Alternative Promoters Regulate Transcription of the Mouse GATA-2 Gene*

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Transcription factor GATA-2 has been shown to be a key regulator in hematopoietic progenitor cells. To elucidate how the expression of the GATA-2 gene is controlled, we isolated the mouse GATA-2 (mGATA-2) gene. Transcription of mGATA-2 mRNAs was found to initiate from two distinct first exons, both of which encode entirely untranslated regions, while the remaining five exons are shared by each of the two divergent mRNAs. Reverse transcriptase-polymerase chain reaction analysis revealed that GATA-2 mRNA initiated at the upstream first exon (IS) in Sca-1+/c-kit+ hematopoietic progenitor cells, whereas mRNA that initiates at the downstream first exon (IG) is expressed in all tissues and cell lines that express GATA-2. While the structure of the IG exon/promoter shows high similarity to those of the Xenopus and human GATA-2 genes, the IS exon/promoter has not been described previously. When we examined the regulation contributing to IS transcription using transient transfection assays, we found that sequences lying between −79 and −61 are critical for the cell type-specific activity of the IS promoter. DNase I footprinting experiments and electrophoretic mobility shift assays demonstrated the binding of transcription factors to this region. These data indicate that the proximal 80 base pair region of IS promoter is important for the generation of cell type-specific expression of mGATA-2 from the IS exon.

GATA-2 is a member of the GATA transcription factor family that binds to a common consensus sequence motif (A/TGATA/A/G) through a highly conserved zinc finger DNA binding domain (1). The founding member of the GATA family is GATA-1 (2–4), whose expression is restricted to erythroid, megakaryocytic, mast and eosinophil hematopoietic cell lineages (4–6), and Sertoli cells in the testis (7, 8). Targeted disruption of the gene revealed that loss of GATA-1 function results in a severe deficit in both primitive and definitive erythropoiesis (9–11).

The tissue distribution of GATA-2 is wider than that of GATA-1, i.e. GATA-2 is expressed not only in several hematopoietic lineages that also express GATA-1 but in hematopoietic stem and progenitor cells, in the embryonic brain, and in endodermal cells, fibroblasts, kidney, liver, and cardiac muscle (1, 6, 12–14). The expression profile of GATA-2 during the development of hematopoietic tissues has been studied most extensively in Xenopus and zebrafish. In Xenopus, low levels of maternal GATA-2 are present in early embryogenesis (15) while zygotic expression of GATA-2 is localized in the ventrolateral ectoderm and mesoderm, in blood cells, and in the central nervous system (16–18). A ventralizing transforming growth factor (TGF)-β family member, BMP-4, induces Xenopus GATA-2 expression (19). In zebrafish, GATA-2 is initially detected in the ventral ectoderm (20). High levels of GATA-2 are expressed at the boundary of the embryo and the yolk syncytial layer, and then by the 2–5 somite stage, in the presumptive hematopoietic progenitors that border the anterior and posterior of the embryo (20). Thus, zygotic GATA-2 induction in vertebrates is coincident with the commencement of hematopoiesis, suggesting that GATA-2 is intimately involved in the development of hematopoietic cell lineages.

Analysis of chimeric mice generated from GATA-2 (−/−) embryonic stem (ES)1 cells revealed that GATA-2 (−/−) cells do not contribute to any hematopoietic lineage (21). GATA-2-null mutant mice die of severe anemia at approximately 10 to 11 days post coitus during embryonic development (21) and manifest a broad hematopoietic deficit. These studies demonstrated that GATA-2 plays a unique and crucial role in the differentiation of hematopoietic progenitor cells.

It should be noted that GATA-2 expression is not found in mature hematopoietic cells (22, 23). In a human bone marrow cell culture system or in an in vitro ES cell differentiation, GATA-2 expression was down-regulated coordinately with the progression of erythroid and myeloid cell differentiation (12, 13, 24). Furthermore, forced expression of GATA-2 in an avian erythroid cell line caused the proliferation of immature cells and markedly inhibited terminal erythroid differentiation (25). These results taken together suggested that the expression of GATA-2 is related to proliferative versus differentiated capacity of hematopoietic progenitor cells and that suppression of GATA-2 activity might be essential for hematopoietic differentiation. Therefore, elucidation of the mechanisms controlling GATA-2 transcription may offer insight into the earliest aspects of hematopoietic cellular differentiation and may also give rise to a means of experimentally manipulating the proliferation versus differentiation of hematopoietic stem cells.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AB007371 and AB009972.

