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Identification of 5′ Flanking Sequence of *RH50* Gene and the Core Region for Erythroid-Specific Expression[1]

Sadahiko Iwamoto,[2] Toshinori Omi, Masako Yamasaki, Hiroshi Okuda, Masaki Kawano, and Eiji Kajii

Department of Legal Medicine and Human Genetics, Jichi Medical School, Minamikawachi-machi, Tochigi 329-04, Japan

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The Rh blood group antigens are carried by two distinct but homologous membrane proteins encoded by two closely related genes, *RHCE* and *RHD*. Rh50 glycoprotein is the membrane protein tightly associated with Rh polypeptides and is critical for expression of Rh antigens. The amino acid sequence and predicted membrane topology of Rh50 glycoprotein are significantly homologous with those of the Rh proteins. Northern blot analysis of leukemic cell lines showed that expression of *RH50* gene is restricted to cells with erythroid features. HEL and K562 cells showed a transcription levels ratio of 1 to 9.9 for Rh50, and 12.3 to 1 for Rh. The nucleotide sequence of 5′ flanking region of *RH50* gene and functional promoter assays also supported the erythroid-specific regulation of the gene, whereas the sequence had lower homology with the promoter sequence of *RH* genes. Seven GATAs, nine E-boxes, two CACCCs, one YY1, and one October motif were identified in the 1868bp 5′ flanking sequence. The core promoter of *RH50* gene was located within 68bp length from the translation start position, which included an inverse GATA motif, although obvious motifs for Sp1 or erythroid Krüppel-like factor were lacking. The inverse GATA motif was the target sequence of GATA-1 protein, and disruption of the motif abolished the transactivating activity of erythroid cells. These studies confirm the erythroid-specific expression of Rh antigens, but suggest distinct regulatory mechanisms for *RH* vs *RH50* genes.

The Rh blood group antigens have crucial importance in transfusion medicine and new-born hemolytic diseases because of their high antigenicity (1). The Rh antigens are carried by two distinct but homologous integral membrane proteins with Mr 30-32 kD, which were isolated by immunoprecipitation studies using anti-Rh antibodies (2), anti-c, -E, and -D. Two cDNAs corresponding to these proteins have been cloned, *RHCE* (3,4) and *RHD* (5,6,7). These cDNAs differ only in 31–35 amino acids out of total 417 residues, and were mapped in tandem on chromosome 1q34.3–36.1. In addition to the 30-32 kD Rh polypeptides, anti-Rh antibodies simultaneously precipitated a glycoprotein with apparent Mr 45-100 kD (the Rh50 glycoprotein) (8,9). The co-precipitation of these proteins suggests that they are closely associated in the erythrocyte membrane. The cDNA of Rh50 glycoprotein was cloned by Ridgwell et al (10). Rh50 showed similarity to the Rh proteins in both predicted amino acid sequence and predicted membrane topology.

Rh deficiency syndrome (Rhnull phenotype) is characterized by morphological and functional abnormalities associated with lack of expression of all Rh antigens (11). Two genetic backgrounds have been proposed for Rhnull phenotype (12), the most common type by Rhnull '*regulator*' gene and second type by '*amorph*' gene resulting from homozygosity of a silent allele at the *RH* locus. It was proposed recently that the *RH50* gene acts as the '*regulator*' gene, mainly because mutant alleles of *RH50* gene were found in five unrelated Rhnull cases (13). Therefore, the Rh50 glycoprotein is regarded as a critical co-expressed factor on erythrocytes, and expression of Rh antigens is presumably regulated by at least these two distinct loci.

A clinical procedure to suppress Rh antigen expression could prevent harmful hemolytic reactions. In order to clarify the regulatory mechanism of Rh antigen expression, we have now identified the 5′ flanking region of *RH50* gene. Distributions of *RH* transcripts have been shown for cells with erythroid features in hematopoietic lineages (3). The promoter sequences of *RH* genes support the erythroid-specific expression of Rh antigens (14). Whereas promoter and Northern analyses indicated similar erythroid-specific expression of *RH50* and *RH* genes, the promoter sequences

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[2] Correspondence. Fax: 0285-44-4902; e-mail: siwamoto@ms.jichi.ac.jp.

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and the transcription levels of these genes were distinct.

MATERIALS AND METHODS

Cell culture. Human leukemic cell lines with erythroid and megakaryoblastic features (HEL and K562), promyelocytic cell line HL60, and B lymphoma cell line Raji, were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan) and maintained in RPMI 1640 medium containing 10% FCS. Epithelial human cell line HeLa was obtained from HSRBB and maintained in DMEM containing 10% FCS. Total RNA was extracted from cells in exponential growth phase by the acid-phenol guanidinium method (15).

RNA analysis by Northern blot hybridization. Rh50 glycoprotein cDNA was amplified by RT-PCR from reticulocyte RNA with a primer set (sense, GTGCCTCTGTCCTTTGCCAC, corresponding to nucleotides −24 to −5; antisense, TGGTCACCATGTCCATGGAA, complementary to nucleotides 1242 to 1262). The PCR product was subcloned into pCRII vector (Invitrogen, San Diego, CA) and used as a Rh50 full length probe. Rh polypeptide cDNA probe was obtained as described previously (7).

