We have successfully identified novel erythroid-specific enhancer for ALAS2 expression, and have identified disease causative mutations of this enhancer in patients with CSA. Despite that about fifty missense or non-sense mutations of ALAS2 gene have been reported as disease causative mutations in patients with XLSA,[3,30] the mutation in the regulatory region for the transcription of ALAS2 gene was rarely reported to date. For example, Ducamp et al. reported that the 48-bp deletion of the ALAS2 gene at the proximal promoter region (c.-91_-44del) in a patient with XLSA, and they proposed that the identified deletion should cause XLSA, since ALAS2 mRNA level in proband's bone marrow was lower than that of normal controls.[31] In this context, it has been reported that the deleted region contained functionally important element for ALAS2 transcription.[16] In addition, Bekri et al. reported the C-to-G transversion at nucleotide -206 (c.-258C>G) from the transcription start site in the proximal region of human ALAS2 gene in patients with XLSA,[24] however, May et al. further demonstrated that this mutation was identified in the normal individuals from South Wales at the rate of 0.05, indicating that this promoter mutation should be a polymorphism.[32]

In conclusion, we have identified the novel erythroid-specific enhancer in the

first intron of human ALAS2 gene, the enhancer function of which may be directed by GATA1 with other transcription factors, such as EKLF and AP-1 binding proteins. Furthermore, we identified the loss-of-function mutation of ALAS2int1GATA, the GATA element within this enhancer, in five within eleven patients with congenital sideroblastic anemia, in whom responsible gene could not be identified. Thus, the intronic region containing ALAS2int1GATA of the ALAS2 gene should be examined in patients with XLSA or nfCSA, in whom the responsible gene for sideroblastic anemia is unknown.

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Authorship and Disclosures

K.F. and H.H. designed the study; R.K. and H.I. recruited the patients; K.K., T.F. and K.F. performed experiments; K.K., K.F., T.F., R.K. and H.I. prepared figures and tables; K.F., K.K., T.F., R.K., H.I. H.H. and S.S. wrote the paper.

There are no relevant conflicts of interest to disclose.

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Table 1. Hematologic status of each proband on diagnosis of the disease

	onset of the anemia	Age on diagnosis of SA	family history for XLSA	Hb (g/L)	MCV (fl)	MCH (pg)	platelet (x10E9/L)	serum iron (μmol/L)	ferritin (pmol/L)
Proband 1	0 y.o. (4 months)	0 y.o. (4 months)	yes	39 [136-183]	65 [83-101]	18.7 [28-35]	246 [140-379]	63.9 [10.7-37.6]	399.7 [49.4-270]
Proband 2	4 y.o.	4 y.o.	no	84 [126-165]	73.4 [87-104]	22 [29-35]	610 [138-309]	49.1 [12.5-25.0]	670.1 [67.4-725]
Proband 3	2 y.o.	19 y.o.	no	78 [120-165]	73.9 [80-100]	22.2 [28-34]	373 [160-420]	39.6 [14.3-21.5]	2489.7 [40.4-288]

SI unit and normal value of each clinical examination was in parentheses and brackets, respectively.

Figure legends

Figure 1. Identification of a functional GATA1 element in the first intron of ALAS2 gene.

A: Chromatin immunoprecipitation assay. Fragmented genomic DNA segments were immunoprecipitated with anti-GATA1 antibody or control IgG, and then precipitated fragments were quantified using real-time PCR as described in "supplemental methods". PC or NC indicates positive control or negative control, respectively, for ChIP assay using anti-GATA1 in K562 cells.[22] One GATA element is present in the proximal promoter region and 17 GATA elements in the first intron (black symbols). Shaded double arrow indicates the region corresponding to ChIP-peak.