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1 The abbreviations used are: ES, embryonic stem; IS, upstream first exon; IG, downstream first exon; bp, base pair(s); kb, kilobase pair(s); FACs, fluorescence-activated cell sorter; RT, reverse transcriptase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; EMSA, electrophoretic mobility shift assay.

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In this study, we have isolated and characterized the murine GATA-2 gene. We found that GATA-2 expression is regulated by two distinct promoters. One is used in general in the tissues and cell lines that express GATA-2, so we refer to this as the IS promoter. The structure of the IS promoter is homologous to that of the previously reported Xenopus and human GATA-2 gene promoters (26, 27). The other promoter regulates the expression of GATA-2 specifically in hematopoietic cells, thus we refer to this as the IS promoter. Since this promoter has not been described elsewhere and displays lineage-restricted utilization, we have also characterized the regulatory domains that specify IS promoter activity.

EXPERIMENTAL PROCEDURES

Isolation and Subcloning of Genomic Clones—A C57Bl/2J mouse genomic library (CLONTECH) was screened with a random primed 0.6-kbp SacI fragment, encoding the 5′ region of mGATA-2 cDNA, and a 0.8-kbp fragment from the HindIII site in exon VI to the poly(A) of mGATA-2 cDNA. Both fragments were derived from an mGATA-2 cDNA clone, which we previously isolated from a fetal mouse liver cDNA library (DBJ/GenBankTM/EBI accession number AB000096).6 Hybridization and washing were carried out as described previously (23). Positive clones were characterized by restriction enzyme mapping and Southern blot hybridization, and DNA in the positive phages was subcloned into pBluescript SK (+) vector (Stratagene). Sequences for the exon-intron boundaries and promoter and upstream regions were determined using a Taq polymerase cycle sequencing system and an automated DNA sequence (Perkin-Elmer Corp.).

Southern Blot Hybridization Analysis of Mouse Genomic DNA—Genomic DNA (25 μg) was isolated from an adult C57Bl/2J mouse and digested with BamHI and SacI. After electrophoresis in a 0.8% agarose gel, the DNA fragments were transferred hydrodynamically to a Zeta Probe GT membrane (Bio-Rad, Hercules) using a solution containing 0.4 M NaOH and 0.6 M NaCl. The DNA fragments were cross-linked to the membrane by UV irradiation and hybridized to a 32P-labeled mGATA-2 cDNA fragment (0.8-kbp HindIII fragment corresponding to a part of exon VI). Hybridization and washing of the membranes were performed at 65 °C following the manufacturer protocol.

Cell Culture—A mouse mast cell line P815 (generously provided by Dr. A. Ichikawa) and T cell line BW5147 (from ATCC) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (JRH Bioscience). Murine erythroleukemia (MEL) cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (JRH Bioscience) and then slowly cooled to room temperature in 150 m M KCl, 10 mM Tris-CI (pH 8.3), and 1 mM EDTA. Reverse transcription was initiated using 10 units of avian myeloblastosis virus reverse transcriptase (Promega) in a solution containing 50 mM KCl, 23 mM Tris-CI (pH 8.3), 20 mM MgCl2, 5 mM dithiothreitol, 0.15 mM dNTPs and 150 μg of actinomycin-D/ml and incubated for 60 min at 42 °C. The template was then digested with RNase A (2 μg) for 15 min at 37 °C followed by phenol-chloroform extraction, ethanol precipitation, and size fractionation on a denaturing polyacrylamide gel. The TaqTrack sequencing system (Promega) was used to generate a sequence ladder of the IS exon with the same primer.

