Deletion of the RUNX1 binding site in the erythroid cell-specific regulatory element of the ABO gene in two individuals with the A_m phenotype

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ABSTRACT

Background and Objectives: An erythroid cell-specific regulatory element, referred to as the +5.8kb site, had been identified in the first intron of the human *ABO* blood group gene. Subsequent studies revealed that either a 5.8-kb deletion including the +5.8kb site or disruption of a GATA factor binding motif at the site was present in all B_m and AB_m individuals examined. We investigated the molecular mechanism of the A_m phenotype which is analogous to the B_m phenotype. **Materials and Methods:** Genomic DNAs were prepared from peripheral blood of two A_m individuals, and the nucleotide sequences were investigated using PCR and direct sequencing. Electrophoretic mobility shift assay (EMSA) and promoter assay with K562 cells were carried out. **Results:** A novel 23-bp nucleotide deletion was found at the +5.8kb site in both individuals. EMSAs demonstrated binding of the transcription factor RUNX1 to the nucleotides within the deletion. Promoter assays showed that the deletion reduced the transcriptional activity of the +5.8kb site. **Conclusion:** Deletion of the 23-bp nucleotides including the RUNX1 binding site decreases transcription of the *A* allele, resulting in reduction of A antigen expression in the A_m phenotype.

INTRODUCTION

The ABO blood system discovered by Karl Landsteiner is of great importance in the context of blood transfusion and organ transplantation. The system is composed of complex carbohydrate structures that are biosynthesized by A and B transferase encoded by the *A* and *B* genes, respectively.[1] Since Yamamoto and colleagues clarified the molecular genetic basis of the ABO system, a number of weak phenotypes or subgroups have been found to be due to single nucleotide polymorphisms (SNPs), hybrid formation between the common alleles, and mutations outside the catalytic domain of the enzyme.[2-9] However, no mutation has been reported in the coding regions or splicing sites in some weak phenotypes such as A_{el}, A_m and B_m,[2,10,11] although mutations in the exons have been revealed in some of them.[2,12-16]

Recently, we have identified an erythroid cell-specific regulatory element in the first intron between positions +5653 and +6154 3' to the ATG translation start site of *ABO*, and this regulatory activity was found to depend on binding sites for GATA transcription factors.[17,18] We referred to this element as the +5.8kb site. A 5.8-kb deletion in intron 1, including the +5.8kb site, was observed in genomic DNAs obtained from 110 of 111 B_m and AB_m individuals, whereas the exceptional B_m individual showed a nucleotide substitution of the GATA factor binding motif at the +5.8kb site.[17,18] Conversely, the deletion was never found in 1005 individuals with the common phenotypes.[17] Because the 5.8-kb deletion and disruption of the GATA motif at the +5.8kb site abrogated the erythroid cell-specific enhancer activity of this site, it is suggested that these mutations down-regulate transcription from those *B* alleles, leading to reduction of B antigen expression in cells of erythroid lineage, but not in mucus-secreting cells, resulting in the B_m phenotype.

In the A_m phenotype, which is analogous to B_m, expression of the A antigen is reduced on red blood cells (RBCs), while a large amount of A substance is present in the saliva of secretors.[1] However, A_m erythrocytes contain abundant H sites. A-

transferase activity was detected in serum of A_m individuals, although the activity was distinctly reduced in all cases. Hansen *et al.* assumed that A_m results from a reduction of *A* gene expression in bone marrow cells, but not in mucus-secreting cells.[10] In contrast, several mutations in exon 7 have been revealed in A_m.[14-16] However, considering the loss-of-function of the +5.8kb site in B_m, the A_m phenotype could be caused by a similar deletion or mutation(s) of the transcription factor binding motif at the +5.8kb site.

Here we report a novel 23-bp deletion at the +5.8kb site in two individuals with A_m. Electrophoretic mobility shift assay (EMSA) demonstrated that the region of the 23-bp deletion comprised a binding motif for the transcription factor Runt-related transcription factor 1 (RUNX1), and transient transfection experiments with luciferase reporters into K562 cells showed that the deletion reduced the transcriptional activity of the +5.8kb site. These results indicate that the deletion could reduce the enhancer activity of the +5.8kb site, leading to reduction of A antigen expression in erythroid lineage cells of individuals with A_m.

MATERIALS AND METHODS

ABO grouping and genotyping. ABO phenotypes, including the common types and subgroup A_m, were serologically determined on the basis of their features.[1] A_m was determined using agglutination tests with anti-A, anti-B, and anti-A,B antibodies as well as *Ulex europaeus*, adsorption-elution tests with pooled antisera prepared from twenty individuals with B,[19] agglutination inhibition tests for ABH substances in saliva, and plasma A transferase assay.