B: Nucleotide sequence of ChIPmini. The GATA binding site, ALAS2int1GATA, is located in the center of ChIPmini (boxed). Box also indicates the consensus for E-box that is bound by Scl/TAL1[22]. The sequence of ChIPmini was further analyzed for putative transcription factor binding sites using GeneQuest software (DNASTAR Inc., Madison, WI), and the results are indicated with the horizontal bar. Yi-consensus, Yi transcription

factor consensus site[33]; BRE, transcription factor IIB binding site[34]; EKLF, erythroid/kruppel like factor consensus site[35]; Sp1, stimulatory protein 1 binding site[36]; P300, P300 transcriptional coactivator consensus site[37]; AP-2, AP-2 beta consensus site[38]; Hox-TF, C1 element binding factor binding site[39]; OctT3, OctT3 binding site[40]; Runx, Runx proteins binding site[41]; AP-1, Activator Protein 1 binding site[42]; and AP4/E1247, AP4/E1247 binding site[43]. The sequence for the wild-type probe used in EMSA is indicated with the dashed line. A double arrow indicates the deleted region of delGATA mutation.

Figure 2. Functional analyses of ChIPmini present in the first intron of ALAS2 gene.

A: Electrophoretic mobility shift assay (EMSA). Wild-type (wt) probe was incubated with nuclear extracts prepared from K562 cells (lanes 2-4) or HEK293 cells expressing GATA1-FLAG (lanes 6-9). HEK293 cells were transfected with mock vector (lane 5) or FLAG-fused GATA1 expression vector before preparation of nuclear extracts. The protein-probe complex was detected as a retarded band (arrow). An excess amount of

unlabeled probe (lanes 3, 7), anti-GATA1 antibody (G1) (lanes 4, 8) or anti-FLAG antibody (F) (lane 9) was included in the reaction mixture. Lane 1 shows the control without nuclear extracts. Asterisk indicates the super-shifted band (lane 9).

B. Functional analysis of ChIPmini as an enhancer for ALAS2 gene. Details for the fragments consisting each plasmid, such as intron1, ChIP-peak and ChiIPmini, was described in Methods. Each DNA fragment was inserted at the upstream of ALAS2 proximal promoter or at the downstream of luciferase cDNA, indicated as (U) or (D), respectively. Results are expressed as a relative activity compared to that of pGL3-AEpro(-267), and are presented as the mean ± standard deviation (S.D.) of three independent experiments.

C: Functional analysis of ChIPmini as an enhancer for non-erythroid gene promoter. The enhancer activity of the first intron was examined using the herpes simplex virus TK promoter as a non-erythroid promoter. ChIP-peak or ChIPmini was inserted at the downstream of luciferase gene of pGL3-TKpro, yielding pGL3-TKpro+ChIP-peak(D) or pGL3-TKpro+ChIPmini(D). Each of these reporter vectors was introduced into K562 cells or HEK293 cells for measuring enhancer activity. Results are expressed as a relative

activity compared to that of pGL3-TKpro, and are presented as the mean ± S.D. of three independent experiments.

Figure 3. Family trees of three unrelated probands

Shown are the family trees of proband 1 with XLSA (A), proband 2 with nfCSA (B), and Proband 3 with nfCSA (C). Shaded boxes indicate affected individuals in each pedigree. Asterisks indicate the individuals in whom mutation in the first intron of ALAS2 gene was detected.

Figure 4. Identification of mutations in the first intron of ALAS2 gene in a patient with XLSA and two patients with nfCSA.

A. ALAS2 mutations of three probands. Upper, middle and lower panels show the sequences of the flanking regions of ALAS2int1GATA (boxed in the wild-type sequence) in ALAS2 gene of probands 1, 2 and 3, respectively. Asterisk indicates the T to C transition in the sense strand identified in ALAS2 gene of proband 1 and proband 2 with CSA. Broken line between middle and lower panels indicates the deleted region

identified in proband 3 with CSA. Solid horizontal bar in each panel indicates the sequence of the sense strand of each probe used for EMSA (see Fig. 3A and 5B).

