The Transcription Factor Sp1 Regulates the Myeloid-specific Expression of the Human Hematopoietic Cell Kinase (HCK) Gene through Binding to Two Adjacent GC Boxes within the HCK Promoter-Proximal Region*

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The human hematopoietic cell kinase (HCK) is a member of the src family of protein tyrosine kinases specifically expressed in myeloid cells and to a minor extent in B-lymphoid cells. HCK expression is up-regulated at the transcriptional level during myeloid differentiation of hematopoietic cells. To elucidate the molecular basis of the differential HCK gene expression, the genomic region containing the HCK promoter was isolated and functionally characterized. A DNA fragment containing 101 base pairs of the 5'-flanking sequence showed strong promoter activity in the macrophage cell line RAW264 but was inactive in the non-myocytic cell lines HUT-78 and NIH-3T3. Site-directed mutagenesis of the proximal promoter region showed that two GC-rich sequence elements are essential for transcriptional activity in myeloid cells. Electrophoretic mobility shift analysis using nuclear extracts obtained from RAW264 cells and from the promonocytic cell line U-937 revealed the formation of at least three distinct protein-DNA complexes at each of these sites, one of which was found to contain the transcription factor Sp1. Expression of a reporter gene linked to the −101 HCK promoter region was up-regulated by Sp1, but not by other members of the Sp1 family of transcription factors, in Drosophila Schneider cells. A synergistic effect on HCK promoter activity was observed at high concentrations of Sp1. Our results show that Sp1 plays an essential role in the regulation of the differential gene expression of the HCK gene.

The HCK protein interacts physically with numerous receptors (8–10) and its catalytic activity is stimulated upon engagement of myeloid-specific receptors (11–14). Inactivation of the HCK gene by homologous recombination in ES cells results in a mild phenotype in HCK-deficient mice, characterized by impaired phagocytosis in macrophages (15).

Like many of the other src-related kinases, HCK expression has been detected in cells of the granulocytic and monocytic lineage (1, 2) and at low levels in B lymphocytes (16) and NK cells (17). The expression of the HCK gene is regulated at the level of transcription (18) and increases during myeloid differentiation of normal and leukemic hematopoietic cells. In addition, the expression of HCK is induced in normal human macrophages and bone marrow macrophages after exposure of cells to bacterial lipopolysaccharide (19–21) while interferon-γ treatment of the cells itself does not alter HCK expression, but primes macrophages toward a stronger induction of HCK gene expression after LPS stimulation (20, 21).

Transfections of reporter gene constructs into cultured cells have been widely used to delimit the promoter regions required for tissue-specific expression of myeloid genes. These experiments have shown that the transcriptional control elements of these genes are clustered within the promoter proximal region of the genes. For example, the first 92 bp of the CD11b promoter are sufficient to restrict the expression of reporter gene constructs to myeloid cells, at least in vitro (22). Similar findings have been reported for the genes encoding the membrane glycoprotein CD14 and for several genes encoding myeloid-specific receptors, like the granulocyte-macrophage colony forming factor receptor or for the granulocyte colony-stimulating factor receptor (see Ref. 23 for review). Moreover, several myeloid-specific genes do contain multiple start sites of transcription due to the lack of a TATA box or defined initiator sequences within their promoter region. In most of these genes, binding sites for either myeloid-specific (like PU.1) or ubiquitous (Sp1) transcription factors or a combination of both are found in close proximity to the major transcriptional start site. Through interaction with additional factors of the basal transcriptional machinery (i.e. TATA-binding protein), these proteins contribute alone or in combination with other factors to the cell specific expression of myeloid genes (see Ref. 23 for review).

In this paper we report the functional analysis of the HCK promoter region. We show that the activity of the HCK promoter depends on sequence elements located in direct vicinity of the transcriptional start site. Moreover, site-specific mutagenesis and electrophoretic mobility shift assays revealed that the transcription factor Sp1 is essential for the myeloid-specific expression of the HCK gene.