Genomic DNA was prepared from peripheral blood samples from the above individuals by standard phenol-chloroform extraction. The DNA samples of A_m were from a library of donors available at the Japanese Red Cross Kanto-Koshinetsu Block Blood Center. Fresh blood samples were taken after obtaining informed consent, and this study was approved by the Ethics Committees of Gunma University Graduate School of Medicine and the Japanese Red Cross Society. *ABO* genotyping was determined by direct sequencing of PCR products comprising exons 6 and 7 according to the methods described previously.[20] The number of CBF/NF-Y enhancer repeats was examined according to the method of Shimada et al.[21] The ABO alleles are presented according to the Blood Group Antigen Gene Mutation Database (http://www.ncbi.nlm.nih.gov/projects/gv/mhc/xslcgi.cgi). In addition, the Blood Group Allele Terminology used by the International Society of Blood Transfusion is also used[22]: *A102*, *O01* and *O02* are referred to as ABO*A1.02, ABO*O.01.01 and ABO*O.01.02 in brackets, respectively.

PCR. Genomic DNA (100 ng) was mixed in a 50-μl final volume containing 1×PrimeSTAR® GXL buffer with PrimeSTAR® GXL DNA Polymerase (TaKaRa, Shiga, Japan), 0.2 mmol per L dNTP, and 0.2 μmol per L each primer. Primers used for PCR20 were ABO–4138S and ABO+19676AS (Table 1, Figure 1). The PCR conditions used for PCR20 were 94ºC for 3 min, 35 cycles of 98ºC for 10 sec and 68ºC for 10 min, followed by incubation at 68ºC for 7 min. Subsequently, direct sequencing of the PCR20 product was performed using a BigDye® Terminator v1.1

Cycle Sequencing Kit (Applied Biosystems, Foster City, California) with specific primers for targets such as the promoter, exons 1 to 7, and exon-intron boundaries. The sequencing run was performed on a Genetic Analyzer (model 310, Applied Biosystems). PCR10 was carried out (Figure 1), followed by cloning into the vector pUC118 using a Mighty Cloning Reagent Set (Blunt End) (TaKaRa) and determination of the nucleotide sequences of the clones obtained, according to the method described previously.[18]

For peptide nucleic acid (PNA)-mediated PCR clamping or PCR21, genomic DNA (100 ng) was mixed in a 50-μl final volume containing 1×PrimeSTAR® GXL buffer with PrimeSTAR® GXL DNA Polymerase, 0.2 mmol per L dNTP, 0.2 μmol per L each primer, and 1.6 μmol PNA. Primers ABO–4138S and ABO+17819AS as well as PNA ABO+267CL were used for PCR21 and those sequences are shown in Table 1 and Figure 1. The PCR conditions used for PCR21 were 94ºC for 3 min, 35 cycles of 98ºC for 10 sec, 60ºC for 15 sec and 68ºC for 10 min, followed by incubation at 68ºC for 7 min. Subsequently, direct sequencing of the PCR21 product was performed with specific primers for targets such as exon 6 and the +5.8kb site (Table 1).

Preparation of nuclear extracts and EMSA. Nuclear extracts were prepared from human erythroleukemia cell line K562 (JCRB0019) using a Nuclear Extraction Kit (Panomics, Fremount, California) in accordance with the manufacturer's instructions. EMSA was carried out according to the method described by Li et al.[23] RUNX1 consensus oligonucleotide Runx was obtained by annealing two chemically synthesized strands, 5'–CGAGTATTGTGGTTAATACG–3' and 5'–CGTATTAACCACAATACTCG–3'. A mutated version of the RUNX1 consensus oligonucleotide, mRunx/6, was obtained by annealing two chemically synthesized strands, 5'–CGAGTATAAACGGGAATACG–3' and 5'–CGTATTCCCGTTTATACTCG–3'. The underlining indicates the specific mutation sites in oligonucleotide mRunx/6. The double-stranded oligonucleotide wt21 was obtained by annealing two chemically synthesized strands, 5'–GCTCACTAGCCACAGAAAAA–3' and 5'–GTTTTTCTGTGGCTAGTGAG–3'. A

mutated version of oligonucleotide wt21, m21/6, was obtained by annealing two chemically synthesized strands, 5'–GCTCACTCCCGTTTGAAAAA–3' and 5'–GTTTTTCAAACGGGAGTGAG–3'. The underlining of oligonucleotide m21/6 indicates the specific mutation sites similar to those in oligonucleotide mRunx/6. Probes were prepared as described previously.[17] A 100-fold molar excess of unlabeled competitor over the radiolabeled probe was used for competition analyses. Two microliters of anti-RUNX1(N-20) antibody (sc-8563x, Santa Cruz Biotechnology, Dallas, Texas), anti-FOXO3 antibody (FKHRL1(H-144), sc-11351x, Santa Cruz Biotechnology) or anti-CTCF(C-20) antibody (sc-15914x, Santa Cruz Biotechnology) was added to the nuclear extracts overnight at 4 ºC prior to addition of the radiolabeled probe. The DNA-protein complex was quantified with a BAS-1800 imaging analyzer (Fuji Film, Tokyo, Japan).