Rapid Amplification of cDNA Ends Assay—Poly(A)+ RNA was prepared from P815 cells with Oligo-dT-29 (TaKaRa). 5′-Rapid amplification of cDNA ends (5′-RACE) assay was performed utilizing the AmplifiFINDER 5′-RACE system (CLONTECH). Two μg of poly (A)+ RNA were reverse transcribed into mGATA-2 cDNA with IS transcription initiation site was amplified using oligonucleotide antisense primer mG23 (5′-AGCGGCGTGGCTGTCGCCC-3′, corresponding to nucleotides 211 to 192 of the IS exon) and primers for the tailed linker sequence. Another antisense primer used for PCR amplification was mG26 (5′-GGCGCCGACGCTGTGCCC-3′, corresponding to nucleotides 124 to 105 of the IS exon). PCR products were subcloned into a pGEM7Z-NI vector (Promega), and their sequences were determined.

Transient Transfection Assays—Plasmids for functional assay of the IS promoter and upstream regions were constructed as follows. A KpnI-NotI fragment that covers from 6.0 kbp to +177 bp region of IS exon was set as +1 ligated to pGL2-Basic plasmid (Promega), which was cleaved with both KpnI and SacI, resulting in pGL-6.0IS. pGL2-Basic contains the firefly luciferase (LUC) gene as a reporter. Similarly, HindII-NotI (1580 to +177), EcoRI-NotI (1670 to +177), Pstl-NotI (165 to +177), Smal-Notl (86 to +177) fragments were ligated to the KpnI/SacI double digested pGL2-Basic, generating pGL-1580IS, pGL-670IS, pGL-165IS, pGL-145IS, and pGL-86IS, respectively. pGL-50IS containing 2.0 kbp upstream from the IG exon, 2.0 kbp downstream from the IG exon, and the sequence 3′ to the IS exon, and the other was a 760-bp BamHI-SacI fragment containing the 5′ flanking region, IG exon, and the sequence 3′ to the exon IG. The constructs were linearized by SpeI and BamHI, respectively, and a labeled antisense RNA was synthesized from the T7 promoter of the plasmid using T7 RNA polymerase (Promega, Madison, Wis.). Total RNA samples (100 μg) from P815 cells or Ba/F3 cells were hybridized to the labeled transcript for 12 h at 45 °C. The RNA samples were digested with an RNase mixture (Ambion), and the sizes of the RNA hybrids were determined by denaturing polyacrylamide gel electrophoresis. Yeast tRNA (Sigma) and total RNA from DS19 cells were used as negative controls to detect nonspecific bands. Radiolabeled RNA size markers were synthesized using an RNA marker template set (Wako Pure Chemicals).

Primer Extension Assay—An oligonucleotide, 5′-CCGGCTCCTCGGC-CCTCTGATCCCTC-3′, was labeled with [32P]ATP using T4 polynucleotide kinase (Toyobo, Osaka). The labeled primer (1 pmol) was annealed to 50 μg of total RNA from P815 cells or tRNA for 90 min at 65 °C and then slowly cooled to room temperature in 150 mM KCl, 10 mM Tris-CI (pH 8.3), and 1 mM EDTA. Reverse transcription was initiated using 10 units of avian myeloblastosis virus reverse transcriptase (Promega) in a solution containing 50 mM KCl, 23 mM Tris-CI (pH 8.3), 20 mM MgCl2, 5 mM dithiothreitol, 0.15 mM dNTPs and 150 μg of actinomycin-D/ml and incubated for 60 min at 42 °C. The template was then digested with RNase A (2 μg) for 15 min at 37 °C followed by phenol-chloroform extraction, ethanol precipitation, and size fractionation on a denaturing polyacrylamide gel. The TaqTrack sequencing system (Promega) was used to generate a sequence ladder of the IS exon with the same primer.

RT-PCR Analysis of the Expression from IS and IG Exons—Ten μg of RNA from normal mouse tissues or total RNA from 105 cells of each FACS-sorted lineage marker-positive fraction and from 5 × 106 cells of Sca-1+/c-kit+ and/or Lin− fractions were reverse transcribed by SuperScript RT (Life Technologies, Inc.) with random hexamer or oligo-dT primer. Products were purified by phenol-chloroform extraction, etha- nol precipitation, and one-tenth of the products was used in each PCR reaction. PCR reactions (35 cycles at 96 °C for 20 s and 68 °C for 60 s) were performed using primer (5′-ACAAAGGTGTTGCTGTCG-CAGG-3′), primer 2 (5′-CCACCCCTCCTGGTATCAGG-3′), and primer 3 (5′-AGCTGTGCTGTCCTAGATTTAAT-3′).