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§ The abbreviations used are: HCK, hematopoietic cell kinase; bp, base pair(s); CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.
Experimental Procedures

Cloning of the 5'-Flanking Region of the Human HCK Gene and Construction of HCK Reporter Plasmids—A human genomic DNA library constructed in bacteriophage APhIX II\textsuperscript{sp} (Stratagene) was screened with an oligonucleotide (CGCTACAGCTTGGAGATCCCGCGCTGC-CCGGGAGGAGC) derived from the first exon of the HCK gene (nucleotides 95 to 132 of the sequence published by N. Quintrell et al. (1)). Three clones containing HCK sequences were identified. For restriction mapping, human DNA inserts were cleaved from the phage arms with NotI and subjected to partial digestion with restriction enzymes. Restriction fragments were identified by Southern blotting using either T3 or T7 \textsuperscript{32}P-labeled oligonucleotide probes.

Plasmid p-4950/CAT, which contains the transcriptional start site of the HCK gene (18), was constructed by cloning a 5-kilobase DNA fragment generated by a partial XbaI digestion of bacteriophage AHCK3 into the XbaI site of the reporter plasmid pCAT Basic (Promega). All further reporter gene constructs were generated after cleavage of p-4954/CAT with appropriate restriction enzymes and cloning into pCAT-Basic using standard protocols (24).

Site-directed mutagenesis was carried out by polymerase chain reaction-based strategies. For mutations within the distal GC-rich element (75 to 190), a 125-bp DNA fragment was amplified from plasmid p-101/CAT with the primers HCKM1 (CAGCTCGGGAGCACATCGAAGCTTAACTAGTGATGGGAAGG) and HCKCATu (CAGATCTCTAAGACCTTGC). The polymerase chain reaction product was cloned into the pGEM-T vector (Promega) and sequenced. An AucLXbaI DNA fragment derived from this construct was isolated and used to replace a similar DNA fragment in p-101/CAT, thereby generating the Mutant plasmid p-101/CATM.

Mutations in the proximal GC-rich box were carried out by using a similar strategy. A 127-bp DNA fragment containing the GC-proximal box was amplified with primers HCKM2 (CTTCTTCTGAACTACTAGGCGGCGAGCAGTCTAGGGGAGG) and HCKCAT (AGCATCTCTAAGACTTTCGAGG), cloned into pGEM-T vector and sequenced. The HCK mutant reporter gene construct p-101MI2 CAT was generated as described before for p-101MI1 M1 CAT.

Cell Culture, Transfections, and Reporter Gene Assays—RAW264, a murine leukemic cell line with monocyte-macrophage characteristics (25), was obtained from European Collection of Animal Cell Cultures (Porton Down, Salisbury, United Kingdom) and cultivated in RPMI 1640 supplemented with 10% fetal bovine serum. The murine embryonic fibroblast cell line NIH3T3 (26) was obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Drosophila Schneider cells (27), kindly provided by G. Suske (University of Marburg), were cultivated in Schneider medium with 10% heat inactivated insect cell qualified fetal bovine serum. U-937 and HUT-78 were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 15% fetal bovine serum, respectively.

Drosophila Schneider cells were transfected by calcium phosphate precipitation exactly as described (30) with 20 μg of reporter plasmid/25 cm\textsuperscript{2} flask. DNA mixtures for transfection of mammalian cells contained 5 μg of control plasmid (RSV-LacZ) and equal molar amounts of reporter gene constructs (10 to 20 μg). The mixture was completed to 25 μg of DNA with pUC18. NIH3T3 cells were transfected by calcium phosphate precipitation with a total of 25 μg of plasmid DNA mixture/25 cm\textsuperscript{2} flask. U-937 and HUT-78 were transfected by electroporation. 4 × 10\textsuperscript{6} cells were resuspended in 375 μl of serum-free RPMI 1640 containing 10 mM HEPES (pH 7.0), mixed with a total of 25 μg of plasmid DNA and subjected to the electrical pulse (960 microfarads, 3.3 μF) and lysed by three freeze-thaw cycles. The mixture was completed to 25 μl of MEM with 10% fetal bovine serum. U-937 (28) and HUT-78 (29) were obtained from the Type Culture Collection and maintained in Dulbecco’s modified Eagle’s Schneider medium with 10% heat inactivated insect cell qualified fetal bovine serum.