RNAs of HeLa, Raji, HL60, HEL, and K562 cells (30μg per lane) was separated with RNA size markers (Life Technologies Inc, Gaithersburg, MD) on 1.0% formaldehyde/agarose gel and transferred to a Hybond-N^+ nylon membrane (Amersham Japan, Tokyo, Japan). The blot membrane was hybridized with Rh50 cDNA probe for 12 h at 65°C and washed in 0.2 × SSPE (1 × SSPE=150mM sodium chloride, 10mM sodium phosphate, 1mM sodium EDTA) with 0.1% SDS for 30 min at 65°C. After autoradiography, the Northern blot membrane was stripped in boiling 0.1% SDS and hybridized to RhcE cDNA probe. To normalize the quantity of blotted RNA, the blot membrane was probed again with GAPDH probe (Clontech, Palo Alto, CA). After final washing, the blot membrane was exposed to X-ray films with intensifying screen for 24 h at −80°C. These probes were labeled with (α-^{32}P)dCTP using a random primer labeling kit (Amersham Japan).

Cloning of exon 1 and the flanking sequence of RH50 gene. Genomic organization of *RH50* gene was studied by Southern blot analysis. Genomic DNA (10μg) was digested with sufficient units of restriction enzymes, *Eco*RI, *Hind*III, *Pst*I or *Bam*HI. DNA fragments were resolved on 0.8% agarose gel and transferred to a Hybond-N^+ nylon membrane (Amersham Japan). The blot membrane was hybridized with Rh50 full length cDNA for 24 h at 65°C and washed in 0.1 × SSPE with 0.1% SDS for 30 min at 65°C. After autoradiography, the blot membrane was stripped and rehybridized with a PCR probe encoding Rh50 cDNA from nucleotides −24 to 100, and then with a probe encoding from nucleotides 101 to 623.

Results of Southern blot analysis indicated that exon 1 of *RH50* gene is encoded by 2.9-kb *Hind*III fragment. This fragment was amplified from genomic DNA prepared from a donor with DCcEe phenotype, using the inverse polymerase chain reaction (IPCR) technique described by Triglia et al (16), with some modification. Genomic DNA (1μg) was digested *Hind*III, and the resulting fragments were ligated by T4 DNA ligase (Takara, Kyoto, Japan) to form monomeric circular forms in 100μL ligation reaction mixture, and heat treated. To amplify the 2.9-kb *Hind*III fragment, two oligonucleotides were prepared (sense, GTTCTCGAGCAGCTCAACATC, corresponding to nucleotides 93 to 113; antisense, TGGCAATTTCCAGGACTATAGC, complementary to nucleotides 45 to 24). PCR amplification was performed with *rTth* DNA polymerase using GeneAmp XL PCR kit (Perkin Elmer, Foster City, CA). After 30 cycles of amplification, the PCR product was ligated in pCRII vector and sequenced on both strands by dideoxy termination method (17). Analysis of the nucleotide sequences was performed with the INHERIT670 system (Perkin Elmer).

RNA analysis by RACE. To determine the complete sequences of 5′-untranslated regions (UTR), we performed 5′ RACE from the total RNA of K562 cells according to the procedure of Frohman et al. (18), with some modifications. An antisense Rh50 glycoprotein-specific primer (complementary to nucleotides 1242 to 1262) was used to synthesize cDNA. The cDNA was purified by ultrafiltration to remove excessive primer and nucleotides, followed by poly-(A) tailing with terminal deoxynucleotidyl-transferase (Life Technologies Inc, Gaithersburg, MD). The poly-(A) tailed cDNA was amplified with a nested antisense primer (GATAGAGGAACCCAGTGCAGCT, complementary to nucleotides 1106 to 1085) and an anchor primer (ATCCGTCGACATCGATACGTAA(T)17). Second nested primers (TCTGAGTAGTATGCGGACTC, complementary to nucleotides 623 to 603, and ATCCGTCGACATCGATACGT) were used in the following PCR under stringent conditions. The PCR band was ligated in pCRII vector and sequenced.

Construction of CAT reporter gene. The predicted promoter sequence of *RH50* gene was amplified by PCR with a primer set (sense, GTCTAAGCTTATGTGAAGAAC, corresponding to nucleotides −1868 to −1852; antisense, ACCTGCAGTTTGTGGCAAAGGACA, complementary to nucleotide −1 to −17 with *Pst*I linker) from Rh-positive genomic DNA. The initiating ATG codon was converted into *Pst*I restriction site. The PCR product was inserted at *Hind*III and *Pst*I sites of the pCAT-basic plasmid (Promega, Madison, WI). The nucleotide sequence of the constructs were determined to rule out the possibility of additional mutations generated through PCR. The pCAT-basic plasmid contains the chloramphenicol acetyltransferase-encoding ORF (CAT) without a promoter or enhancer, and was used as an negative control. pCAT vector with cytomegalovirus promoter at *Xba*I site was used as a positive control (pCAT-CMV). Serial deletion mutants were generated from pCAT-1868RH50 vector using exonuclease III and mung-bean nuclease system (Deletion kit for kilo-sequence; Takara, Kyoto, Japan). Short core constructs with or without mutation at GATA motif were generated by PCR with −68 primers (CACAAAGCTTTCCTTTGTTCTT**A**TCAA or CACAAAGCTTTCCTTTGTTCTT**G**TCAA, corresponding to −68 to −52 with *Hind*III linker).

