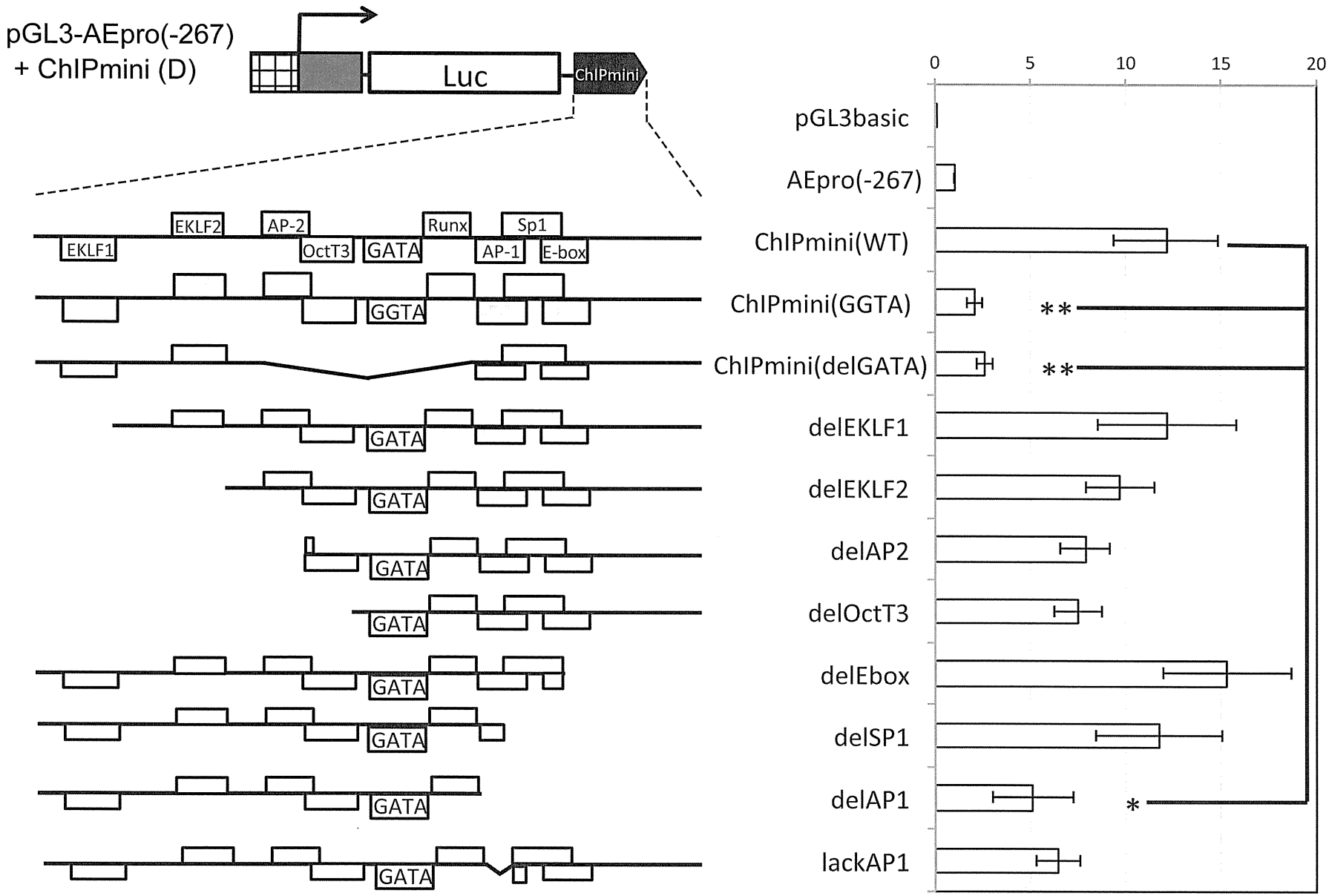
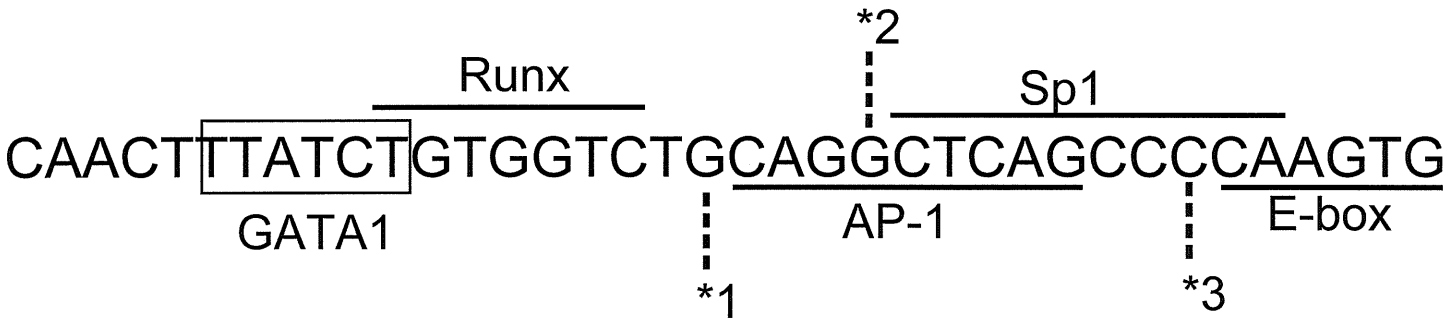