Preparation of Nuclear Extracts—Nuclear extracts were prepared essentially as described by Gorski et al. (32). Briefly, 5 × 10\textsuperscript{6} cells were washed with cold phosphate-buffered saline and collected by centrifugation at 300 × g. Cells were suspended in 30 ml of homogenization buffer (10 mM Tris, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 mM sucrose, 10% glycerol) supplemented with protease inhibitors (1 μg/ml antipain, 4 μg/ml aprotonin, 0.5 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride), grinded in a Dounce homogenizer and layered on top of 10 ml homogenization buffer in a SW27 Beckmann tube. After sedimentation at 100,000 × g for 30 min at 2 °C in a SW27 rotor nuclear pellets were suspended in nuclease lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 1 mM diethiothreitol, 10% glycerol) supplemented with protease inhibitors, homogenized until if necessary diluted to 10 A\textsubscript{260} units/ml. Nuclear proteins were extracted by adding 0.1 volume of 4 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} slowly over a 30-min period. The suspension was cleared by centrifugation (120,000 × g, 0 °C, 60 min). Proteins were precipitated by addition of 0.3 g of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}/ml and centrifugation at 120,000 × g for 30 min at 0 °C in a swing-out rotor. The protein pellet was suspended in dialysis buffer (25 mM HEPES, pH 7.8, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM diethiothreitol, 0.05% Nonidet P-40, and protease inhibitors) and dialyzed two times for 2 h each against the same buffer. Precipitates were removed by centrifugation and the clear supernatants kept frozen in liquid nitrogen until use.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were preincubated on ice with 1 μg of poly(dI-dC)-in shifting buffer (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 20 mM KCl, 4 mM MgCl\textsubscript{2}, 2 mM spermidine, 2 mM diethiothreitol, 17.5% glycerol) in a total volume of 20 μl. Whenever indicated, reaction mixture was supplemented with competing oligonucleotides. After 15 min, 1.25 fmol of a \textsuperscript{32}P-labeled DNA fragment (10,000 to 20,000 cpm) was added to the incubation mixture. After 30 min on ice, the reaction mixture was loaded onto an 8% polyacrylamide gel in 45 mM Tris borate, 1 mM EDTA and separated at 200 V at room temperature. For supershift studies, 2 μg of monoclonal Sp1 antibody (Santa Cruz Biotechnology) was added 15 min after addition of the probe to a standard binding reaction and incubation was carried out for additional 30 min at room temperature.

Results

Cloning and Transcriptional Activity of the HCK Promoter—To identify regions responsible for the cell-type specific expression of the HCK gene, we cloned human genomic sequences preceding the first exon of the human HCK gene. For this, a AfphIX II\textsuperscript{sp} genomic library (Stratagene) was screened with an oligonucleotide derived from the first exon of the HCK gene (18). Three clones spanning a total of 22.5 kilobase pairs of DNA were identified. Restriction mapping of the DNA inserts showed that two of the three phases (HCK3, HCK5) contained identical inserts (results not shown). A 5-kilobase DNA fragment, spanning the first 36 nucleotides of exon 1 and upstream sequences, was isolated from HCK3 by partial XbaI restriction enzyme digestion and cloned into the XbaI restriction site in plasmid pCAT-Basic to generate p-4954CAT. A series of reporter gene constructs containing successive 5'-3' deletions within the HCK promoter region were derived from p-4954/CAT by restriction enzyme digestion (Fig. 1A).

The reporter gene constructs were transfected into the HCK positive murine monocyte cell line RAW264 (25) as well as into the HCK negative cell lines HUT-78 (29) and NIH3T3 (26). Transfection efficiency was monitored by co-transfection of an RSV-LacZ plasmid. A basal level of activity was observed in RAW 264 cells transfected with constructs containing HCK promoter sequences up to position -36 (1.6-fold above background), while extension of the flanking sequence up to position -101 enhanced the relative promoter activity by 8-fold (Fig. 1B). Constructs containing sequences up to position -422 showed similar CAT activities. However, the inclusion of further upstream sequences (up to position -813) caused a dramatic decrease in promoter activity (1.3-fold above background). The addition of sequences up to 3.6-kilobase did not lead to an increase in CAT expression. Promoter activity was partially restored by inclusion of sequences up to position...
None of these constructs showed any significant activity in the non-myeloid cell lines HUT-78 and NIH3T3 (Fig. 1B). The lack of HCK-driven CAT activity in these cells was not due to low transfection efficiencies, since the relative CAT activity of a CMVtk-CAT reporter gene construct in NIH3T3 cells was 70 to 320% (mean 160%, n = 4) and in HUT-78 cells 70 to 80% of that observed in RAW 264 cells (results not shown).