Transfection and CAT-ELISA assay. Each constructed plasmid (1μg) was co-transfected with 1μg of pSV-β-Gal vector (Promega) to normalize the transfection efficiency. Transfection was performed on HeLa, K562, and HEL cells in 6-well culture plate by Lipofectamine (Life Technologies Inc, Gaithersburg, MD) according to instructions. Each transfection study was repeated five times. Forty-eight hours after the start of transfection, cells were harvested and CAT and β-galactosidase protein expression was assayed using CAT-ELISA and β-Gal-ELISA kits (Boehringer Mannheim, Germany) according to instructions.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from K562 cells were prepared by the method of Schreiber (19). The 5′ flanking sequence of *RH50* gene with intact GATA motif was PCR-amplified from nucleotide −68 to −1. The PCR product was digested by *Hind*III and then labeled with (^{32}P)dCTP and Klenow fragment. Nuclear extract (2μg protein each) was incubated with 1ng of probe (5000 cpm) and 1μg of poly(dI)-poly(dC) (Pharmacia, Uppsala, Sweden) in buffer containing 12mM HEPES-KOH (pH 7.9), 60mM KCl, 4mM $MgCl_2$, 1mM EDTA, 12% glycerol, 1mM DTT, and 0.75mM phenylmethylsulfonyl fluoride (PMSF). Cold 68bp PCR fragments with wild GATA motif (GATAwild) and with mutation at the motif (GATAmutant) were used as specific competitors. Anti GATA-1 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for supershift study. Samples were incubated for 30 min at room temperature and analyzed on 4% polyacrylamide gel in 1 × TAE buffer. The gel was pre-electrophoresed at 150V for 30 min and electrophoresed at the same voltage for 3 h at 4°C. After electrophoresis, the gel was dried and autoradiographed for 24 h.

RESULTS

RH50 gene expression is restricted to cell lineages with erythroid features. Total RNAs from human au-

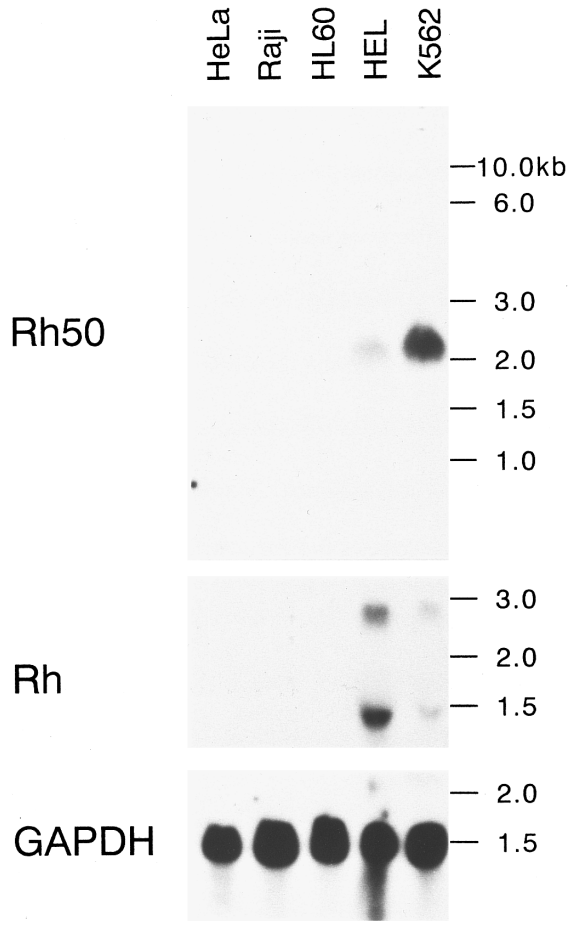

FIG. 1. Northern blot analysis. Total RNAs (30 μg per lane) of HeLa, Raji, HL60, HEL, and K562 cells were separated on 1% denaturing agarose gel and transferred to membrane. The blot was hybridized initially with Rh50 cDNA probe. The membrane was stripped and rehybridized secondly to *RH* probe and thirdly to GAPDH probe. RNA standards are indicated on the right.

topsy tissues (lung, kidney, spleen, bone marrow, brain) were insufficient to show positive bands by Northern analysis with Rh50 probe. RT-PCR study showed that Rh50 RNA was expressed predominantly in bone marrow, but very weak bands were also observed after 30 cycles of PCR amplification in every other tissue (not shown). It was impossible to know whether the PCR bands were amplified from RNA of residual RBC or from ubiquitously distributed Rh50 mRNA. To clarify Rh50 distribution in hematopoietic cells, we carried out Northern blotting study in leukemic cell lines. The Rh50 probe gave a weak band on HEL cell RNA and a strong band on K562 cell RNA (Fig 1). In HeLa, Raji, and HL60 cells, no positive bands were observed even after prolonged exposure. The densitometric band intensity ratio between HEL and K562 cells was 1 to 9.9. Reprobing study with Rh probe gave a strong band from HEL cells and a weak band from K562 cells, with intensity ratio 12.3 to 1. The Rh probe failed again to show positive bands in other cell lines, despite almost identical expression of GAPDH in all cell lines. RT-PCR study of Rh50 gave no bands in T-cell line MOLT4 or myeloblastic cell line KG-1 also. All cell lines with erythroid features studied (HEL, K562, KU812, JK-1) showed PCR products (data not shown). These findings suggest that *RH50* gene expression in hematopoietic tissues is restricted to cells with erythroid features, although transcription levels vary.

Cloning and sequence analysis of exon 1 and the flanking sequence of RH50 gene. In order to clarify the regulation mechanism of erythroid-specific *RH50* gene expression, we identified the DNA fragments encoding exon 1 and the flanking region. When genomic DNA was cut with *Hind*III and hybridized to Rh50 full length probe, 24kb and 2.9kb fragments were observed. In subsequent hybridization procedure with probe corresponding to nucleotides −24 to 100, the 2.9kb fragment reappeared but the 24 kb fragment disappeared. Conversely, the probe corresponding to nucleotides 101 to 623 gave intense 24kb band and weak 2.9kb band (Fig 2). These hybridization patterns were completely reproducible in three different DNA samples, suggesting that the 2.9kb *Hind*III fragment encodes exon 1 of *RH50* gene.