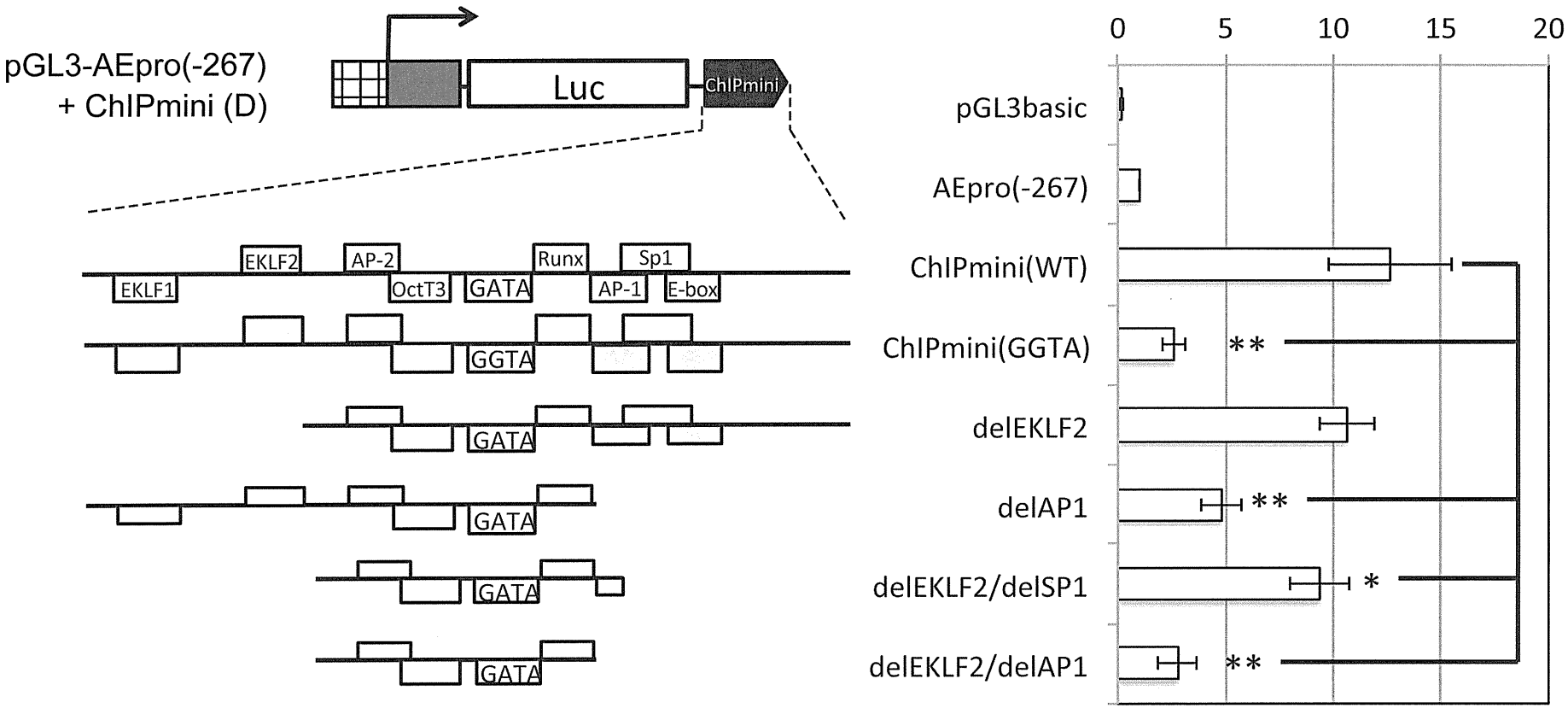