Sequence Elements Responsible for Promoter Activity—Within the first 101-bp 5′-flanking region two GC-rich sequences resembling binding sites for the Sp1 family of transcription factors were identified by sequence homology analysis (Fig. 2A). The distal sequence GAGGCGAG between base pairs 258 and 251 and the proximal sequence CGCCCGCGGC between base pairs 216 and 27 both fit to known Sp1 consensus binding sites (33, 34). In order to analyze the role of these elements in the differential expression of the HCK gene, a site-directed mutagenesis of the GC boxes was performed. Within the distal site, the three central nucleotides were mutated (GAGGCGAG to GACTAGAG), since mutation of similar nucleotides in GC-rich regions has been shown to abolish transcription factor binding and transcriptional activation by members of the Sp1 family (35). Similarly, a reporter plasmid was constructed where the proximal GC-rich sequences was mutated to TAATGCATTA (Fig. 2A).

Reporter gene constructs containing wild type or mutations in either of the GC boxes were analyzed by transient transfection into the monocytoid cell line RAW264. As shown in Fig. 2B, mutations in either of the GC boxes severely reduced promoter
activity. The wild type activity was reduced 5.3-fold by mutations within the distal GC box, while a larger decrease in promoter activity (8.4-fold) was observed in constructs containing alterations within the proximal GC box. None of mutant promoter constructs showed CAT activity above background levels in NIH3T3 cells (Fig. 2B).

Multiple Proteins Binding the GC Box Elements of the HCK Promoter—Since the GC boxes in the promoter region of the human HCK gene are essential for promoter function, we looked for binding of nuclear proteins to HCK-derived fragments by EMSA. A DNA fragment spanning the distal GC-box (from −77 to −10; HCK F1 in Fig. 3A) was used to probe nuclear extracts derived either from: (i) RAW 264 cells, (ii) the human promonocytic cell line U-937 (28), or (iii) U937 cells after 12-O-decanoylphorbol-13-acetate stimulation. Nuclear extracts from HeLa cells were used as a negative control. Two distinct bands, designated D1 and D3, were visible upon incubation of the HCK fragment F1 with nuclear extracts derived from U-937 cells (Fig. 3B). The interaction between U-937 nuclear proteins and the HCK F1 fragment could be competed with either a 100-fold molar excess of a tetramerized Sp1-binding site (36) or a 100-fold molar excess of unmethylated F1 fragment (Fig. 3B), suggesting the involvement of Sp1 or closely related proteins in these interactions. In contrast, no competition was observed upon preincubation of nuclear extracts with two oligonucleotides containing either a wild type or a mutated PU.1-binding site derived from the SV40 promoter (37). Similar complexes were observed when the F1 fragment was incubated with nuclear extracts obtained either from phorbol ester-induced U-937 cells or from the murine macrophage cell line RAW264 (Fig. 3C).

To delimit the promoter region involved in protein-DNA interaction more precisely, a 22-bp oligonucleotide (F3) centered on the transcription initiation site was synthesized and used as a probe in EMSA experiments. Similarly, an oli-
A 32P-labeled HCK F2 wt-oligonucleotide (1.25 fmol) was incubated in the absence (lane 1) or presence of 10 μg of bovine serum albumin (BSA) (lane 2) or nuclear extracts prepared from U-937 (B, left), HeLa (B, right), 12-O-decanoylphorbol-13-acetate (TPA)-induced U937 (C, upper panel), or RAW264 (C, lower panel) cells (lanes 3–7). The unlabeled double stranded competitor oligonucleotides were added at 100-fold molar excess as described in the legend to Fig. 3. P1 and P3 refer to the DNA-protein complexes formed with nuclear extracts from U-937 and HeLa cells, P2 refers to the HeLa specific complex. D, mutations within the proximal GC box abolishes binding of nuclear factors. A 32P-labeled HCK F2 wt-oligonucleotide (1.25 fmol) was incubated in the absence (lane 1) or presence of 10 μg of bovine serum albumin (lane 2) or nuclear extracts prepared from U-937 (lanes 3–6) or HeLa cells (lanes 7–10). A mutated F2 oligonucleotide was added to the samples at 125-fold (lanes 4 and 8), 250-fold (lanes 5 and 9), or 500-fold (lanes 7 and 9) molar excess. The complexes P1, P2, and P3 are described above.