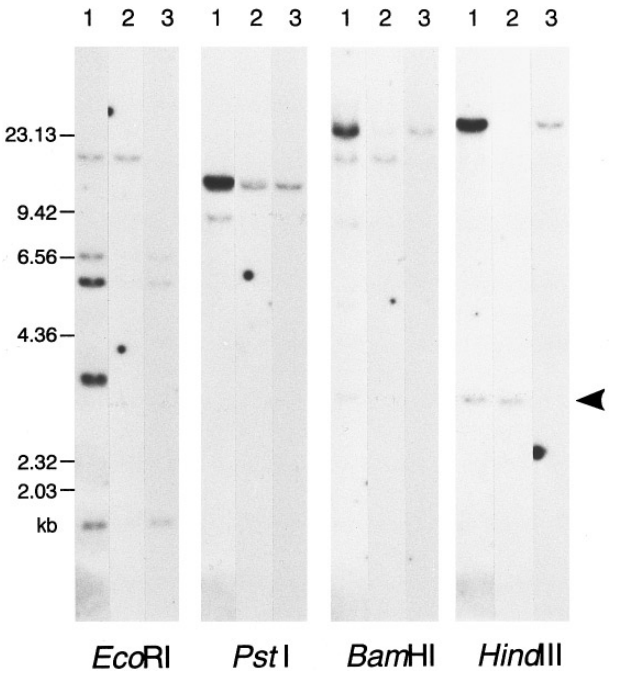

FIG. 2. Southern blot analysis of *RH50* gene. Genomic DNAs were digested with *Eco*RI, *Pst*I, *Bam*HI, or *Hind*III, electrophoresed on 8% agarose gel, and transferred to nylon membrane. The blotted membrane was hybridized with full length cDNA probe (lane 1). After autoradiography, the membrane was stripped and rehybridized to the exon1 PCR probe from nucleotide −24 to 100 (lane 2), and subsequently to the probe from nucleotide 101 to 623 (lane 3). Arrowhead indicates the 2.9 kb *Hind*III fragment revealed by the exon1 probe.