pGL3-AEpro(-267)
+ ChIPmini (D)
Luc
ChIPmini
EKLF2
AP-2
Runx
Sp1
EKLF1
OctT3
GATA
AP-1
E-box
GGTA
GATA
GATA
GATA
GATA
GATA
GATA
GATA
0
5
10
15
20
pGL3basic
AEpro(-267)
ChIPmini(WT)
ChIPmini(GGTA)
ChIPmini(delGATA)
delEKLF1
delEKLF2
delAP2
delOctT3
delEbox
delSP1
delAP1
lackAP1
**
**
*

Figure 6

C


*2
Runx
Sp1
CAACTTTATCTGTGGTCTGCAGGCTCAGCCCCAAGTG
GATA1
AP-1
E-box
*1
*3

D


pGL3-AEpro(-267)
+ ChIPmini (D)
Luc
ChIPmini
EKLF2
AP-2
Runx
Sp1
EKLF1
OctT3
GATA
AP-1
E-box
GGTA
GATA
GATA
GATA
GATA
0
5
10
15
20
pGL3basic
AEpro(-267)
ChIPmini(WT)
ChIPmini(GGTA)
delEKLF2
delAP1
delEKLF2/delSP1
delEKLF2/delAP1
**
**
*
**

Supplemental methods

Reagents

Chemical reagents were purchased from SIGMA-ALDRICH (St. Louis, MI), Nacalai Tesque (Kyoto, Japan) and Wako pure chemicals (Osaka, Japan).

Identification of the mutations of ALAS2 gene

Genetic analyses performed in this project had been approved by the ethical committee of Tohoku University School of Medicine. Blood samples were withdrawn from the probands and the family members after informed consent. Genome DNA was then extracted using QIAamp DNA Blood Midi Kit (QIAGEN GmbH, Hilden, Germany). All exons including exon-intron boundaries, the proximal promoter region and the erythroid enhancer in intron 8 of ALAS2 gene (GeneBank genomic: NG_8983.1, GenBank mRNA: NM_000032.4, GenBank: protein; NP_000023.2) were amplified using ExTaq DNA polymerase. Sequences of primers and the conditions for PCR were reported previously.[1] In addition, the first intron of ALAS2 gene was amplified with PrimeStar DNA polymerase. Amplified DNA fragments were purified using QIAquick gel purification kit (QIAGEN GmbH), and the sequences of each amplified DNA were determined directly using BigDye terminator sequencing kit ver. 1.1 with ABI3100 genetic analyzer (Applied Biosystems, Foster city, CA). The sequences of primers for amplification of the first intron of ALAS2 gene are listed in supplemental table 1.