FIG. 4. Binding of nuclear factors to the proximal GC box of the human HCK promoter. A, schematic representation of the HCK F2 oligonucleotide (−44 to +3) used for EMSA. The HCK promoter region is drawn as a line, GC-rich elements are marked by boxes. The transcriptional start point is indicated by an arrow. The probe fragment is shown as a bar below. Binding of nuclear factors to the HCK F2 oligonucleotide are shown in B and C. A 32P-labeled F2 oligonucleotide (1.25 fmol) was incubated in the absence (lane 1) or presence of 10 μg of bovine serum albumin (BSA) (lane 2) or nuclear extracts prepared from U-937 (B, left), HeLa (B, right), 12-O-decanoylphorbol-13-acetate (TPA)-induced U937 (C, upper panel), or RAW264 (C, lower panel) cells (lanes 3–7). The unlabeled double stranded competitor oligonucleotides were added at 100-fold molar excess as described in the legend to Fig. 3. P1 and P3 refer to the DNA-protein complexes formed with nuclear extracts from U-937 and HeLa cells, P2 refers to the HeLa specific complex. D, mutations within the proximal GC box abolishes binding of nuclear factors. A 32P-labeled HCK F2 wt-oligonucleotide (1.25 fmol) was incubated in the absence (lane 1) or presence of 10 μg of bovine serum albumin (lane 2) or nuclear extracts prepared from U-937 (lanes 3–6) or HeLa cells (lanes 7–10). A mutated F2 oligonucleotide was added to the samples at 125-fold (lanes 4 and 8), 250-fold (lanes 5 and 9), or 500-fold (lanes 7 and 9) molar excess. The complexes P1, P2, and P3 are described above.

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with 160 nM 12-O-decanoylphorbol-13-acetate for 24 h and from RAW264 cells (Fig. 4C). Binding of nuclear factors to the F2-oligonucleotide was not competed by a mutated F2 oligonucleotide (Fig. 4D), indicating that the GC box within the F2 oligonucleotide is essential for binding activity.

Differential Binding Affinity of Nuclear Factors to the Proximal GC Box—During the course of the competition experiments we realized that the factor(s) involved in complexes P1 and P2 on the F2 oligonucleotide were competed differentially with the wt-F2 oligonucleotide or the Sp1 tetramer. To verify this, a 32P-labeled HCK F2 oligonucleotide was incubated with either U-937 or HeLa nuclear extracts in the presence of increasing molar amounts of unlabeled F2 competitors at 15-, 75-, and 125-fold molar excess (lanes 2–4, respectively). B, similar to A, but with increasing amounts of Sp1 tetramer as competitor. C, ratio between complexes P1 and P2. The intensity of the complexes P1 and P2 obtained with HeLa extracts were quantitated with a laser scanning densitometer. The intensity ratio of band P1 to band P2 was plotted as a function of molar excess competitor. Open triangle, HCK F2 wt competitor; open square, Sp1 tetramer competitor.

Fig. 5. Nuclear factors compete for binding to the proximal GC box. A 32P-labeled F2 oligonucleotide was used as a probe in EMSA experiments with nuclear extracts of U-937 or HeLa cells as indicated above the panels. A, EMSA reactions containing 1.25 fmol of 32P-labeled probe and 10 μg of nuclear extract and either no competitor (lane 1) or increasing amounts of unlabeled F2 competitor at 15-, 75-, and 125-fold molar excess (lanes 2–4, respectively). B, similar to A, but with increasing amounts of Sp1 tetramer as competitor. C, ratio between complexes P1 and P2. The intensity of the complexes P1 and P2 obtained with HeLa extracts were quantitated with a laser scanning densitometer. The intensity ratio of band P1 to band P2 was plotted as a function of molar excess competitor. Open triangle, HCK F2 wt competitor; open square, Sp1 tetramer competitor.