Plasmids. Luciferase reporter plasmids SN, C(*01*)/SN and C(*04*)/SN were described previously.[18] In reporter plasmid SN, the *ABO* proximal promoter located between –150 and –2 relative to the translation start site was subcloned upstream of the luciferase gene, whereas the erythroid cell-specific regulatory element between +5653 and +6154 or the +5.8kb site with haplotype *ABOInt1*01* was subcloned upstream of the *ABO* promoter in reporter C(*01*)/SN. Subsequent to PCR10 and cloning into the pUC118 vector, the +5.8kb site prepared from an A_m secretor individual was inserted at the *Sac*I and *Mlu*I sites just upstream of the promoter in construct C(A_m)/SN. Deletion between +5892 and +5914 as well as substitution of AGCCACA with CCCGTTT between +5901 and +5907 at the +5.8kb site in the background of haplotype *ABOInt1*01* were generated using overlapping PCR mutagenesis in constructs C(Δ23)/SN and C(mR6)/SN, respectively. For all constructs, sequencing was performed over the entire region of the amplified sequences as described above. Plasmid DNA was purified using a HiSpeed® Plasmid Maxi Kit (QIAGEN GmbH, Hilden, Germany).

Transfection and luciferase assay. Transient transfection into K562 cells was performed with Lipofectamine LTX reagent™ (Invitrogen Corp., Carlsbad,

California) as reported previously[24]; 2.5 μg of firefly luciferase reporter plasmid and 0.01 μg of pRL-SV40 *Renilla* luciferase reporter vector were used for each analysis. After collecting the cells, cell lysis and luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) to measure the activities of firefly and *Renilla* luciferase. Light emission was measured using a Wallac 1420 ARVOMX (Perkin Elmer, Turku, Finland). Variations in transfection efficiency were normalized to the activities of *Renilla* luciferase expressed from the cotransfected pRL-SV40 *Renilla* luciferase reporter.

RESULTS

Serological tests and phenotypes. Serological tests were performed for two individuals without any sanguineous relationship. Their RBCs were not agglutinated with anti-A and anti-A,B, whereas a positive reaction was observed in an adsorption and elution test with anti-A. Both individuals had anti-B but not anti-A in their plasma, and the activities of plasma A-transferase were comparable to those of blood group A_1. One of the individuals with the Le(a–b+) phenotype had both A and H substances in saliva, while no substance was observed in the other individual with the Le(a+b–) phenotype. Thus, the results of these tests indicated that the individuals had the A_m phenotype.

Presence of a 23-bp nucleotide deletion 3' adjacent to a GATA motif at the +5.8kb site in genomic DNAs derived from two A_m individuals. Further investigation was carried out to clarify the mechanism responsible for reduction of A antigen expression in the above A_m individuals. Both of the *A102* (ABO*A1.02) and *O02* (ABO*O.01.02) alleles were demonstrated by direct sequencing of the PCR products comprising exons 6 and 7 using the genomic DNA prepared from one secretor individual, while the *A102* (ABO*A1.02) and *O01* (ABO*O.01.01) alleles were confirmed in the other non-secretor (data not shown). PCR20 was performed to determine the sequences of the promoter region, exons 1 to 7, and the exon-intron boundaries (Figure 1). Direct sequencing of each PCR product demonstrated that the nucleotide sequences of exons 1 to 7 entirely matched those of *A102*/*O02* or *A102*/*O01* and did not show any mutation in either the promoter region or the exon-intron boundaries. In addition, the numbers of CBF/NF-Y enhancer repeats were found to be one and four in each individual.

Since the A_m phenotype could be explained by loss-of-function of the erythroid cell-specific regulatory element or +5.8kb site, PCR10 and subsequent cloning into a vector were performed to examine the entire sequence of the +5.8kb site in the genomic DNAs derived from both individuals (Figure 1). Since single-nucleotide

polymorphisms (SNPs) are present in the +5.8kb site, we previously referred to the haplotypes of those SNPs as *ABOInt1*01* to **06*.[18] In one secretor individual, nucleotide determination of the +5.8kb site in the clones obtained indicated that one allele contained a deletion between +5892 and +5914 as well as nucleotide substitutions of G with A at +5733 and T with C at +5980 relative to the translation start site of *ABO* in the genomic DNA. The haplotype of these nucleotide substitutions at +5733 and +5980 in the +5.8kb site was typed into *ABOInt1*04*.[18] Another allele matched the nucleotide sequence corresponding to haplotype *ABOInt1*05*.[18] In the other non-secretor individual, one allele contained the same sequence as that with the 23-bp deletion, while the other matched the nucleotide sequence of haplotype *ABOInt1*01*.[18] Thus, a 23-bp deletion at the +5.8kb site was verified in the background of *ABOInt1*04* in both individuals (Figure 2).

To examine the allele in which the deletion was located, *A* or *O*, PNA−clamping PCR21 was performed using PNA ABO+267CL complementary to the deletion of G at nucleotide 261 on the cDNA in order to exclusively amplify the DNA region ranging from the upstream region to exon 6 on the *A* allele (Figure 1). The PCR product of approximately 22kb was barely detectable in a control individual with blood group O, whereas the products were observed in a control group A_1 individual as well as the secretor individual with A_m (Figure 1), suggesting that the 22kb-PCR product was likely to be derived from the *A* allele. PCR21 was followed by direct sequencing. Sequence determination of exon 6 demonstrated G at position 261 and A at position 297 on the cDNA, while that of the +5.8kb site revealed the 23-bp deletion (Figure 1). Similar observations were obtained in the other non-secretor individual with A_m. Thus, it appears that the deletion is present at the +5.8kb site of *ABOInt1*04* on *A102* in both A_m individuals.