ChIP-quantitative PCR analysis

Real-time-PCR-based quantitative chromatin immunoprecipitation (ChIP) analysis was conducted essentially as described,[2] K562 cells were cross-linked with 1% formaldehyde (Sigma) for 10 min at room temperature. The nuclear lysate was sonicated to reduce DNA length, and the protein-DNA complexes were then immunoprecipitated with anti-GATA1 antibody (ab11963, Abcam, Cambridge, United Kingdom) or control rabbit IgG and Protein A Sepharose (Sigma). Immunoprecipitated DNA fragments were quantified by real-time PCR using the primer set listed in Fig. 2A and supplemental table 1. Product was measured by SYBR Green fluorescence (QIAGEN) in a reaction mixture of 20 μL, and the amount of product was determined relative to a standard curve generated from titration of input chromatin. Analysis of post-amplification dissociation curves showed that primer pairs generated single products.

Identification of the mutation of the mutation of SLC25A38, ABCB7, GLRX5, PUS1 and SLC19A2 gene

All exons encode each protein were amplified with their exon-intron boundaries using ExTaq DNA polymerase. Primer sequence for the amplification of SLC25A38, ABCB7, GLRX5 and SLC19A2 genes were listed in supplemental table 2, 3, 4 and 5, respectively. Primer sequence for PUS1 gene was reported previously.[3]

Cell culture

K562 erythroleukemia cells were maintained in RPMI-1640 medium (WAKO Pure

Chemical Industries Ltd.), and HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium with high glucose (WAKO). Both mediums were supplemented with 10% heat-inactivated FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin.

Electrophoretic Mobility Sift Assay (EMSA)

EMSA was performed using "DIG Gel Shift Kit, 2nd Generation" (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. Sequences of oligonucleotides for probes are indicated by the horizontal bar in Fig. 2B and Fig 4A, and are listed in supplemental table 1. Nuclear extracts were prepared, as described previously,[4] from K562 cells or HEK293 cells that were transfected with GATA1 expression vector or its backbone vector. For construction of the GATA1 expression vector that expresses GATA1 as a FLAG-tagged protein, human GATA1 cDNA was amplified by PCR, and was then cloned into pFLAG-CMV5a plasmid (SIGMA). The resulting FLAG-fused GATA1 expression vector or pFLAG-CMV5a was introduced into HEK293 cells using Lipofectamine 2000 transfection reagent (Invitrogen) at 48 hours before harvest.

Promoter/enhancer activity assay.

The human ALAS2 proximal promoter region (g.4820_5115, between -267 and +29 from the transcription start site) was amplified by PCR, and cloned between BglII and HindIII site of pGL3basic plasmid (Promega Corporation, Madison, WI). The resulting plasmid was referred as pGL3-AEpro(-267), which was used as a parent plasmid. For examining the

enhancer activity of the first intron, a single DNA fragment (5.2 kbp), carrying ALAS2 proximal promoter, first exon, first intron and the untranslated region of second exon, was amplified by PCR, and was subcloned between KpnI and HindIII sites of pGL3basic plasmid (referred as pGL3-AEpro(-267)+intron1). A DNA fragment containing GATA1 binding region in the first intron of ALAS2 gene, which was defined by ChIP-seq analysis,[2] was amplified by PCR using genome DNA prepared from healthy volunteer, proband 1 or proband 3, and was cloned into pCRII-blunt-TOPO vector (Invitrogen). Each DNA fragment obtained after the digestion with appropriate enzymes, which corresponds to g.7488_7960 for wild-type, was referred as ChIP-peak(WT), ChIP-peak(GGTA) or ChIP-peak(delGATA), respectively. The length of ChIP-peak(WT) is 473 bp. In addition, a 130-bp fragment containing ALAS2int1GATA in ChIP-peak was amplified by PCR using genome DNA prepared from healthy volunteer, proband 1 or proband 3, and referred as ChIPmini(WT), ChIPmini(GGTA) or ChIPmini(delGATA), respectively, and cloned into pCRII-blunt-TOPO. Primer sets for PCR were listed in supplemental table 1. Each of these DNA fragments was inserted at the upstream of ALAS2 proximal promoter or the downstream of the luciferase gene of pGL3-AEpro(-267) vector using appropriate restriction enzymes. pGL3-TKpro plasmid was constructed by transferring herpes simplex virus thymidine kinase (HSV-TK) promoter from pRL-TK plasmid (Promega) to the upstream region of luciferase gene of pGL3basic plasmid. Then, ChIP-peak(wt) or ChIPmini(WT) was inserted at the downstream of the luciferase gene of pGL3-TKpro, resulting in pGL3-TKpro+ChIP-peak or pGL3-TKpro+ChIPmini, respectively. To examine the erythroid-specific or non-specific