B: ALAS2 mRNA expression in erythroblasts of proband 3. ALAS2 mRNA levels were determined in purified erythroblasts isolated from proband 3 and three independent normal individuals using real-time PCR. Results are expressed as the mean ± S.D. of independent three experiments.

Figure 5. Effects of the mutations of ALAS2int1GATA on the GATA1-binding activity.

A: DNA probes used in EMSA. Shown are the nucleotide sequences in the antisense strand of the probes. The position of each probe is also indicated in the Figure 1B as the solid horizontal bar. ALAS2int1GATA is boxed in the sequence of WT probe, and the single nucleotide transition (GGTA mutation) is underlined in the sequence of GGTA probe. The delGATA probe represents the 5'- and 3'-flanking sequences of the deleted 35-bp segment (see Fig. 3B).

B: Effect of each mutation of ALAS2int1GATA on the GATA1-binding activity.

Wild-type probe (lanes 3-7) or each mutant probe (lanes 8, 9) was incubated with the nuclear extracts prepared from HEK293 cells transfected with GATA1-FLAG expression vector. An excess amount of unlabeled wild-type probe (lane 5), each of unlabeled mutant probes (lanes 6, 7), or anti-FLAG antibody (lane 4) was included in the reaction mixture. Lane 2 shows the negative control with nuclear extracts from HEK293 cells transfected with mock vector.

Figure 6. Identification of cis-elements essential for erythroid-specific enhancer activity of ChIPmini.

A: Effect of each mutation of ALAS2int1GATA on the enhancer activity of ALAS2 ChIPmini. The region corresponding to +intron1, ChIP-peak or ChIPmini, derived from proband 1 or proband 3, was subcloned into pGL3-AEpro(-267) for constructing the reporter vector containing the GGTA mutation or the deletion of ALAS2int1GATA, respectively.

B: Effect of the deletion at the 5'- or 3'-flanking region of ALAS2int1GATA on the enhancer activity of ChIPmini.

The 5'- and 3'-flanking regions of ALAS2int1GATA contain potential transcription factor-binding sites (cis-elements), and a portion of each flanking region was deleted, as schematically shown. The enhancer activity of each deletion mutant was determined in K562 erythroleukemia cells.

C: The nucleotide sequence of the 3'-flanking region of ALAS2int1GATA. Note that the Sp1 site is overlapping with AP-1 site and E-box. Each number, *1, *2 or *3, indicates the nucleotide at the 3' end of the deletion mutant, delAP1, delSP1 or delE-box, respectively. Thus, delSP1 also lacks the 3' portion of the AP-1 site.

D: Effect of deletion of the 5'- and 3'-flanking regions of ALAS2int1GATA on the enhancer activity of ChIPmini.

The construct, delEKLF2/delSP1, lacks two EKLF sites in the 5'-flanking region and both Sp1 element and E-box in the 3'-flanking region. The AP-1 element at the 3'-flanking region was deleted from delEKLF2/delSP1, yielding delEKLF2/delAP1.

Results are expressed as a relative activity compared to that of pGL3-AEpro(-267), and are presented as the mean ± S.D. of at least three independent experiments.