Binding of a nuclear protein, RUNX1, to the region between +5892 and +5914 at the +5.8kb site. Inspection of the nucleotide sequence between +5892 and +5914 revealed a putative binding site for the hematopoietic transcription factor RUNX1 (Figure 2). To examine whether the region binds RUNX1, EMSAs were performed

with the labeled wild-type probe wt21 containing the +5894 to +5914 sequence. The oligonucleotide wt21 probe produced one major up-shifted band when the probe was incubated with the nuclear extracts from K562 cells (Figure 3A, lane 1). Formation of the up-shifted complex was diminished by addition of competing unlabeled self-oligonucleotide and oligonucleotide Runx containing the consensus motif for RUNX1 (lanes 2 and 3), but not by addition of oligonucleotide mRunx/6 containing six substitutions in the consensus motif (lane 4). Consistently, formation of the DNA-protein complex was abolished when an oligonucleotide m21/6 probe with the six mutations in the RUNX1 binding motif was incubated with the nuclear extracts (lane 5). These results suggested that the +5892 to +5914 sequence could bind RUNX1 or RUNX1-related factors. To investigate whether RUNX1 would bind to the oligonucleotide wt21 probe, an anti-RUNX1 antibody was added to the nuclear extracts. This resulted in loss of the DNA-protein complex and the appearance of a supershifted complex (Figure 3B, lane 3), whereas no change was observed after addition of the anti-CTCF or anti-FOXO3 antibody (Figure 3B, lanes 2 and 4). Consistent results were obtained in three consecutive experiments for both EMSA and supershift assays. These results indicate that RUNX1 likely binds to the region between +5892 and +5914.

Reduced transcriptional activity of the +5.8kb site by the 23-bp nucleotide deletion found in two A_m individuals. To examine whether the +5.8kb site found on the *A* allele in both individuals with A_m would have reduced potential to enhance the promoter activity in comparison with the original sequence of the +5.8kb site with *ABOInt1*01*,[18] we prepared a reporter construct C(A_m)/SN carrying the +5.8kb site with the 23-bp deletion and two SNPs at +5733 and +5980. Transient transfection into K562 cells demonstrated an 82% reduction of the enhanced luciferase activity attributable to the +5.8kb site, when compared with the original sequence (Figure 4). To investigate whether the deletion was responsible for the reduced activity, we prepared a reporter construct C(Δ23)/SN carrying the 23-bp deletion in the background of the +5.8kb site of *ABOInt1*01*. Transient transfection experiments

demonstrated a 72% reduction of the enhanced luciferase activity attributable to the +5.8kb site. In contrast, transient transfection experiments with C(*04*)/SN carrying the substitutions at +5733 and +5980 of *ABOInt1*04* demonstrated an increase of the promoter activity similar to that of the reporter plasmid C(*01*)/SN. These observations suggested that the transcriptional activity of the +5.8kb site was down-regulated by the 23-bp deletion. To investigate whether the decrease in activity was due to reduction of RUNX1 binding to the +5.8kb site, we prepared a reporter construct C(mR6)/SN carrying the same mutations as those used in EMSA to abrogate RUNX1 binding to the +5.8kb site in the background of the +5.8kb site of *ABOInt1*01*. Transient transfection experiments demonstrated an 85% reduction of the enhanced luciferase activity attributable to the +5.8kb site. Therefore, it is plausible that the transcriptional activity of the +5.8kb site may be down-regulated by deletion of the RUNX1 binding sequence at the +5.8kb site on the *A* allele, leading to reduction of A antigen expression in erythroid lineage cells of individuals with A_m.

DISCUSSION

In this study, we found a novel 23-bp deletion 3' adjacent to the GATA motif in the +5.8kb site on the *A* allele in two individuals with the A_m phenotype. EMSAs and promoter assays demonstrated that RUNX1 binds to the +5.8kb site through the 23-bp region, the deletion of which reduced the transcriptional activity of the +5.8kb site. These results suggested that the 23-bp deletion abrogates RUNX1 binding to the +5.8kb site, leading to reduced transcriptional activity at the site, followed by down-regulation of A antigen expression on erythroid lineage cells in affected individuals. This mechanism is similar to that of the B_m phenotype because disruption of the GATA motif at the +5.8kb site, which was found in an individual with the B_m phenotype, abrogated the erythroid cell-specific enhancer activity of the site.[18] Thus, reduced activity of the +5.8kb site seems to be associated with the A_m and B_m phenotypes. In fact, in these *in vitro* promoter assays we did not demonstrate abrogation of the regulatory activity of the site by the 23-bp deletion. As promoter assays have several limitations in terms of precise replication of *in vivo* transcription, this might explain the discrepancy between the slight activity remaining at the +5.8kb site after the deletion in promoter assays and the degree of reduction of A antigen expression on A_m phenotype erythrocytes. The actual effect of the deletion on the enhancer potential of the +5.8kb site might be clarified by further investigations using *ex vivo* culture of erythroid lineage progenitor cells obtained from A_m individuals.