enhancing activity of each DNA fragment on ALAS2 promoter or HSV-TK promoter, each reporter vector (0.075 pmol/well) and pEF-RL[5] (5 ng/well) were introduced into K562 human erythroleukemia cells or HEK293 human embryonic kidney cells using X-tremeGENE HP transfection reagent (Roche) or Fugene HD transfection reagent (Roche), respectively. Cells were incubated for 24 hours after transfection and were collected to determine luciferase activity using Dual-luciferase reporter system (Promega). Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity.

Measurement of ALAS2 mRNA in purified erythroblasts

Bone marrow aspirates were overlayed on Ficoll-Paque PLUS (GE Healthcare UK Ltd, Buckinghamshire, England) to obtain mononuclear cells, and glycophorine A-positive cells were then separated using MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Total RNA was extracted from these cells with TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized from total RNA using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). Reaction mixtures (20 μL) for real-time quantitative RT-PCR consisted of 2 μL of cDNA, 10 μL of SYBR green master mix (QIAGEN) and appropriate primers. Product accumulation was monitored by measuring SYBR Green fluorescence and normalized with GAPDH (MIM# 138400) mRNA.

References

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5. Muto A, Hoshino H, Madisen L, Yanai N, Obinata M, Karasuyama H, et al. Identification of Bach2 as a B-cell-specific partner for small maf proteins that negatively regulate the immunoglobulin heavy chain gene 3' enhancer. EMBO J. 1998;17(19):5734-43.

Supplemental table 1

		forward (sense) primer	reverse (antisense) primer
Amplification of proximal promoter and intron 1 of ALAS2 gene		GGTACCTCAAGGTATCCCAGCTCTATG	AAGCTTGAACCTAAAGTCCTGCAGAAGAC
Amplification of ALAS2 GATA1 ChIP-peak		GCCAAAAGCCCCCTAGTAATGATGT	CAGAAGGGTTGTTGGAGGGACTAAAT
Primer set used in Figure 2A	1	GGGGTACACTAGAGGGAGGGGC	GGACGAACGAATGACAGGTGGGT
	2	GGCAACAGGTAAGAGCTGCTTTCAG	TCAGATCTACTCAGACCAGAGAGTCA
	3	ACTGACTCTCTGGTCTGAGTAGAT	TCAGAGAATGATGAAATGAAATGAATGATC
	4	TTTCATTTCATCATTCTCTGACACTCTTGC	GGAGAGAGGAATGGGGAATTGCAG
	5	GGCTTGGCCTGTAATTTCCACCACC	TCCTAGTACCCCTTTCCCACCATGA
	6	TGGGTGCTGACACAAGAGAGGATT	TGTCACCAATGCCTTACCAAAGGAACA
	7	AAGAGGCCTATGTTAGTGCAGCAGA	TGCCAGGCCGTGTTCTCATGT
	8	TGAGAACACGGCCTGGCACA	GAACGTACAGCCAAGGGAAGTCACA
	9	TGTGACTTCCCTTGGCTGTACGTT	AACAAGGACAATCTGCATCACAGGAA
	10 (ChIPmini)	TCTCCCACGCCCTGGTCTCA	TTCCCTTCCCCTGCCTGCTTGT
	11	CCTCAAAACATAAGGCTGAGGGTGC	GCAGGGGGATGAGGGTTAAGAGGGGTG
	12	TGTCCCTTTCGTGCCCCTTGG	GGTAGAGGGAAAGGGAGCAGGGT
	13	ACCCTGCTCCCTTTCCCTCTACCT	AGCAAATGACACACAGGCACTCAA
probe for EMSA	wild type	TAAACTCTGGCAACTTTATCTGTGGTCTGCAGGCTC	GAGCCTGCAGACCACAGATAAAGTTGCCAGAGTTTA
	GGTA mutant	TAAACTCTGGCAACTTTACCTGTGGTCTGCAGGCTC	GAGCCTGCAGACCACAGGTAAAGTTGCCAGAGTTTA
	delGATA mutant	TGGGGAGTGGTCAGACCCCTGCAGGCTCAGCCCCAA	TTGGGGCTGAGCCTGCAGGGGTCTGACCACTCCCCA