RNase Protection Assay—Total RNA was extracted from cultured cell lines and normal mouse tissues using RNAzol B (TEL-TEST) or ISO- GEN (Wako Pure Chemicals, Osaka, Japan). Two mGATA-2 genomic fragments were subcloned into pBluescript KS (+); one was a 610-bp PstI-NotI fragment containing the 5′ flanking sequence, IS exon, and the sequence 3′ to the IS exon, and the other was a 760-bp BamHI-SacI fragment containing the 5′ flanking region, IG exon, and the sequence 3′ to the exon IG. The constructs were linearized by SpeI and BamHI, respectively, and a labeled antisense RNA was synthesized from the T7 promoter of the plasmid using T7 RNA polymerase (Promega, Madison, Wis.). Total RNA samples (100 μg) from P815 cells and Ba/F3 cells were hybridized to the labeled transcript for 12 h at 45 °C. The RNA samples were digested with an RNase mixture (Ambion), and the sizes of the RNA hybrids were determined by denaturing polyacrylamide gel electrophoresis. Yeast tRNA (Sigma) and total RNA from DS19 cells were used as negative controls to detect nonspecific bands. Radiolabeled RNA size markers were synthesized using an RNA marker template set (Wako Pure Chemicals).

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To begin to analyze the regulatory mechanisms controlling transcription in the mGATA-2 gene, genomic DNA fragments containing the gene were cloned and characterized. Using the 5′ end region of the mouse GATA-2 cDNA clone, which was previously isolated from a fetal liver cDNA library, a mouse genomic library was screened, and the recovered recombinants were plaque-purified. Twelve independent mGATA-2 bacteriophage λ genomic clones were characterized by restriction enzyme site mapping and Southern blot hybridization. The genomic phage clones were found to be largely overlapping and to cover the entire coding region of the mGATA-2 locus of approximately 14 kbp (Fig. 1A).

We had previously isolated human GATA-2 cDNA and genomic clones and also determined the gene structure including a first exon (23). We found that the 5′ end sequence of the human GATA-2 cDNA, corresponding to the first exon, did not show high similarity with that of mouse GATA-2 cDNA, whereas other regions show remarkable sequence similarity. Indeed, the structures of the Xenopus (26) and human GATA-2 genes (27) were recently reported, and these were found to be highly homologous to each other. However, we did not find strong similarity in either of those structures to the mouse first exon sequence. For this reason, we speculated that there might be an alternative first exon for the mGATA-2 gene in addition to the one we had identified in both genomic and cDNA clones. We therefore searched the human IS promoter/exon region by Southern blotting of the hGATA-2 genomic phage clones, using the mouse IS promoter/exon fragment as a probe. However, we could not find the IS exon in our hGATA-2 genomic phage clones (data not shown), probably because those genomic clones contained only 4-kbp sequences upstream of the IS exon and, therefore, insufficient 5′ sequences to include the IS promoter and exon.

The mGATA-2 gene is therefore composed of seven exons
(Fig. 1A). The two distinct first exons are named IS and IG for the upstream and downstream exons, respectively, and both encode only 5' UTR sequence. The common second exon contains the initiation codon for translation of the mGATA-2 protein. The amino and carboxyl zinc fingers are encoded in exons IV and V, respectively. The basic amino acid tail region (36), 3' UTR, and the two polyadenylation signals (37) are located within exon VI. The exon-intron boundaries conform to the general splicing consensus (Fig. 1B). Southern blot analysis of genomic DNA confirmed the presence of bands of the predicted size from the restriction map of the phage clones (Fig. 1C).