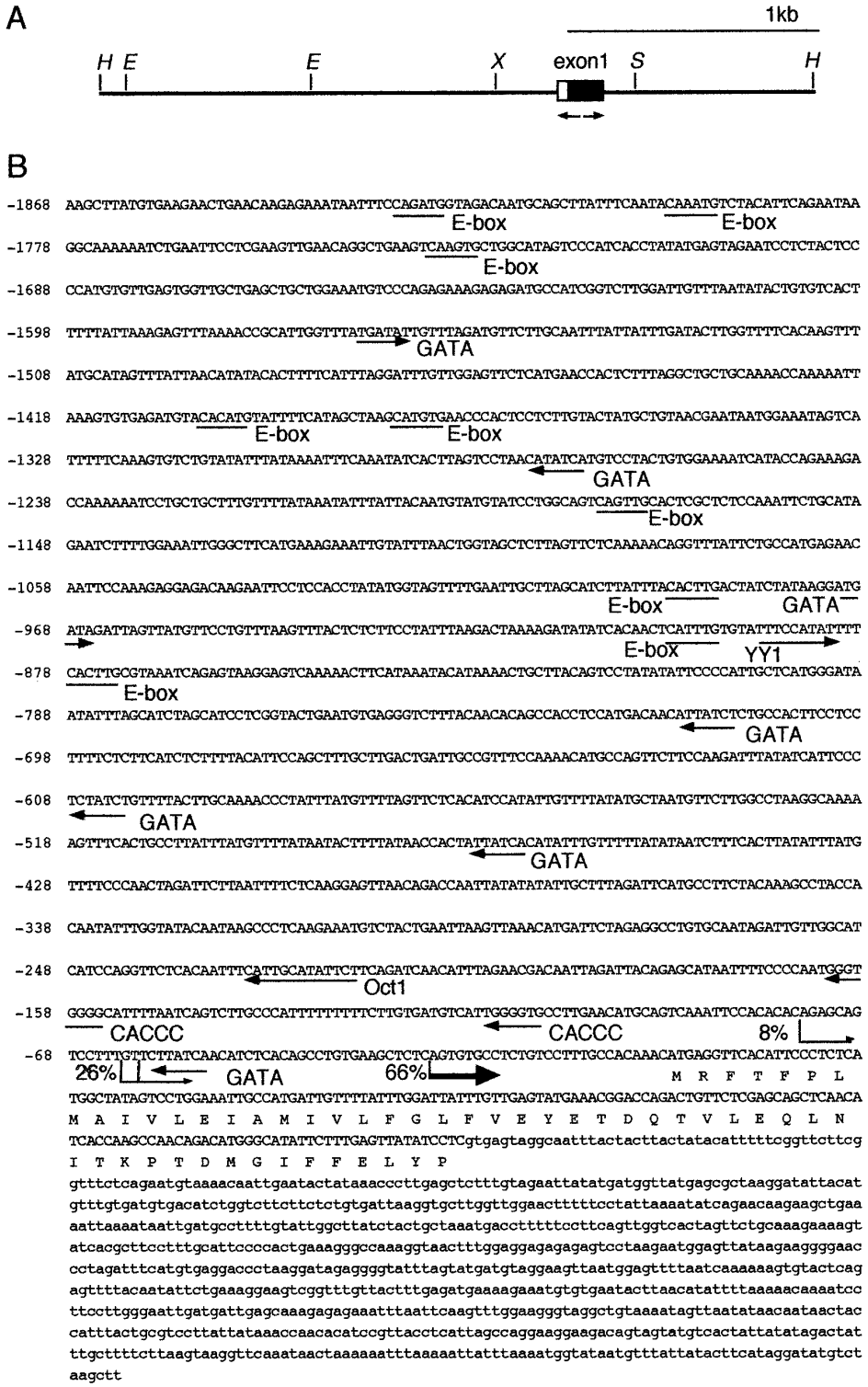

FIG. 3. The exon1 and flanking sequences of *RH50* gene. (A) Scheme showing restriction sites, cloning strategy, and the oligonucleotide primers. The 2.9 kb HindIII fragment was amplified by IPCR procedure with outside-facing primers located in exon1 (arrows). H, *Hind*III; E, *Eco*RI; X, *Xba*I; S, *Sac*I. (B) Complete gene sequence. The 5′ flanking and exon1 sequences are capitalized; intron1 sequence is in lowercase letters. Nucleotide numbering corresponds to distance from first ATG codon. Putative regulatory elements representing binding sites are indicated by underlines or arrows. Angled arrows are transcription start sites determined by 5′RACE procedure. Percentages represent proportions of the clone starting from the position, out of total studied clones.

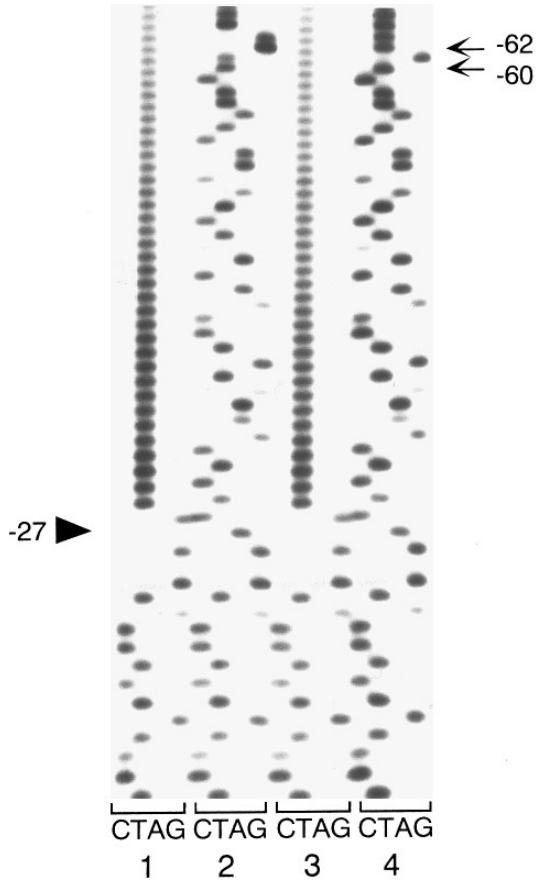

FIG. 4. The 5′RACE product from K562 cells was subcloned and sequenced. Thymine stretches indicate the poly(A) tail at the cDNA terminal. Four representative clones are shown. Arrowhead and thin arrows indicate the major TSP (−27) and minor TSPs (−60 and −62) respectively.

A DNA fragment with 2.8kb length was amplified by IPCR procedure from covalently circular *Hind*III-digested DNA as illustrated in Fig3A. In the negative control study, no PCR products were observed from untreated genomic DNA. The IPCR products included the published Rh50 cDNA sequences from nucleotides 93 to 156 at the 5′ end and from nucleotides −27 to 45 at the 3′ end. The following sequence from nucleotide 156 was matched to the 5′ end of intron 1 reported by Cherif-Zahar et al (13). A cutting site of *Hind*III appeared at 856bp downstream from the 5′ terminus of intron 1. A novel upstream sequence with 1868 bp length was identified from the translation-initiating codon to the *Hind*III site (Fig3B). The DNA sequence was confirmed by usual PCR and direct sequence analysis from nucleotide −1868 to −1 with untreated genomic DNAs.

Identification of the transcription start position (TSP) of Rh50 mRNA in erythroid cells. To identify the 5′ flanking sequence of exon 1 as the erythroid promoter, we performed 5′-RACE and isolated 50 positive clones from K562 RNA. Thirty-three clones (66%) initiated at −27, identically to the cDNA clone isolated by Ridgwell et al (10). Thirteen clones started from −60bp and four clones from −75bp. Thus, −27 was regarded as the major TSP of *RH50* gene and the 5′ flanking sequence was identified as the promoter region in erythroid cells (Fig. 4).

Computer-assisted analysis was performed to identify the putative cis-regulatory elements in the 1868bp upstream sequence of the translation start codon, using the transcription factor database (20,21). The promoter sequence was AT-rich (66%), with no obvious TATA or CCAAT boxes present. However, several cis-regulatory elements were identified (marked in Fig3B with arrows and horizontal bars): GATA, CACCC box, Oct-1, YY1, and E-box. The most proximal GATA motif was located at nucleotides −58 to −53 with inverse form.

Deletion analysis of RH50 promoter defines a region involved in its erythroid expression. To map the core region involved in erythroid-specific transcriptional activation, we constructed sequential 5′ deletion mutants of *RH50* promoter linked to the CAT coding sequence. These constructs were introduced by transient transfection in HEL, K562, or HeLa cells, together with a plasmid expressing β-galactosidase as an internal control for transfection efficiency. The ratio (ng/ng) of CAT to β-galactosidase in each sample served as a measure of normalized CAT value. The longest construct showed very weak transactivation activity in HEL and K562 cells, rather than in HeLa cells. As the construct became shorter, the CAT value increased in HEL and K562 cells, but not in HeLa cells (Fig 5). The shortest −68 construct, which contained the proximal inverse GATA motif, showed maximal activity in both HEL and K562 cells, whereas the construct with a mutation at the GATA motif showed almost entire loss of activity. Inconsistent with the results of Northern blotting, HEL cells showed CAT value almost twice that of K562 cells in all the *RH50* promoter constructs.