The present results suggest that RUNX1 is involved in the regulation of *ABO* gene expression. RUNX1, also known as acute myelogenous leukemia-1 (AML1), was initially found at the translocational breakpoint of chromosome 21 in the t(8;21)(q22;q22) chromosomal translocation most frequently found in acute myelogenous leukemia (AML).[25] In hematopoietic cells, RUNX1 is constitutively expressed in all lineages except for mature erythroid cells,[26] and regulates the expression of hematopoiesis-specific genes including cytokine receptors and cytokines through binding to its consensus sequence TGTGGT or TGCGGT.[27] In this

report, we revealed that the enhancer activity of the +5.8kb site was reduced without the RUNX1 binding site, although the 5' adjacent GATA motif was completely retained. On the other hand, in the B_m phenotype, we had clarified that disruption of the GATA motif led to loss of activity of the site while the RUNX1 consensus sequence was fully retained.[18] These results suggested that both transcription factors GATA-1/-2 and RUNX1 played a significant role in *ABO* transcription. Indeed, association between GATA-1 and RUNX1 have been reported.[28] Thus, involvement of these associations between RUNX1 and GATA factors in the enhancer potential of the +5.8kb site remains to be further investigated.

On the one hand, *RUNX1* is involved in a number of chromosomal translocations found in leukemias such as AML and myelodysplastic syndrome (MDS), and is also mutated in various types of leukemia. Most of these mutations result in loss or impairment of RUNX1 function. Thus, RUNX1 acts as a critical regulator of hematopoiesis, and alteration of its function results in leukemogenesis. On the other hand, alteration of ABH antigens in hematological malignancy was first reported by van Loghem et al., who described very weak A antigen expression on RBCs of a patient with AML, who had previously shown normal A antigen expression.[29] Later, using a flow cytometric method, Bianco et al. demonstrated frequent loss of the A, B, and H antigens on RBCs in myeloid malignancies including AML and MDS.[30] Combining the present observation that RUNX1 is involved in transcriptional regulation of *ABO*, dysfunction of RUNX1 accompanying AML and MDS can decrease the regulatory activity of the +5.8kb site, leading to reduction of A and/or B antigens from the surface of RBCs. In **the** future, this possibility may be examined using clinical samples from patients with AML and MDS, even though DNA methylation of the *ABO* promoter is suggested to cause loss of *ABO* allelic expression in a significant proportion of leukemic patients.[31]

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FIGURE LEGENDS

Figure 1. Schematic representation of the genomic organization of the human *ABO* gene with locations of the erythroid cell-specific regulatory element and PCR. The top diagram indicates *ABO* exons 1 to 7 as solid boxes. The clear box indicates the location of the erythroid cell-specific regulatory element or the +5.8kb site. Positions are shown relative to the ATG translation start site in exon 1. Under the diagram, PCR-amplified fragments in PCR10, 20 and 21 are indicated by thick lines. PCR21 is PNA-mediated PCR clamping, and PNA is indicated by a triangle. The upward triangle of ABO+267CL is complementary to the upper strand. PCR20 and 21 products were directly sequenced. The left-hand panel at the bottom represents an electrophoretogram of PCR21 products in 0.5% agarose gel stained with ethidium bromide. The center and right-hand electropherograms at the bottom were derived from the upper strand of PCR21 in the A_m secretor individual. Numerals over the nucleotides represent their positions relative to the ATG translation start site in exon 1 on the genomic DNA, and the numbers in parentheses are their positions on the cDNA. Nucleotide G with an asterisk at position 261 on the cDNA is associated with the *A* allele. Location of the deleted sequences between +5892 and +5914 is indicated as Δ +5892: +5914.

Figure 2. Nucleotide sequence of the +5.8kb site. The sequence of haplotype *ABOInt1*01* is given from +5653 to +6154 relative to the ATG translation start site of the *ABO* gene. Sequences typed in bold indicate the location of the nucleotide deletion between +5892 and +5914 at the +5.8kb site, which was found in two individuals with A_m. The sequences corresponding to oligonucleotides wt21 and m21/6 used in EMSA are indicated by lines. The positions and identities of mutations in the RUNX1 binding motif used in oligonucleotide m21/6 are represented by underlining and arrows. The motifs for several relevant transcription factors and the E-box are indicated by overbars. Dots over nucleotides indicate those with SNPs.

Figure 3. RUNX1 specifically binds to the sequence between +5892 and +5914 at

the +5.8kb site. A. Oligonucleotide corresponding to the +5894 to +5914 sequence binds a nuclear protein. EMSAs were performed using nuclear extracts from K562 cells. DNA-protein interaction was investigated using radiolabeled probes wt21 and m21/6 in the presence or absence of a 100-fold molar excess of competing unlabeled oligonucleotides. Oligonucleotide Runx contained the consensus motif for RUNX1, while oligonucleotide mRunx/6 included six substitutions in the motif. The mutated version of oligonucleotide wt21, m21/6, contained the same substitutions between +5901 and +5907 as mRunx/6. B. Anti-RUNX1 antibody supershifts the DNA-protein complex. Anti-RUNX1 antibody, anti-CTCF antibody or anti-FOXO3 antibody was added to the nuclear extracts prior to addition of the radiolabeled probe wt21.