Figure 1A

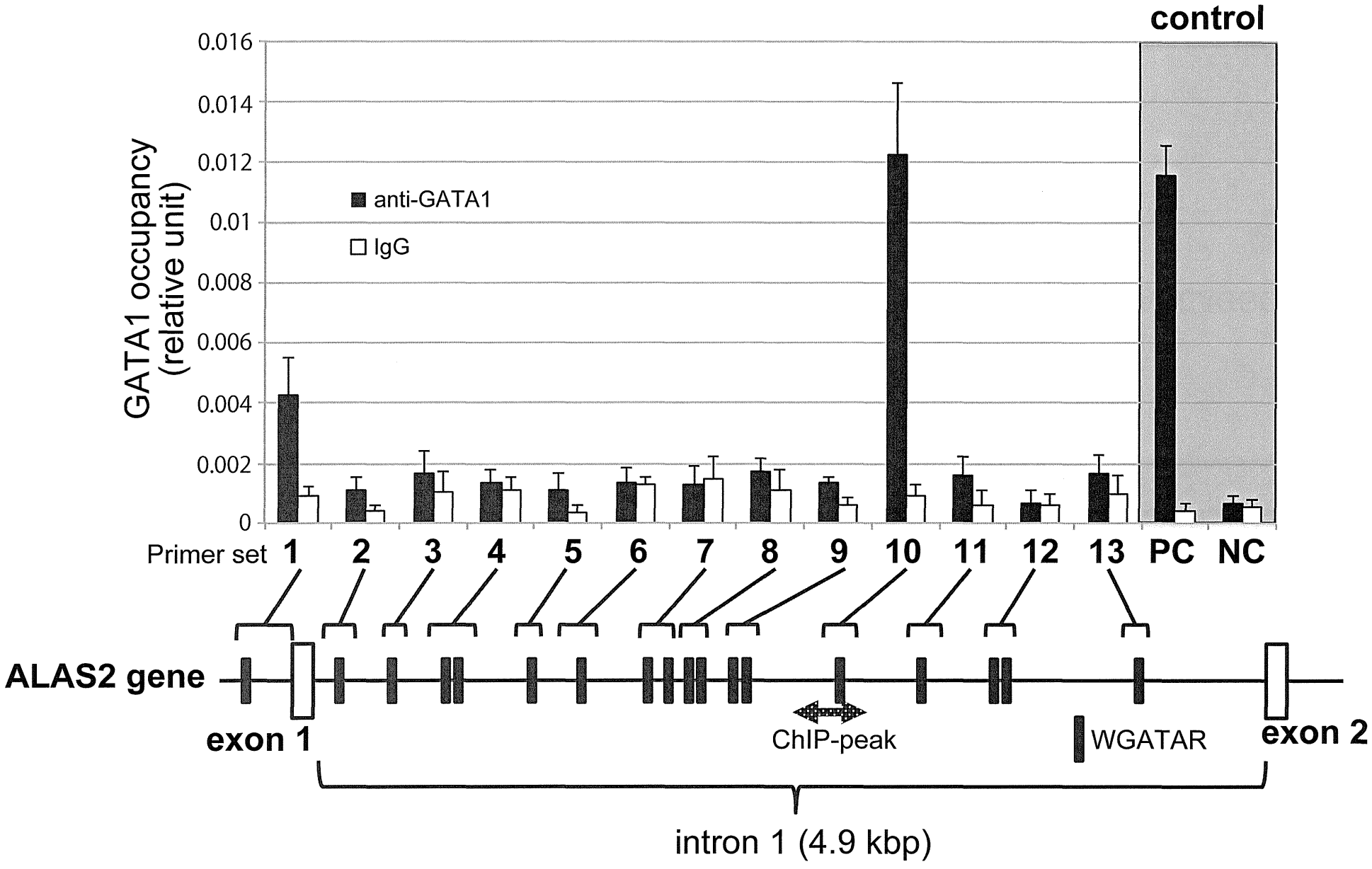
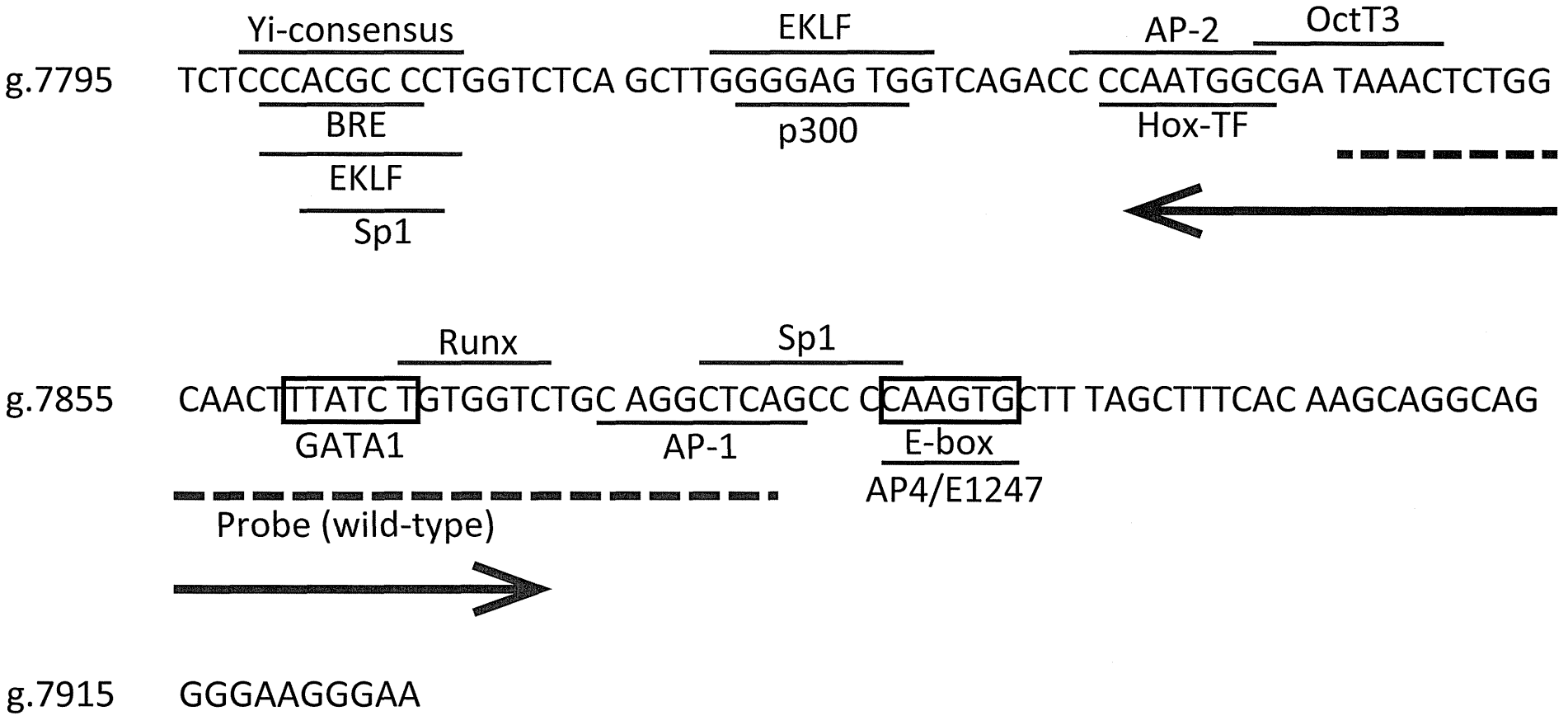

control
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
GATA1 occupancy (relative unit)
anti-GATA1
IgG
Primer set 1 2 3 4 5 6 7 8 9 10 11 12 13 PC NC
ALAS2 gene
exon 1
ChIP-peak
WGATAR
exon 2
intron 1 (4.9 kbp)

Figure 1B


Yi-consensus
EKLF
AP-2
OctT3
g.7795 TCTCCCACGC CCTGGTCTCA GCTTGGGGAG TGGTCAGACC CCAATGGCGA TAAACTCTGG
BRE
EKLF
Sp1
p300
Hox-TF
Runx
Sp1
g.7855 CAACTTTATC TGTGGTCTGC AGGCTCAGCC CCAAGTGCTT TAGCTTTCAC AAGCAGGCAG
GATA1
AP-1
E-box
AP4/E1247
Probe (wild-type)
g.7915 GGGAAGGGAA