The most proximal inverse GATA motif binds GATA-1. The DNA fragment with 68bp length, which spanned the region from nucleotide −68 to −1 of the *RH50* gene with intact inverse GATA motif, was used for a probe in EMSA. Nuclear extract of K562 cells showed an intense shifting band, which was competed out by cold GATAwild probe but not by GATAmutant probe, and was super-shifted by anti-GATA-1 mAb (Fig6). The proximal GATA motif in the 5′ flanking region of *RH50* gene was thus revealed as the target sequence of GATA-1 in erythroid cells.

DISCUSSION

In this study, we identified the cis-acting elements of the human *RH50* gene involved in erythroid-specific expression. The 5′ deletion mutagenesis study revealed

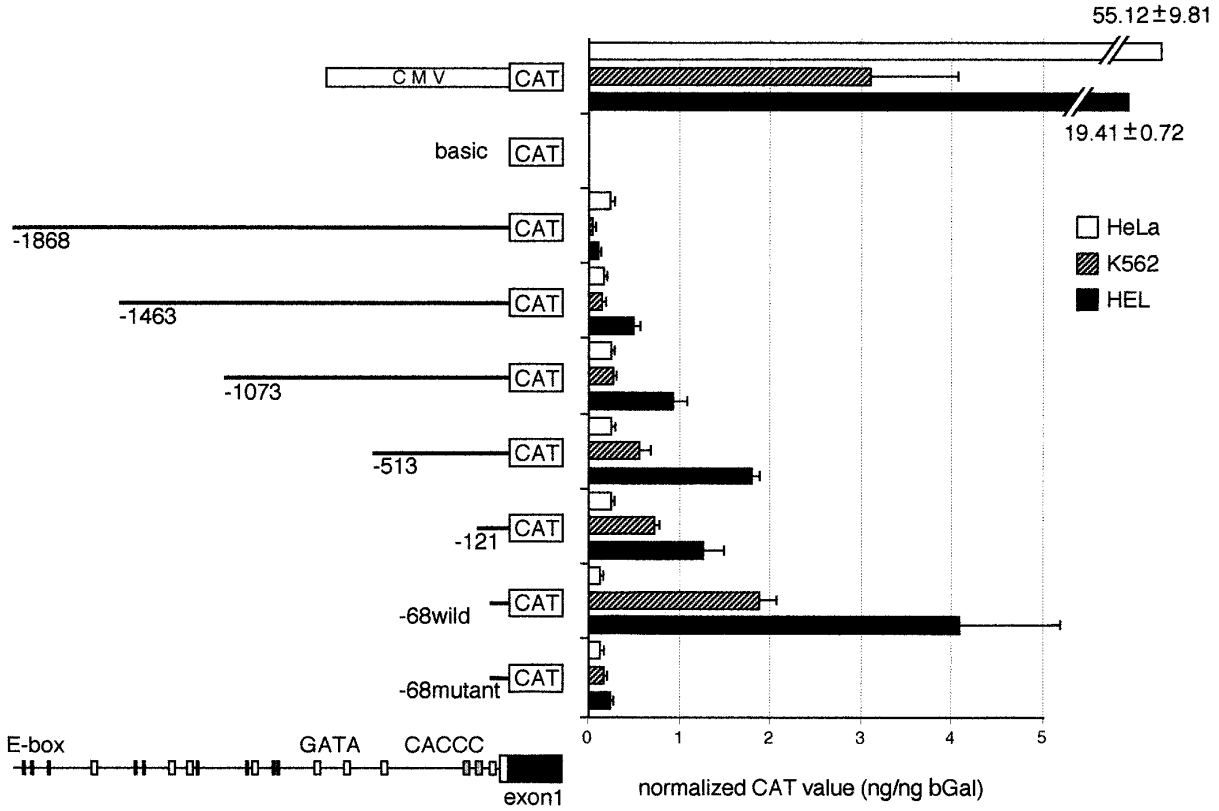

FIG. 5. Characterization of functional promoters for *RH50* gene. Gene map is shown at bottom. The positions of GATA sites are indicated by open boxes, CACCC motifs by two shaded boxes, and E-boxes by vertical bars. The longest −1868 promoter connected with CAT gene was used as original construct, and five progressive deletions were constructed from this original. The −68 mutant clone has a A→G mutation at −56, disrupting the GATA element. Results are expressed as means of five experiments together with standard error of the mean.

that the shortest construct, which encodes 68bp upstream from the first ATG codon, acts as a core promoter in erythroid but not in HeLa cells. The 68bp included an inverse GATA motif, to which GATA-1 bound. Mutation at the motif disrupted transactivation in HEL and K562 cells. GATA-1 protein is the transcription factor distributed to erythroid-megakaryocytic lineage cells and Sertori cells in testis (22). The importance of GATA-1 function in erythropoiesis was confirmed by targeted disruption of GATA-1 gene in chimeric mice (23). These GATA-1 knock-out mice lost erythroid development but retained development of other hematopoietic lineages. For maturation of megakaryocytes, the p45 subunit of NF-E2 has been shown to be more essential (24). In cis-regulatory sequence of erythroid- and megakaryocte-specific genes, the GATA motif has been consistently identified (23). However, the GATA motif appears not to be sufficient for activity of the promoters of megakaryocte-specific αIIb (25) and thrombopoietin receptor (26); in these cases coregulation with Ets seems more essential. The *RH50* core promoter encodes a GATA motif, but other consensus motifs were not identified by computer study. Therefore, the proximal inverse GATA motif at nucleotides −53 to −58 is presumably involved in erythroid-specific *RH50* gene expression.