With the same procedure explained above, we performed EMSA experiments with nuclear extracts of U-937 or HeLa cells in the presence or absence of 2 μg of a monoclonal anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as indicated. The supershifted band is marked as SS, the remaining complexes are labeled as described in Fig. 5. B, same experiment as described in A but at higher resolution, showing that band D1 contains two protein-DNA complexes (D1a and D1b). Except for electrophoresis time the experimental conditions were identical to those in panel A. C, 10 μg of nuclear extracts of U-937 or HeLa cells were incubated with 1.25 fmol of 32P-labeled HCK F2 probe (−44 to +3) in the presence or absence of 2 μg of a monoclonal anti-Sp1 antibody. Electrophoresis time was comparable to the experiment shown in panel B. P1a and P1b refer to the complexes formerly referred as P1.

P1. In contrast, complex P1 was effectively competed by a 15-fold molar excess of the Sp1 tetramer, while complex P2 was not fully competed even at a 125-fold molar excess of Sp1-binding sites (Fig. 5B), indicating a much lower affinity of the protein(s) forming complex P2 to a consensus Sp1 site than to the HCK sequence.

Sp1 Binds to the GC Boxes within the HCK Promoter—Sequence comparison and EMSA competition experiments suggested that both GC-rich elements within the HCK promoter are targets for the Sp1 family of transcription factors. To prove this, nuclear extracts prepared from U-937 or HeLa cells were incubated with the F1 DNA fragment in the presence of 2 μg of a specific Sp1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). In the presence of the antibody an additional complex with slower migrating properties was evident in the EMSA gel (denoted SS in Fig. 6A), with a simultaneous decrease in the intensity of the D1 complex. At higher resolution it was evident that the complex D1 consisted of two bands migrating closely together and that only the faster migrating band within complex D1 (D1b) was shifted by the anti-Sp1 antibody (Fig. 6B). The nature of the remaining complexes D1a, D3, and the HeLa specific complex D2 remains unknown. Similar observations were made when the oligonucleotide F2 was used as probe in the EMSA reaction (Fig. 6C).
The HCK Promoter Is Activated by Transcription Factors of the Sp1 Family in Drosophila Schneider Cells—To investigate directly the role of Sp1 in HCK promoter activity, the reporter plasmid p(-101)CAT, containing the HCK promoter fragment from nucleotide -101 to +36, was transfected together with the Sp1 expression vector pPac-Sp1 (38) in cells lacking endogenous Sp1 activity (38). As shown in Fig. 7A, the HCK promoter was activated almost 9-fold by saturating concentrations of Sp1. The degree of activation by Sp1 seemed to plateau at 5 μg of pPac-Sp1, whereas at higher concentrations of Sp1 a synergistic effect of Sp1 on HCK transactivation was observed (Fig. 7A). In contrast to Sp1, activation of the HCK promoter by the Sp1 related transcription factors Sp3 and Sp4 (39, 40) was minimal (2.9 ± 1.1-fold and 3.0 ± 1.1-fold activation, respectively; see Fig. 7B). Also, activation of the HCK promoter by Sp1 was dependent on intact GC boxes, since mutations of either the distal (M1) or proximal (M2) GC boxes reduced reporter gene expression by more than 50% (Fig. 7C). The reduction in reporter activity was statistically significant according to a Student’s t test (p < 0.02). In summary, our analysis suggests that Sp1 plays a critical role in the differential gene expression of the human HCK gene.

DISCUSSION

In this work, we showed that a 137-bp DNA fragment (-101 to +36) derived from the promoter proximal region of the human HCK gene was sufficient for transcriptional activation of a reporter gene in myeloid cells (RAW264), but was inactive in the non-myeloid cells HUT-78 or NIH3T3. Previously, Lichtenberg et al. (18) failed to demonstrate promoter activity of reporter gene constructs containing the first 662 bp of the HCK promoter region in human myeloid cell lines. Our results are in agreement with this, since we found that the HCK promoter region between nucleotides 422 and 813 markedly reduced promoter activity in myeloid cells to background levels.

