THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 48, Issue of November 27, pp. 31844–31852, 1998
Printed in U.S.A.

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*

(Received for publication, May 21, 1998, and in revised form, September 21, 1998)

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The human hemopoietic 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-monocytic 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 human hematopoietic cell kinase (HCK)[1] belongs to a family of cytoplasmic protein tyrosine kinases for which c-*src* serves as an archetype (1–4). All members of the family share common structural features, including a catalytic domain in their COOH-terminal half, two regulatory domains known as SH2 and SH3 domains, an N-terminal domain unique to each of the family members and a myristylation site at the amino terminus (5–7).

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 lipopolysaccaride (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.

* This work was supported by a grant from the Deutsche Forschungsgemeinschaft (to M. G.). The Georg-Speyer-Haus is supported by the Bundesministerium für Gesundheit and the Hessisches Ministerium für Wissenschaft und Kunst. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] 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 λFIX II™ (Stratagene) was screened with an oligonucleotide (CGCTCAAGCTGCGAGGATCCGGGCTGCCCGCGAGACG) 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 *Not*I and subjected to partial digestion with restriction enzymes. Restriction fragments were identified by Southern blotting using either T3 or T7 ^{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 *Xba*I digest of bacteriophage λ*HCK*3 into the *Xba*I 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 (−58 to −51), a 126-bp DNA fragment was amplified from plasmid p(−101)CAT with the primers *HCK*M1 (CAGCTCGGGAGCACATCAGAGGCTTAGACTAGAGTGGGAAGG) and *HCK*CATu (CAGATCCTCTAGAACTAGAGGCG). The polymerase chain reaction product was cloned into the pGEM-T vector (Promega) and sequenced. An *Ava*I/*Xba*I DNA fragment derived from this construct was isolated and used to replace a similar DNA fragment in p(−101)CAT, thereby generating the *HCK* reporter gene mutant p(−101) M1 CAT.

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 *HCK*M2 (CTTTCTAGAACTAGAGGCGGGGGCGACGCTTAGAGGGGCTTTGGTTAATGCATTATTACTCGTGGTGCTGTCTGAG) and *HCK* 243d (GTCCCAGTCCGGGAGCACATCAG), cloned into pGEM-T vector and sequenced. The *HCK* mutant reporter gene construct p(−101) M2 CAT was generated as described before for p(−101) 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 (28) and HUT-78 (29) were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10 or 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^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^2 flask. U-937 and HUT-78 were transfected by electroporation. 4×10^6 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, 250 V).

Transiently transfected cultures were harvested 48 h after transfection and lysed by three freeze-thaw cycles. β-Galactosidase activity in each mammalian cell extract was used to standardize CAT values to compensate for variability in transfection efficiency between samples and measured by an adaption of established methods (24) for the use in a microplate reader. In brief, 100 μl of reaction mixture containing 10 μl of extract, 100 mM sodium phosphate, pH 7.5, 1 mM $MgCl_2$, 45 mM β-mercaptoethanol, 13,3 mM *o*-nitrophenole-β-D-galactopyranoside was incubated 30 min at 37 °C in a 96-well plate. The reaction was stopped by addition of 200 μl of 1 M Na_2CO_3 and absorption was measured at 420 nm in a microplate reader (SpectraMAX 340, Molecular Devices). CAT assays were performed as described by Seed and Sheen (31). Protein concentrations in the assay were adjusted according to transfection efficiency.