Figure 4. Deletion between +5892 and +5914 results in reduced regulatory activity of the +5.8kb site. K562 cells were transiently transfected with the *ABO* enhancer-luciferase gene constructs C(*01*)/SN, C(A_m)/SN, C(Δ23)/SN, C(mR6)/SN and C(*04*)/SN. Reporter plasmid C(*04*)/SN comprised the +5.8kb site with substitutions of G with A at +5733 and T with C at +5980 in the background of the +5.8kb site with *ABOInt1*01*. Boxes indicate locations of the GATA motif, while open circle represents that of RUNX1. The v-shaped segment indicates the nucleotide deletion between +5892 and +5914. Filled circle represents the nucleotide substitutions at the RUNX1 motif in construct C(mR6)/SN, whereas the ovals indicate SNPs at +5733 and +5980 in haplotype *ABOInt1*04*. The results are expressed as the average fold of the activity observed for the *ABO* promoter. Standard deviations are indicated for a minimum of three experiments.

Table 1. Oligonucleotide primers and PNA used for PCR amplification and direct sequencing.

Primer*	Sequence (5'→ 3' or N → C)	PCR or Target
ABO–4138S	5'-CACCATCCATAGTCAGATTAGCTCTTGCTCCT-3'	PCR20 & 21
ABO+19676AS	5'-GCCTAGGCTTCAGTTACTCACAACAG-3'	PCR20
ABO+17819AS	5'-CTGACTTACTTCTTGATGGCAAACACAG-3'	PCR21
ABO+267CL†	N-AGGGGTACCACG-C	PCR21
ABO-243S‡	5'-CCAAGGTACCAGGGCCACGA-3'	promoter & exon 1
ABO+5743S‡	5'-GACAGTTATTCAGTTAAGACATG-3’	+5.8kb site
ABO+12939S‡	5’-AGGGTGTGATGCCTGAATTACA-3'	exon 2
ABO+13721S‡	5'-GAACTCTTGTGCTCACGCTG-3'	exon 3
ABO+15275S‡	5'-CACTTGTGCCCTAAATCCTGCT-3'	exon 4
ABO+16945S‡	5'-ACAGTGATTCCAGCCTGGGA-3'	exon 5
ABO+17573S‡	5'-AGAAGCTGAGTGGAGTTTCCA-3'	exon 6
ABO+17890AS‡	5'-TGAACACAAGGAGAGACCTC-3'	exon 6
ABO+18736S‡	5'-AGAACGTGGTGCATCTGCTG-3'	exon 7
ABO+18991S‡	5'-GGCAGCTGTCAGTGCTGGAG-3'	exon 7
ABO+19212S‡	5'-GAGGCCTTCACCTACGAGCG-3'	exon 7

* Numerals of primers represent locations of the 5' terminus relative to the ATG translation start site of exon 1 in *ABO* on genomic DNA of NCBI reference sequence NT 035014.4.

† PNA used in PCR21 is indicated.

‡ Oligonucleotides were used for direct sequencing of each target region.