The 68bp sequence includes only GATA motif; consensus motifs for other transcription factors were not identified. GATA-1 has been shown to work synergistically with Sp1 or erythroid Krüppel-like factor (EKLF) for activation of erythroid-specific transcription (27). Inverse CACCC motifs for EKLF in *RH50* promoter exist at 52bp and 102bp upstream of the proximal GATA motif. Deletion of the CACCC motifs activates rather than decreases CAT transcription. It has been proposed that the interval between positioning GATA and Sp-1 or EKLF motifs is always an approximate multiple of 10bp, which is necessary for GATA-1 and Sp-1 proteins on the same face of the DNA helix (28). Synergistic activity of GATA and CACCC motifs was observed when the interval was within 50bp (29). The CACCC motifs in the *RH50* promoter may be too far to achieve synergism with the inverse GATA element. Recently, a non-consensus Sp1 binding site was reported in the promoter of αIIb gene (25), which plays an important role in megakaryocyte-specific expression. The Sp1 site is a GA-rich region 14bp upstream from the TSP of αIIb gene and has a low affinity with

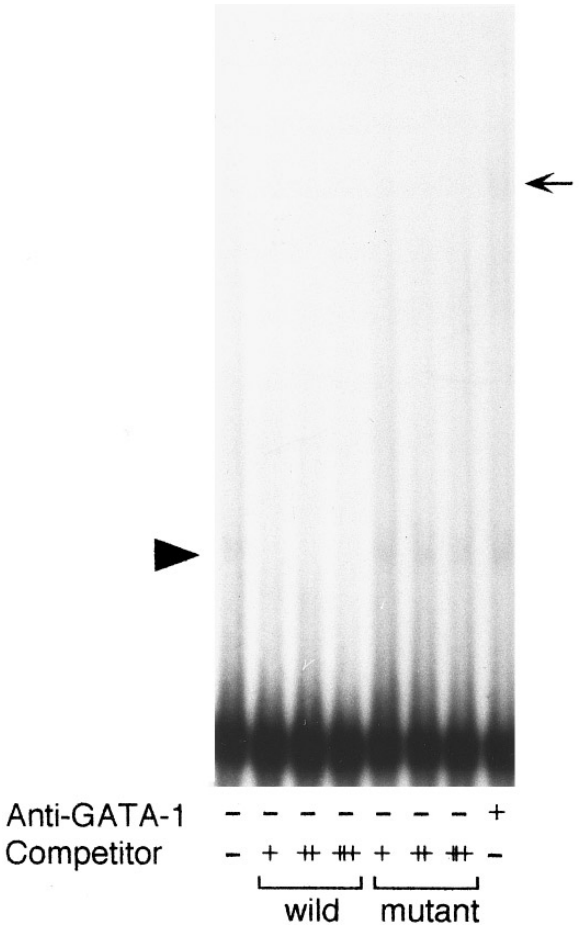

FIG. 6. Electrophoretic mobility shift assay of *RH50* core promoter sequence. PCR fragment of core promoter sequence from −68 to −1 including intact GATA motif was used as probe. Addition of cold competitors (−, without competitor; +, 10-fold molar excess; ++, 25-fold excess; +++, 100-fold excess) and the presence (+) or absence (−) of a specific anti-GATA-1 antibody are indicated below the lanes. Arrowhead represents specific bands with K562 cell extract. Thin arrow indicates the supershift band by anti-GATA-1 antibody.

Sp1 protein, as evidenced by interaction with proteins binding at an Ets consensus site 20bp further upstream. Direct protein-protein interaction between GATA-1 and Sp1 or EKLF mediated by their DNA binding zinc finger domain has also been demonstrated (27). Further studies are needed to clarify whether occlusive binding motifs for Sp1 or EKLF already exist between the inverse GATA motif and the first ATG of *RH50* gene, or whether GATA-1 interacts directly with the co-factors.

The upstream sequence of *RH50* 5′ flanking sequence includes additional consensus motifs for transcription factors: seven GATAs, two CACCCs, nine E-boxes, one Oct-1, and one YY1. However, the longest construct, −1868, lost erythroid-specific promoter activity. The CAT value for −1868 construct in HEL and K562 cells were lower than that in HeLa cells. CAT values in HEL cells with all *RH50* constructs were approximately twice those in K562 cells. These data appear to be inconsistent with the transactivating activity of native *RH50* gene observed in Northern blotting study. HeLa cells do not transcribe *RH50* gene, and HEL cells transcribe less *RH50* gene than K562 cells. It has been suggested that erythroid-specific repressor elements are present in −1868 to −68, or that remote enhancer elements play an important role in regulation of *RH50* gene expression. The sequence from −892 to −876 corresponds to the motif for nuclear factor YY1, which has been reported to act as a developmental repressor factor for human ϵ-globin gene (30). In our preliminary study, *RH50* transcription in K562 cells but not in HEL cells increased by hemin-induced differentiation. For detailed characterization of the *RH50* promoter, the upstream sequence and entire genomic organization should be analyzed. This could provide additional examples of erythroid-specific gene regulation.