Preparation of Nuclear Extracts—Nuclear extracts were prepared essentially as described by Gorski *et al.* (32). Briefly, 5×10^9 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 M sucrose, 10% glycerol) supplemented with protease inhibitors (1 μg/ml antipain, 4 μg/ml aprotinin, 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 layerd 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 nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) supplemented with protease inhibitors, homogenized, and if necessary diluted to 10 A_{260} units/ml. Nuclear proteins were extracted by adding 0.1 volume of 4 M $(NH_4)_2SO_4$ slowly over a 30-min period. The suspension was cleared by centrifugation (123,000 × *g*, 0 °C, 60 min). Proteins were precipitated by addition of 0.3 g of $(NH_4)_2SO_4$/ml and subsequent centrifugation at 123,000 × *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 dithiothreitol, 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 $MgCl_2$, 4 mM spermidine, 2 mM dithiothreitol, 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 ^{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 λFix II™ 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 phages (λ*HCK*3, λ*HCK*5) 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 λ*HCK*3 by partial *Xba*I restriction enzyme digestion and cloned into the *Xba*I restriction site in plasmid pCAT-Basic to generate p(−4954)CAT. 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. 1*A*).

The reporter gene constructs were transfected into the HCK positive murine monocytoid 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 8.6-fold (Fig. 1*B*). Constructs containing promoter 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