In most myeloid-specific promoters, a short promoter region usually spanning the first 100 base pair 5'-flanking sequences confers cell-type specific expression in tissue culture (see Refs. 23 and 48 for review). Also, the lack of a TATA box and initiator sequences have been recognized as hallmark of genes specifically expressed in myeloid cells. Our results add the HCK gene to the list of myeloid promoters containing these characteristics and support the notion that most myeloid promoters underlay similar regulatory control mechanisms. In agreement with this, all myeloid promoters that share common regulatory features are also expressed, albeit at lower levels, in B-cells.

Within the first 100-bp 5'-flanking region of the HCK gene, two GC-rich elements (-58 to -51 and -16 to -7) were identified as being essential for promoter activity by site-directed mutagenesis and transactivation studies. Moreover, we identified Sp1 as one of the transcription factors binding to each of these boxes. The significance of these findings is reinforced by the fact that consensus sequences for potential Sp1-binding sites have been identified at virtually the same positions (centered at -40 and -19) in the murine HCK promoter (19). This conservation during evolution point toward their relevance in promoter function.

Both GC boxes were required for full activity of the (-101)CAT reporter gene construct in myeloid cells. Mutations in any one of the boxes reduced reporter gene expression significantly. The reduction in promoter activity caused by mutations in either of the GC boxes was quantitatively comparable to the reduction observed by deleting the 5'-flanking region up to position -36, thereby removing the distal GC box, or by deleting virtually the entire flanking sequence including both GC-rich elements, suggesting that the only regions involved in the activation of the -101 HCK promoter region in myeloid cells are the GC-rich regions. Besides the GC-rich elements, sequence comparison analysis of the -101 promoter region revealed the presence of a putative PU.1-binding site from position -27 to position -17 (AGGACGAGAAG). However, competition analysis with a PU.1 oligonucleotide derived from the SV40 early promoter region (AAAGAGGAAAC) did not provide any evidence for the binding of the myeloid-specific transcription factor PU.1 to the HCK proximal promoter region. These results are consistent with previous observation indicating that besides the central purine-rich region (consensus sequence GGAA) the adjacent nucleotides to the core region are also essential for PU.1 binding (22). The recent resolution of the x-ray crystallographic structure of PU.1 has confirmed these findings (41).
Beside the human and murine HCK genes, Sp1 sites have been identified in the promoter regions of other myeloid genes and there is growing evidence, that transcription factors of the Sp1 family either alone (42) or in cooperation with other transcription factors are involved in tissue-specific gene expression (43–46). For example, the tissue-specific expression of CD14, the receptor for bacterial lipopolysaccharides which is expressed only in monocytes and macrophages (47), is regulated exclusively by Sp1. Similar to the HCK promoter, the first 128 bp of the CD14 promoter region are sufficient for myeloid-specific expression of reporter genes (42). The only transcription factor binding to this region is Sp1. In several other genes expressed in myeloid cells, like CD11b and the colony stimulating factor receptor, promoter activity is regulated by Sp1 in addition to myeloid-specific transcription factors (reviewed in Refs. 23 and 48). An example of this is the CD11b promoter which is up-regulated in myeloid cell lines by PU.1 and Sp1 (22, 35). In this case however, the promoter retains significant myeloid specific activity after inactivation of the PU.1-binding site. The myeloid-specific gene expression is conferred by binding of Sp1 to a site located at position −60 in the CD11b promoter (22).