Supplemental table 2

Primers for amplification of each exon of human SLC25A38 gene (coding region)

	forward primer	reverse primer
exon 1	GTCGTCCACGCTGGTCTCCA	CCCCGGCAATTCCGCCCTTT
exon 2	TGAGGCACCACCAGGTAAGTGT	GCTGCTCAGGAACGGACCCC
exon 3	AGGAAGTGTTTGAGTGGGGAATTGTTT	AGACCACATAGGTACTCCCACCACT
exon 4	TGGGGTCTTTTGGGAAAACCCAGC	GTGACTCGCATGGAGGCGCT
exon 5	GCCCCATAACCTGCAGTCTGCTT	CACCCTATCCTCACCCCGCCA
exon 6	GGTGGGCAACTTGCACTGACCT	GCCTAGATTTTAACCTGGGCATGGGG
exon 7	ACCCTCACTGTGGTACCAAG	CCTGGTTTTCCAGGTAGGAC

Supplemental table 3

Primers for amplification of each exon of human ABCB7 gene

	Forward primer	Reverse primer
exon 1	ACAGCTGAAGCCTCCTCCCAGG	CCCCGAGGTCAGGAGGGCAA
exon 2	TGATCCGCCCGCCTTAGCCT	TCTCTGCATTTCCAGAAGCAGAAACAT
exon 3	AGTGAATGACACTGGGAAAGCCAG	ACCTTGAAGCACACGCACACACA
exon 4	ACCAAGCCCTCTGCTTTCCTAAAAGG	AGTGATTTACACCAGGCCCAGGA
exon 5	AGCCTGAAATGACAGCTCTCCCA	AACCTCCTTGAAGAAAGTCAACACCTG
exon 6	TCCACAGTAATGCCATGTGGGCT	CCCATGGGCATGCAACAGTACA
exon 7 & 8	CACGTACATAACTTCACGCCACCA	GGGACCAACATCATAGATGCCAAAACA
exon 9	TCAGGGGAAGGCTTTGTGAAGGA	CCAATCAGTGAGTGAGGCAGTGCT
exon 10	GGTGGGTCTTTCCCATTCCTAACG	AGCACCCCCACCCCTGACAA
exon 11 & 12	CCCTCCCCAACCCCACCTCA	GAGGCCCCAGGCCACACAAC
exon 13	ACCCCTGGGAAGGGAATGGGA	ACCCAATCAAATGTGACTCAACGAGCA
exon 14	GCCTCATTCTCATTCTTCCCACCTGC	TGGAAAAAGGGGGATAGGCATTTTGCT
exon 15	AGTTGCCTTCTCTTTTTGCTTTCTCCT	AGGGGCTAAAAACAGAATCGTAACAGG
exon 16	GGCACTGGGTAGCTCAACAGGGA	TGAGCACAACCAGGACAGTGACA

Supplemental table 4

Primers for amplification of each exon of human GLRX5 gene

	forward primer	reverse primer
exon 1	CCGCGCCTCTCCCAGTTGTCT	CCGGCTCGAACCTTCAGACAGAC
exon 2	GGGAAGCCAGGGAGGGACAGTG	CAGGGCTCCAGAGATAGGCAGGTG