**Tissue-specific Utilization of the IS and IG Exons/Promoters**—To elucidate how the promoters preceding the two individual first exons might be differentially utilized, we performed a series of expression analyses. First, RNA blot analysis showed that, of the cell lines examined, GATA-2 mRNA was expressed abundantly in P815 mastocytoma (Fig. 2, lane 1) and Ba/F3 proB lymphocyte (lane 2). In contrast, GATA-2 mRNA levels were undetectable in the DS19 murine erythroleukemia (lane 3) and BW5147 T lymphocyte (lane 4) cell lines. When we carried out additional analyses using IS or IG exon-specific probes, the experiment showed that IS mRNA was transcribed abundantly in P815 cells but only poorly in Ba/F3 cells, whereas IG mRNA is expressed in both P815 and Ba/F3 cells (Fig. 2). RNA blot analysis using these three probes all showed the presence of two mRNAs for mouse GATA-2. This analysis revealed that the IS exon might be used more often in differentiating hematopoietic cells.

We next examined the expression of IS and IG mRNAs by RT-PCR. We used three primers for this purpose: primers 1 and 2 were specific for the sense strand sequence of the IS and IG exon, respectively, and primer 3 for the antisense strand sequence of the common exon II (Fig. 3A). RNA from P815 cells was used as a positive control for the expression of both types of mGATA-2 mRNAs (Fig. 2B). This analysis revealed that the IG mRNA is expressed widely in tissues that express GATA-2, whereas IS mRNA could not be detected in these same tissues (Fig. 3B). Since we isolated cDNA clones containing the IS exon from a fetal liver cDNA library, we tested the possibility that the IS exon might be expressed more often in differentiating hematopoietic cells.

To examine in further detail the possibility that IS and IG mRNAs might be differentially expressed during hematopoietic differentiation, we examined FACS-sorted hematopoietic cell fractions using these same RT-PCR methods. We used antibodies against several lineage markers (Lin) and Sca-1 and c-kit for this purpose (29). Both the IS and IG mRNAs were found to be expressed in the immature hematopoietic Lin- cell population as well as in the even more immature c-kit- /Sca-1- /Lin- cells (Fig. 3C). In addition, IS mRNA was weakly expressed in NK cells. These results show that the GATA-2 IS promoter is active in immature (Lin-) hematopoietic cell lineages.

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(figures and references are omitted for brevity)
bands protected by both probes, suggesting the existence of minor transcription start sites; in particular, the IG protection probe gave rise to bands around 270 bp (lane 11).

The assignment of the start site of the IG exon was confirmed by primer extension analysis (Fig. 4B). The result coincided well with the results from the RNase protection assay. Our assignment of the mouse IG start site is close to the reported transcription initiation sites of Xenopus and human GATA-2 mRNAs (26, 27). The transcription initiation site of the IS exon was confirmed using 5′ RACE, employing poly (A)+ RNA recovered from P815 cells. Six independent transformants were selected from the RACE cloning products, and their sequences were determined (data not shown). All 5′ ends of the selected clones were consistent with the major transcription initiation site determined from the RNase protection assay (6/6). The IS and IG exon transcription initiation sites, superimposed on the exons (boxed areas) and variable amounts of promoter sequence, are shown in Fig. 5 (see below).

Structure of the Promoter and Upstream Regions of the IS and IG Exons.—We next determined the sequences of the promoter and upstream regions of both the IS and IG exons (Fig. 5). We analyzed these sequences by TFSEARCH which searches within sequenced fragments versus the TFMATRIX transcription factor binding site profile data base by E. Wingender, R. Knuettel, P. Dietze, and H. Karas (GBF-Braunschweig). We found a number of possible transcription factors binding sites, including some expressed uniquely in hematopoietic cells, in this search and in other literature searches (described under “Discussion”). These positions of these transcription factor binding sites are indicated in Fig. 5.

In the IS promoter, a TATA box-like AAATAAAA element was found between position −33 to −25 relative to the major IS start site. Four consensus binding sequences for GATA factors can be found in this region, with three of them clustered at a position around −1,050. Of the GATA motifs, two are in a palindrome position separated by only 5 bp relative to one another, whereas two other GATA sites (at −1,095 and −645) are palindromic with incomplete GATA motifs: AGATA and GGATAA, respectively (see underlined sequences in Fig. 5, left). The AGATA motif, in particular, has been shown to be a preferential binding site for GATA-2 and GATA-3 over a GATA-1 preference (38).