Regulation of differential gene expression by ubiquitous expressed transcription factors, like Sp1, can be achieved, among others, by modulation of the transcription factor expression levels or by differential post-translational modification of the factor. Sp1 expression increases during hematopoietic cell differentiation and the highest levels of Sp1 are found in differentiated hematopoietic cells (49). Also, binding of Sp1 to DNA allows considerable variations within the target sequence (34) and increased deviation from optimal binding sequence correlates with a higher dissociation constant of the DNA-protein complex (33, 34, 50) which in turn can determine promoter activity in a differentiation dependent manner (36, 51). Both GC-rich sequence elements within the HCK promoter diverge from the high affinity Sp1 binding consensus sequence (GGGCGG), and thus can be classified as sites with lower binding affinity. According to this, high levels of Sp1 may be required for HCK gene expression. This hypothesis is supported by the titration experiments in Drosophila Schneider cells, which suggest that once both GC boxes are occupied by Sp1, protein-protein interaction between the bound Sp1 molecules and additional factors (or Sp1 itself) cause promoter superactivation. Synergistic effects on transcription through Sp1 binding to adjacent sites have been described previously (39, 52) and is caused by an increase in the number of activation domains over a defined promoter region facilitating therefore the recruitment of factors required by the transcription machinery. In this regard it is of interest to notice that both Sp1-binding sites are on the same face of the DNA separated only by four helix turns.

Beside expression levels, the binding and transactivation properties of Sp1 can also be modulated by post-translational modification, i.e. glycosylation (53) and phosphorylation (54). During terminal differentiation of liver cells, the DNA binding activity of Sp1 decreases as a result of phosphorylation by casein kinase II (55, 56). In contrast, activation of protein kinase A in doxorubicin-resistant HL-60 cells leads to increased Sp1 phosphorylation and as a consequence to increased DNA binding activity and transactivation properties (57). Similar studies have not been done for the hematopoietic system yet, but higher phosphorylation levels of Sp1 in myeloid cells compared with epithelial cells have been reported (42).

In addition, the activity of Sp1 can be regulated by interactions with regulatory proteins (58–60) or by competition between Sp1 and other transcription factors for the same binding site (40, 61, 62). The latter mechanism may be a crucial event in the regulation of HCK expression, since multiple DNA-protein complexes were detected in EMSA with oligonucleotides F1, F2, and F3. In particular, the shifting experiments performed with HeLa cell extracts showed the presence of an additional protein-DNA complex (D2/P2) not found in extracts of HCK expressing cells. Although the nature of this factor is not known, the affinity of the factor for the proximal GC box (complex P2) was clearly higher than that of the factor(s) involved in complex 1. This differential affinity was confirmed by competition experiments under nonsaturating conditions. Complex P2 was competed 15-fold more efficiently than complex P1 when excess unlabeled F2 fragment was used as competitor, while complex P1 was preferentially competed when an Sp1 tetramer oligonucleotide was used as competitor.

This data suggests that the proximal GC-rich region is preferentially occupied by the factor(s) involved in complex P2, leading to the hypothesis that these factor(s) repress HCK promoter activity in non-myeloid cells. However, reporter gene constructs containing mutations in the proximal GC box were inactive in NIH3T3 cells probably due to the failure of transactivators to bind to the mutated GC box. Indeed, mutations within the GC-rich box in oligonucleotide F2 were unable to compete for DNA binding activity. These results suggest a biphasic model for HCK gene regulation. In non-myeloid cells, a factor (repressor?) binds with high affinity to the proximal GC box thereby impairing binding of Sp1 or Sp1-related factors to this site. In myeloid cells, the absence of the negative regulator is not sufficient to activate the HCK promoter. The increase in Sp1 activity during myeloid differentiation gradually activates the HCK promoter until full activity is achieved in terminally differentiated cells as a consequence of high Sp1 levels. Further experiments will certainly be required to prove or disprove this model.

The utility of the −101 HCK promoter region to direct expression exclusively to myeloid cells in vivo remains to be established. Previous studies in transgenic mice have shown that, in addition to tissue-specific transcription factors, other DNA elements, like silencers, matrix attachment regions, or locus control regions, are essential for tissue-specific expression in vivo (22, 63–67). From these studies we would predict that additional regions of the HCK promoter will be required for appropriate expression in vivo. Indeed our analysis showed that the promoter region from position −3602 to −4954 supports high levels of gene expression in myeloid cells. Within these regions putative PU.1-binding sites have been recognized (AAAGAGGTGACAA and AGATGAGGAAA) by sequence analysis homology. Additional studies will be required to solve this issue.

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