Figure 1

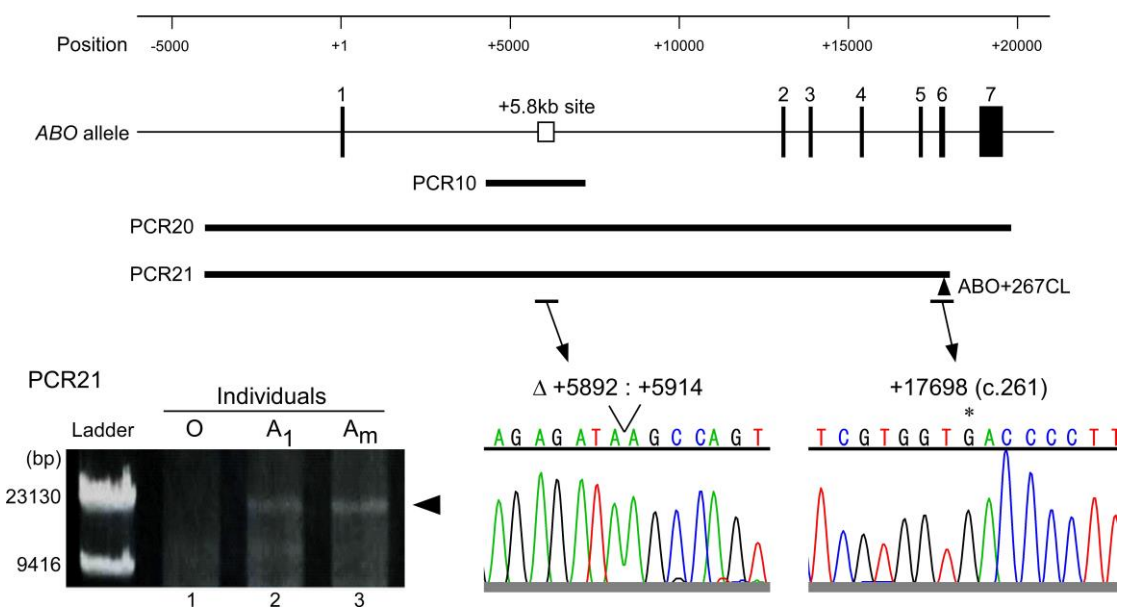

Position
-5000
+1
+5000
+10000
+15000
+20000
ABO allele
1
+5.8kb site
2 3
4
5 6
7
PCR10
PCR20
PCR21
ABO+267CL
PCR21
Individuals
Ladder
O
A1
Am
(bp)
23130
9416
1
2
3
Δ +5892 : +5914
+17698 (c.261)
*

Figure 2

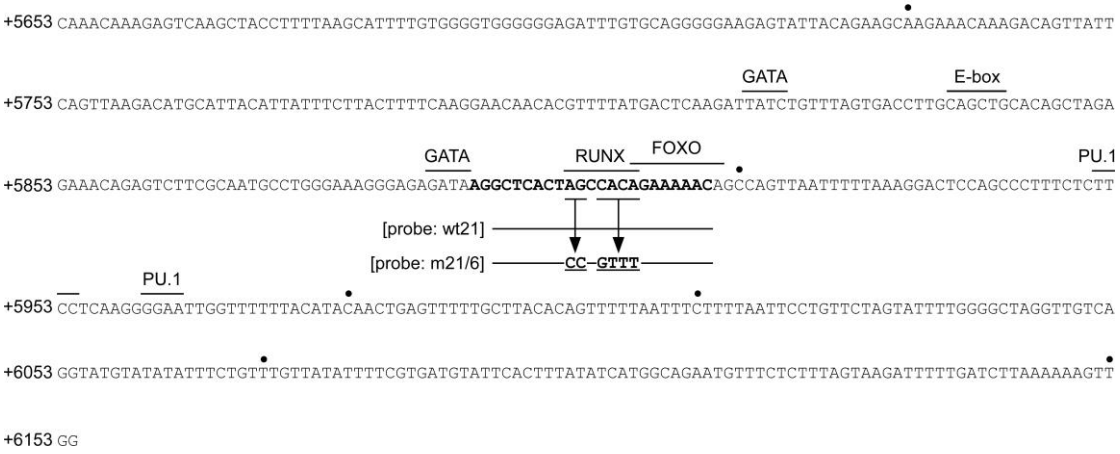

+5653 CAAACAAAGAGTCAAGCTACCTTTTAAGCATTTTGTGGGGTGGGGGGAGATTTGTGCAGGGGGAAGAGTATTACAGAAGCAAGAAACAAAGACAGTTATT
GATA
E-box
+5753 CAGTTAAGACATGCATTACATTATTTCTTACTTTTCAAGGAACAACACGTTTTATGACTCAAGATTATCTGTTTAGTGACCTTGCAGCTGCACAGCTAGA
GATA
RUNX
FOXO
PU.1
+5853 GAAACAGAGTCTTCGCAATGCCTGGGAAAGGGAGAGATAAGGCTCACTAGCCACAGAAAAACAGCCAGTTAATTTTTAAAGGACTCCAGCCCTTTCTCTT
[probe: wt21]
[probe: m21/6]
CC–GTTT
PU.1
+5953 CCTCAAGGGGAATTGGTTTTTTACATACAACTGAGTTTTTGCTTACACAGTTTTTAATTTCTTTTAATTCCTGTTCTAGTATTTTGGGGCTAGGTTGTCA
+6053 GGTATGTATATATTTCTGTTTGTTATATTTTCGTGATGTATTCACTTTATATCATGGCAGAATGTTTCTCTTTAGTAAGATTTTTGATCTTAAAAAAGTT
+6153 GG