Since it had not been previously reported, we also determined the mouse IG exon/promoter sequence. The sequence is highly conserved with that of human GATA-2 (Fig. 5, right) (27). In particular, the double CCAAT boxes that were shown to be important for the expression of Xenopus GATA-2 during early embryonic development are also found in the mouse IG promoter region (26).

Trans-activation Activity of the IS Promoter in P815 Cells.—We next attempted to localize cis-acting elements involved in the determination of the hematopoietic cell-specific expression of the IS promoter. We first examined six constructs bearing rather large deletions of 5′ sequence information containing the IS promoter. The largest construct in this analysis is pGL-6.0IS, containing 6.0 kbp upstream from the transcription initiation site and 177 bp of exon IS (to a Not I site in the IS exon). The shortest construct we examined was pGL-50IS, in which the LUC reporter gene was driven by a proximal promoter fragment extending only to −50 relative to the beginning of exon IS. These reporters were transfected into P815 cells. LUC activity was determined using total cellular extracts and normalized for transfection efficiency based on a cotransfected pENL-β-galactosidase control plasmid.

Fig. 6A shows that all the constructs, except pGL-50IS, yielded high and reproducible LUC activity in cells which normally express GATA-2. Deletion of the upstream sequence from −6.0 kbp to −145 bp resulted in no significant decrease in promoter activity. In contrast, deletion of an additional 95 bp from the pGL-145IS reporter gene resulted in more than a 5-fold reduction of promoter activity. These results seem to exclude our anticipated possibility that the three (complete and incomplete) palindromic GATA binding motifs in the upstream region might significantly influence (either positively or negatively) GATA-2 transcriptional activity.
Since the IS promoter appeared to be significantly influenced by local promoter sequences, we next examined finer mutations in the LUC reporter plasmid pGL-145IS using a PCR method and again transiently transfected these constructs into the same cells. Among these constructs, pGL-79IS showed the strongest transcriptional activity of all the plasmids, but additional deletion of only 18 bp from the pGL-79IS resulted in a consistent 4-fold decrease in LUC reporter activity (Fig. 6B), and similar results were obtained from experiments using Ba/F3 cells (data not shown). These data strongly suggest that a positive regulatory element for IS-directed transcription resides in that 18-bp region.

To refine the position of the important cis-acting positive regulatory elements within the IS promoter region, we prepared a set of substitution mutants and analyzed their effect on expression using P815 as the substrate recipient cells (Fig. 6C). Replacement of the sequences 236 to 239, 242 to 248, and 251 to 254 reduced the transcriptional activity to 52, 29, and 40% of the wild-type pGL-145IS, respectively, indicating that important cis-acting elements exist in this region (proximal to 254). Substitution of the sequence of 255 to 278 did not affect transcriptional activity. Replacement of the sequence between 270 to 273 again affected IS promoter activity, resulting in a reduction to 40% in comparison with the wild-type plasmid. Conversion of the 255 to 278 region of the pGL-145IS to GGTACC (shown as del55–78 in Fig. 6C) caused a reduction to 32% of wild type. Therefore, there is a possibility that the ~55 to ~78 deletion might reflect a function that is elicited through that specific cis-acting element (the ~70 to ~73 mutation).

IS and IG Promoter Activities in P815 Cells—We performed a second set of transient transfection assays using constructs containing sequences lying between the IS promoter and the common second exon transfected into P815 cells (Fig. 7A). Transfection of reporter plasmid pGL-79ISIG-II, containing the IS promoter (79 bp), IS exon, and a part of the second exon, resulted in almost identical LUC activity to that generated by pGL-482IG-II, indicating that genuine IS promoter activity is comparable with the IG promoter activity in P815 cells. pGL-79ISIG-II typically generated lower LUC activity than did pGL-482IG-II. Thus the region upstream from the ~482 to IS exon has no stimulating activity in P815 cells (Fig. 7A).