The Rh50 glycoprotein has been shown to be homologous to the Rh proteins (10), including twelve predicted hydrophobic transmembrane domains. Expression of *RH* gene is restricted to hematopoietic organ and erythroid lineages, e.g. K562 and HEL cells (3). The 5′ flanking region of *RHCE* gene contains the putative erythroid-specific cis-regulatory elements; GATA, Ets, NFE-2, CACCC box, and Sp1. *RHD* gene, which has evolved with *RHCE* gene through duplication of a common ancestor gene, shares identical cis-elements. Only five substitutions were found in the 600bp 5′ flanking region between *RHD* and *RHCE* genes (14). However, neither proximal nor distal promoter sequence of *RH50* gene shows any conserved fragments with *RH* gene (data not shown). Northern blotting study of HEL and K562 cells also suggests the existence of distinct regulatory mechanisms in these homologous genes. The differential expression of transcription factors in HEL and K562 cells, e.g. deficit of EKLF in K562 cells (31), will provide clues to these distinct mechanisms. It is intriguing that these homologous genes have retained erythroid-specific expression, despite losing the homology of 5′ flanking sequences. The erythroid-specific expression in hematopoietic organs of the critical co-expressing factor of Rh polypeptides confirms the erythroid-specific distribution of Rh antigens.

Despite the discovery of *RH* genes and one critical accessory molecule, the physiological role of Rh polypeptides remains unclear. Based on studies of Rhnull erythrocytes, Rh polypeptides were proposed to be involved in maintenance of aminophospholipid asymmetry in the lipid bilayer of erythrocyte membrane (32). Detailed knowledge of the expression mechanisms of Rh and Rh50 proteins will help clarify their physiological functions.

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REFERENCES

1. Mollison, P. L., Engelfreit, C. P., and Contreras, M. (1993) Blood Transfusion in Clinical Medicine, 9th Ed, Blackwell, Oxford.
2. Moore, S., Woodrow, C. F., and McClelland, D. B. L. (1982) *Nature* **295,** 529–531.
3. Cherif-Zahar, B., Bloy, C., Le Van Kim, C., Blanchard, D., Bailly, P., Hermand, P., Salmon, C., Cartron, J-P., and Colin, Y. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6243–6247.
4. Avent, N. D., Ridgwell, K., Tanner, M. J., and Anstee, D. J. (1990) *Biochem. J.* **271,** 821–825.
5. Le Van Kim, C., Mouro, I., Cherif-Zahar, B., Raynal, V., Cherrier, C., Cartron, J-P., and Colin, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10925–10929.
6. Arce, M., Thompson, E. S., Wagner, S., Coyne, K. E., Ferdman, B. A., and Lublin, D. M. (1993) *Blood* **82,** 651–655.
7. Kajii, E., Umenishi, F., Iwamoto, S., and Ikemoto, S. (1993) *Hum Genet* **91,** 157–162.
8. Moore, S., and Green, C. (1987) *Biochem. J* **244,** 735–741.
9. Avent, N. D., Judson, P. A., Parsons, S. F., Mallinson, G., Anstee, D. J., Tanner, M. J. A., Evans, P. R., Hodges, E., Maciver, A. G., and Holmes, C. (1988) *Biochem. J* **251,** 499–505.
10. Ridgwell, K., Spurr, N. K., Laguda, B., Macgeoch, C., Avent, N. D., and Tanner, M. J. A. (1992) *Biochem. J* **287,** 223–228.
11. Agre, P., and Cartron, J-P. (1991) *Blood* **78,** 551–563.
12. Tippett, P. (1990) *Transfusion Medicine Rev.* **4,** 56–68.
13. Cherif-Zahar, B., Raynal, V., Gane, P., Mattei, M-G., Bailly, P., Gibbs, B., Colin, Y., and Carton, J-P. (1996) *Nature. Genet.* **12,** 168, 1996.
14. Cherif-Zahar, B., Le Van Kim, C., Rouillac, C., Raynal, V., Cartron, J-P., and Colin, Y. (1994) *Genomics* **19,** 68–74.
15. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
16. Triglia, T., Peterson, M. G., and Kemp, D. J. (1989) *Nucleic Acids Res.* **16,** 8186.
17. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
18. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
19. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419.
20. Ghosh, D. (1990) *Nucleic Acids Res.* **18,** 1749–1756.
21. Faisst, S., and Meyer, S. (1992) *Nucleic Acids Res.* **20,** 3–26.
22. Orkin, S. H. (1995) *J. Biol. Chem.* **270,** 4955–4958.
23. Bronson, S. K., and Smithies, O. (1994) *J. Biol. Chem.* **269,** 27155–27158.
24. Shivdasani, R. A., Rosenblatt, M. F., Zucker-Franklin, D., Jackson, C. W., Hunt, P., Saris, C. J., and Orkin, S. H. (1995) *Cell* **81,** 695–704.
25. Block, K. L., Shou, Y., and Poncz, M. (1996) *Blood* **88,** 2071–2080.
26. Deveaux, S., Filipe, A., Lemarchandel, V., Ghysdael, J., Romeo, P-H., and Mignotte, V. (1996) *Blood* **87,** 4678–4685.
27. Merika, M., and Orkin, S. H. (1995) *Mol. Cell. Biol.* **15,** 2437–2447.
28. Mignotte, V., Eleouet, J. F., Raich, N., and Romeo, P-H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6548–6552.
29. Frampton, J., Walker, M., Plumb, M., and Harrison, P. R. (1990) *Mol. Cell. Biol.* **10,** 3838–3842.
30. Raich, N., Clegg, C. H., Grofti, J., Romeo, P. H., and Stamatoyannopoulos, G. (1995) *EMBO J.* **14,** 801–809.
31. Bieker, J. J. (1996) *DNA Cell Biol.* **15,** 347–352.
32. Schroit, A. J., Bloy, C., Connor, J., and Cartron, J-P. (1990) *Biochemistry* **29,** 10303–10306.