Figure 3A

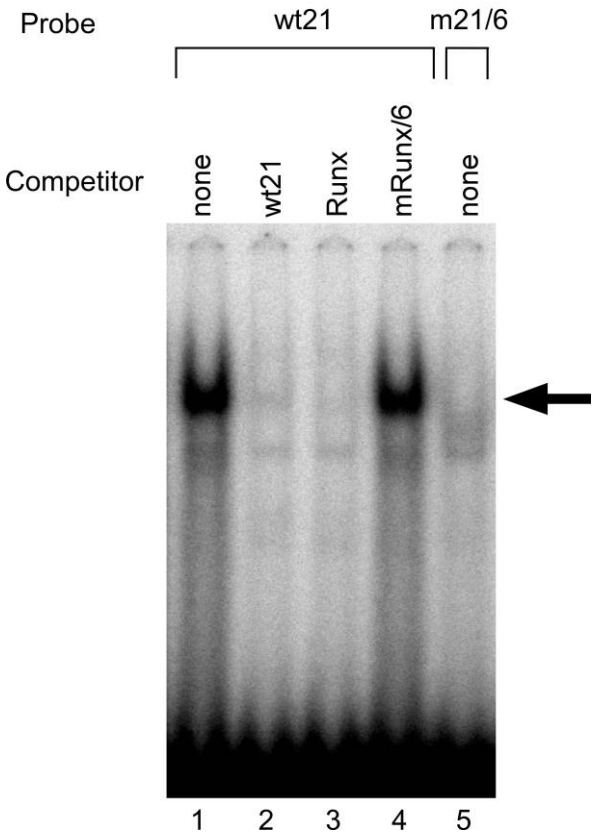


Figure 3B

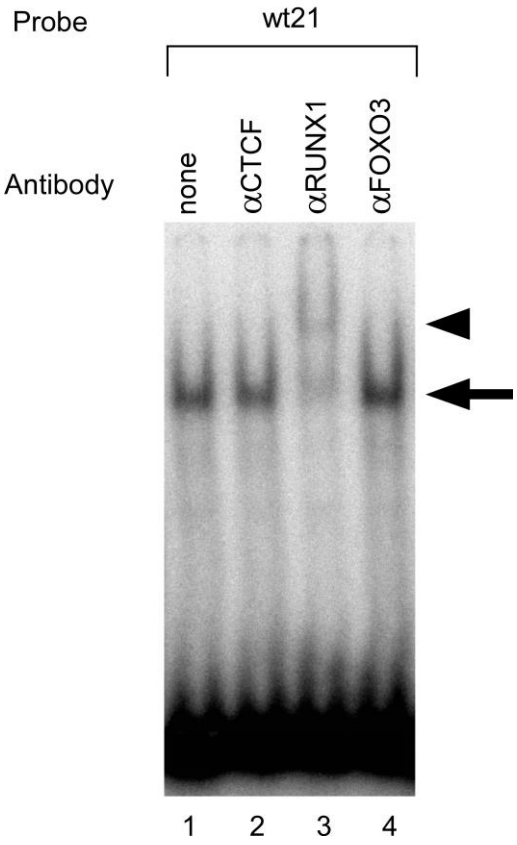
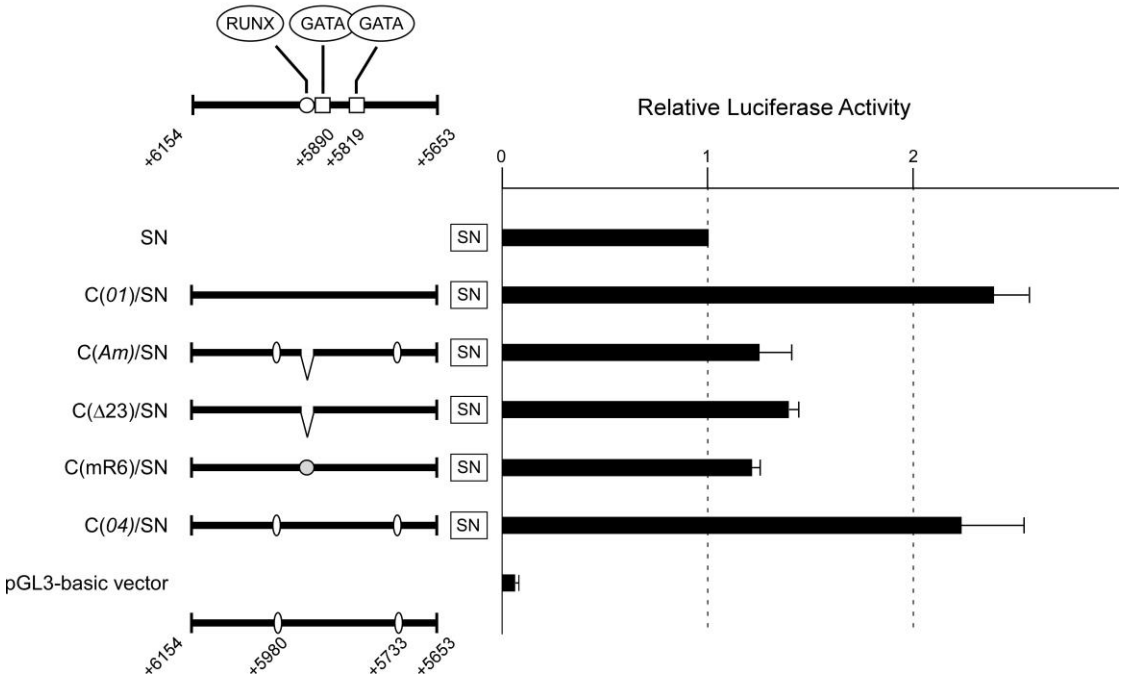


Figure 4


RUNX
GATA
GATA
+6154
+5890
+5819
+5653
Relative Luciferase Activity
0
1
2
SN
C(01)/SN
C(Am)/SN
C(Δ23)/SN
C(mR6)/SN
C(04)/SN
pGL3-basic vector
SN
SN
SN
SN
SN
SN
+6154
+5980
+5733
+5653