We also prepared a series of deletion mutants of IG promoter and transfected the constructs into P815 cells. This analysis revealed that the region spanning ~103 to ~31 is important for IG promoter activity. The 72-bp region contains double CCAAT boxes that are conserved between the human (27) and Xenopus (26) GATA-2 gene promoters, both of which appear to correspond to the mouse IG promoter. Therefore, we introduced a mutation into the proximal CCAAT box of mGATA-2 IG promoter and found that the mutation resulted in approximately 70% decrease of the promoter activity (data not shown). This

![Fig. 5. Nucleotide sequences of the IS and IG exons, promoters, and upstream regions of mGATA-2 gene. Transcription initiation sites are indicated by arrows. The major transcription start sites are numbered as +1. Sequence motifs that match consensus binding sites of known transcription factors are underlined. Consensus GATA binding sites are circled and CCAAT binding sequences are boxed.](http://www.jbc.org/)

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result is consistent with that of Xenopus GATA-2 promoter analysis and indicates that the CCAAT box is essential for full IG promoter activity.

Cell-type Specificity of Minimal IS Promoter Activity—To test for cell-type specificity of IS promoter activity, the reporter gene activities of pGL-145IS and pGL-50IS were determined in several culture cell lines. LUC activity generated by pGL-145IS in DS19 and BW5147 cells were 27 and 11% of that generated in P815 cells, respectively, whereas LUC activity in Ba/F3 cells was 99% of that in P815 cells (Fig. 8). The former two cell lines do not express GATA-2 mRNA to a detectable level (see Fig. 2). These differences were not observed when we transfected pGL-50IS construct into the same cell lines. These results indicate that the IS promoter indeed contributes primarily, if not exclusively, to the determination of cell-type specificity of mGATA-2 expression from the IS exon.

The Functional Cis-elements Are Protected in DNase I Footprinting Analysis—We next tried to identify transcription factors that might bind to the IS promoter. To this end, we first carried out DNase I footprinting analysis of these domains using nuclear extracts prepared from P815 (GATA-2 high) or DS19 (GATA-2 low) cells. As shown in Fig. 9A, two regions (downstream from −54 and from −61 to −79) were protected using a sense probe in combination with P815 nuclear extracts (lanes 4–6). Several hypersensitive sites, another hallmark of specific protein-DNA interactions, were also observed at −51, −68, −75, and −80 in this experiment. In contrast, the DS19 nuclear extract protected only the region downstream from −54 and did not produce strong hypersensitive sites (lanes 9–11). When we carried out the footprinting experiment with the antisense probe, the P815 nuclear extract protected the region proximal of −54 and −65 to −75 from DNase I digestion, whereas the DS19 nuclear extract did not protect these regions effectively (Fig. 9B). Since the DS19 nuclear extract was purified by the same procedure as the P815 nuclear extract and since we used identical amounts of nuclear protein in comparable lanes in this experiment, these results suggest that a transcription regulatory complex is formed more efficiently with the P815 nuclear extract than that with DS19 nuclear extract. The two regions protected by the P815 nuclear extract
(i.e. proximal to −54 and −61 to −79) show very good coincidence with the regions that are important in the transient transfection assays (see above).

**Competitive Transcription Factor Binding to GC-rich Sequences in the IS Promoter**—To further investigate transcription factor binding to the IS promoter region, we next performed EMSA using the P815 and DS19 nuclear extracts. A probe spanning −85 to −51 (Fig. 10A) formed several major bands (bands a to d, see lane 1 of Fig. 10B and lane 8 of Fig. 10C). These bands could be competed with a 100-fold excess of unlabeled competitor (lane 2 of Fig. 10B), indicating that the binding of transcription factors to this probe is specific.

Competition experiments with a series of mutant oligonucleotides suggested that several independent transcription factors bound to this region since no single mutant oligonucleotide competed for all the bands (Fig. 10B, lanes 3 to 6). The mutations at −70 to −73 were previously shown to be important for IG promoter activity in transient transfection assays (see above). Competition with the oligonucleotide containing this mutation left bands b and d. EMSA with DS19 cell nuclear extracts exhibited a different pattern of retarded bands (Fig. 10B, lane 7), without obvious bands corresponding to b and d. Addition of an anti-Sp1 antibody to the reaction resulted in supershift of a component of band a (lane 9), whereas addition of an anti-Ap2 antibody did not influence the banding pattern (lane 10). The intensity and mobility of bands b and d were not influenced by the addition of these antibodies, indicating that these bands did not contain Sp1, AP-2, or other transcription factors cross-reactive to these antibodies. These results indicate that transcription factors in bands b and/or d may be directing the IS promoter function in hematopoietic cells. The identity of these two factors remains to be clarified.