Supplemental table 5

Primers for amplification of each exon of human SLC19A2 gene

	forward primer	reverse primer
exon 1	CAATGGAAGAGCAGGCAAGT	CGCTTTTCTCGGTCCTCTCT
exon 2	CCAGGTCCTTTCATCACTAATGT	GCCCCCATAGTAGCAATTACA
exon 3	TGGGCCTGTAAATTGCTTTC	CAAATTTGGGAGGGGTGAAT
exon 4	GCAACAGCATTTGTGTAGCA	ACAATGCTTCCTCCCATTTG
exon 5	CATTTGGTTGGAAAGGCAAT	TCACCCTGATCAAGTCACACA
exon 6	GGCACGTGGTGTAAGTATGC	TGCTGTGCAGAGTTCTTGCT

Differential regulation of 5-aminolevulinate synthase isozymes in vertebrates

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X. Summary and perspectives for the future

I. Introduction

Heme plays an essential role in several biological pathways, such as hemoglobin formation, electron transfer in mitochondria and xenobiotic metabolism, as a prosthetic group of several proteins[1]. On the other hand, excess amount of heme might produce reactive oxygen species; therefore heme production in mammalian cells is tightly regulated. In this chapter, it will be discussed how heme biosynthetic pathway is regulated in animal cells by focusing the regulatory mechanisms for the expression of 5-aminolevulinate synthase (ALAS), which is the first and rate-limiting enzyme of heme biosynthetic pathway.

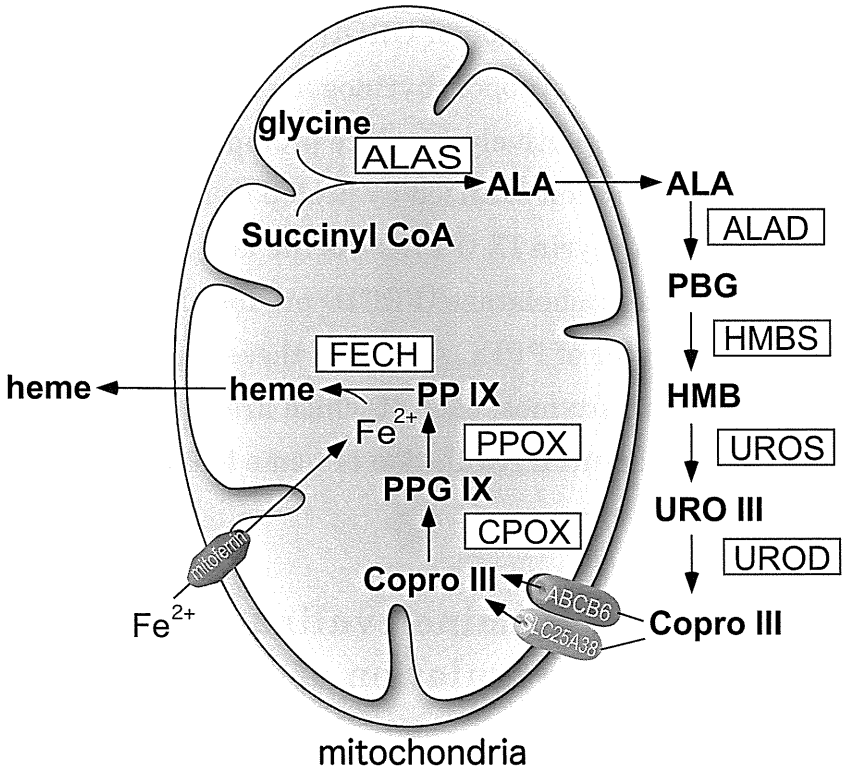

Figure 1. Heme biosynthetic pathway in vertebrates (Adopted from ref. 1)

ALA, 5-Aminolevulinic acid; PBG, Porphobilinogen; HMB:, Hydroxymethylbilane; UROIII, Uroporphyrinogen III; COPRO III, Coproporphyrinogen III; PPG IX, Protoporphyrinogen IX; PP IX, Protoporphyrin IX; ALAS, 5-Aminolevulinate synthase; ALAD, 5-Aminolevulinate dehydratase; HMBS, Hydroxymethylbilane synthase (= PBGD: Porphobilinogen deaminase; UROS, Uroporphyrinogen III synthase; UROD, Uroporphyrinogen decarboxylase; CPOX, Coproporphyrinogen oxidase; PPOX, Protoporphyrinogen oxidase; FECH, ferrochelatase

II. Biosynthetic pathway of heme in vertebrate

Eight enzymes are involved in heme biosynthetic pathway in mammals[2]. As shown in Figure 1, heme biosynthesis is initiated by ALAS, which condensates glycine and succinyl Coenzyme A (CoA) to form aminolevulinic acid (ALA). This reaction is catalyzed in mitochondrial matrix where ALAS is localized. Synthetized ALA is exported to cytoplasm; two molecules of ALA are then condensed to produce one molecule of porphobilinogen (PBG), which reaction is catalyzed by ALA dehydratase (ALAD). Then, hydroxymethylvilane synthase (HMBS) (alternatively, porphobilinogen deaminase; PBGD) linearize four molecules of PBG to form hydroxymethylvilane (HMB). HMB is subsequently circularized by uroporphyrinogen III synthase (UROS) to form uroporphyrinogen III (UROIII). Stepwise decarboxylation of UROIII by uroporphyrinogen decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX), which is fifth and sixth enzyme, respectively, produce protoporphyrinogen IX. Protoporphyrinogen IX is then oxidized by protoporphyrinogen oxidase (PPOX) to form protoporphyrin IX (PPIX). As the last step of heme biosynthetic pathway, ferrochelatase (FECH) produce heme by chelating ferrous iron into the center of PPIX. Among of these eight enzymes, only ALAS have two distinct isozymes, each of which are encoded by independent gene, resulting in a differential regulation of heme biosynthesis in erythroid cells and non-erythroid cells.

III. Identification of 5-aminolevulinate synthase (ALAS) and tissue-specific regulation of heme biosynthesis

In 1958, Laver et al.[3] demonstrated the enzymatic formation of 5-ALA using a certain particle fraction, which might be a mitochondria rich fraction, obtained from a lysate of avian erythrocytes. Using this system, Gibson et al. reported that this enzyme condensate glycine and succinyl-CoA to form ALA[4]. Kikuchi et al. independently succeeded the first purification of soluble ALAS from the extracts of Rhodopseudomonas spheroids and

Rhodospirillum rubrum[5]. Thereafter, as a source of ALAS, avian erythrocytes and photo-synthetic bacteria was preferentially used to examine the regulation of ALAS expression in vertebrates and to clarify the mechanisms of enzymatic reaction of ALAS, respectively. As for the regulation of heme biosynthetic pathway in mammals, Granick and Urata first showed the evidences that 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) induced ALAS activity in hepatocyte mitochondria isolated from Guinea pig[6]. Miyakoshi et al. also confirmed that allylisopropylacetamide (AIA) induced the ALA synthesis in rat liver mitochondria[7].

It has been suggested that ALAS is the rate-limiting enzyme of heme biosynthetic pathway in liver[6], as well as in erythroid cells[8], however, Wada et al. reported that ALAS activity in hematopoietic tissue was differently regulated from that in liver[9], when mice were treated with hypoxia or DDC. Briefly, ALAS activity in hematopoietic tissue (spleen), but not in liver, was increased under hypoxic environment, while ALAS activity was greatly induced only in liver by DDC treatment. Moreover, these authors found that hemin treatment could prevent the induction of ALAS activity in liver after DDC treatment, although there was no effect on the induction of ALAS activity in hematopoietic tissues under hypoxia. These results suggested that ALAS activity was independently regulated in hematopoietic tissues or in liver.

IV. Establishment of ALAS isozymes

Although ALAS activity was known to be differentially regulated in liver and in hematopoietic organs (fetal liver and adult bone marrow)[9, 10], it was not clear whether hepatic ALAS and erythroid one was identical or not. Bishop et al. demonstrated that some biochemical properties (e.g., binding affinity to AMP-agarose, Km value for glycine and the inhibition of enzymatic activity by 0.4M sodium chloride) were different between erythroid and non-erythroid ALAS, which were purified from hematopoietic organs and liver of guinea pig, respectively[11]. Then, Watanabe et al. found that in vitro translated products using polysomes isolated from chicken