**DISCUSSION**

Although transcription factor GATA-2 is essential for hematopoiesis (21), its expression is not restricted to hematopoietic lineages. In hematopoietic cells, the expression level of GATA-2 changes dramatically during progressive cell differentiation (12, 39). This suggested that mechanisms could exist for regulating the expression of GATA-2 specifically in the hematopoietic lineage. Consistent with this expectation, we report here the presence and activity of an independent hematopoietic promoter in the mGATA-2 gene. The IS promoter is used specifically in hematopoietic progenitors, whereas the IG promoter seems to be responsible for the expression of GATA-2 in various tissues but also including hematopoietic cells. While we found the structure of the IG promoter to be quite homologous to the previously reported human and *Xenopus* GATA-2 promoters (26, 27), the IS promoter was identified for the first time here.

Determination of the complete structure of the mGATA-2 gene revealed an organization resembling that of the other GATA-1/2/3 factors so far defined (23, 26, 27, 40, 41) (Fig. 11). The restriction enzyme sites within the mGATA-2 gene determined in this study coincide completely with the map previously reported by Tsai and colleagues (21). Genes for vertebrate GATA-1/2/3 factors are generally composed of six exons. In addition, both GATA-1 (7) and GATA-2 (this study) genes contain two alternative first exons (Fig. 11). The chicken GATA-5 gene, which belongs to another subfamily of GATA factors
are rapidly expanding. An assessment of the trans-activation activity in a transgenic mouse system, using constructs containing both the mGATA-2 promoter driving the expression of the Escherichia coli β-galactosidase gene, is now ongoing in this laboratory. Our preliminary data show that the reporter gene expression in the hematopoietic stem cell fraction of bone marrow cells and in aorta/gonad/mesonephros region of mouse embryo is mainly driven by IS promoter.

The basic transcriptional activity of the IS promoter was found to be localized within the proximal 80 bp. Specific transcription factor interactions to the −85 to −50 region of the IS promoter were identified through DNase I footprinting analysis and EMSA using nuclear extracts from P815 cells. Whereas EMSA competition experiments with mutated oligonucleotides showed that transcription factors binding to the −73 to −70 sequence are responsible to the IS promoter activity, no consensus DNA-binding sequence of known transcription factors has been identified for this sequence.

In this regard, we noticed that transcription factors Sp1, MZF-1 (47), CTCF (48), or MAZ/ZF87 (49) has the possibility to bind (48, 51, 52). As c-myc controls differentiation, proliferation, and apoptosis of hematopoietic cells (47, 50), and overexpression of MZF-1 inhibits hematopoietic cell development from ES cells (50). These properties are similar to those of GATA-2. We also have noticed the presence of three CCCTC sequences in the IS promoter (44). These motifs resemble the cis-elements in the c-myc promoter, to which several transcription factors, such as Sp1, ZF78, MAZ, hnRNPK, and CNBP, can bind (48, 51, 52). As c-myc controls differentiation, proliferation, and apoptosis of hematopoietic cells (53), factors binding to the CCCTC sequence may also be related to mGATA-2 gene expression in hematopoietic progenitors.

A 31-bp pyrimidine-rich sequence (5′-TCTGCCGCGCTTT-CTGGCCCCCTCTGCCCCTC-3′, −3831 to −3800 relative to translation start site) in the zebrafish GATA-2 gene was recently reported to be responsible for neuronal expression of the gene (54), although the position of the first exons as well as the

5 J. Ohta, N. Minegishi, and M. Yamamoto, unpublished observation.
specificity. Since GATA-2 is essential for the development of all hematopoietic tissue of the zebrafish. Deletion of 2.5 kbp from the 5′ end of the 7.3-kbp plasmid nearly abolishes the GFP expression specifically in the posterior end of the intermediate cell mass, the early hematopoietic progenitors in mouse bone marrow, we speculate that the upstream 2.5-kbp region may contain a first exon homologous to the mouse IS exon.

We demonstrated here the presence of two mGATA-2 gene promoters. Of the two promoters, the IS promoter is expressed homologous to the mouse IS exon.

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