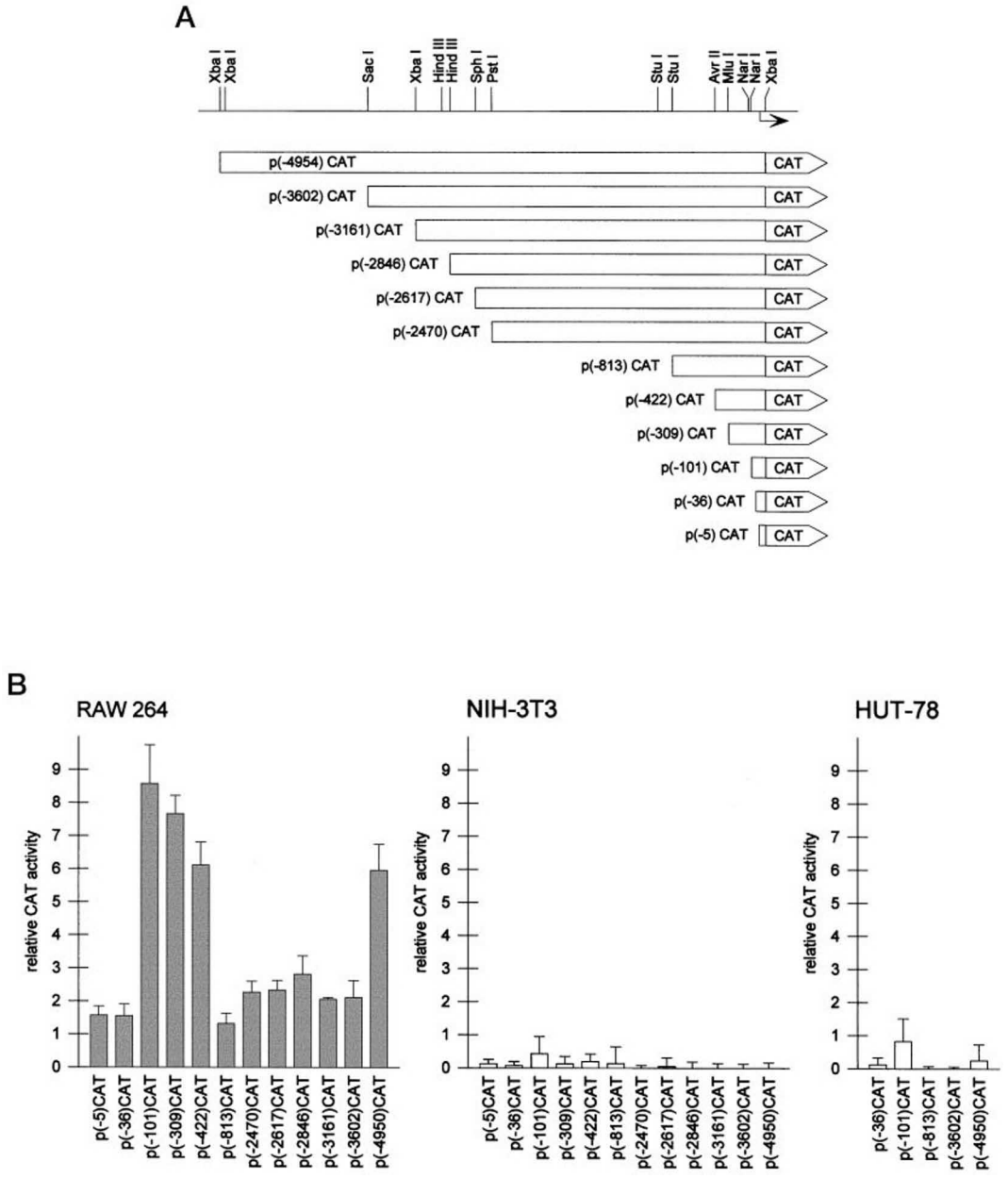

FIG. 1. **Functional analysis of *HCK* reporter gene constructs.** *A,* the human *HCK* promoter proximal region is shown at the *top*. Genomic DNA is represented by a *thin line*, the transcriptional start site is marked by an *arrow*. Restriction sites used for cloning purposes are shown. A schematic representation of CAT reporter plasmids is shown *below*. Denomination of the plasmids corresponds to the most 5′ nucleotide included in the promoter fragment. All plasmids share a common 3′-end at nucleotide +36. *B,* functional characterization of the *HCK* promotor region. A series of 5′-deletions in the *HCK* promoter region were linked to the CAT reporter gene and transiently transfected into HCK positive RAW264 cells, or into the non-myeloid HUT-78 or NIH-3T3 cells together with 5 μg of RSV-LacZ. CAT activity in cell extracts was normalized to β-galactosidase levels and is shown as the CAT activity of the deletion mutants relative to that of the promotorless pCAT-Basic vector. The values represent mean and standard deviation from at least six independent transfections with two different DNA preparations.

−4954. None of these constructs showed any significant activity in the non-myeloid cell lines HUT-78 and NIH3T3 (Fig. 1*B*). 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. 2*A*). The distal sequence GAGGCGAG between base pairs −58 and −51 and the proximal sequence CGCCCGCGGC between base pairs −16 and −7 both fits 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. 2*A*).

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. 2*B*, mutations in either of the GC boxes severely reduced promoter

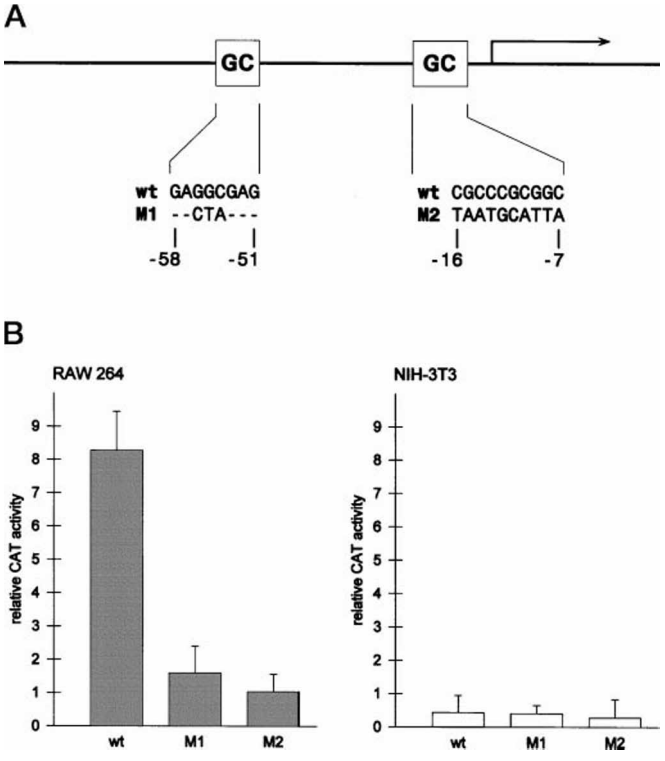

FIG. 2. **Point mutations within the GC-rich elements significantly reduces *HCK* promoter activity.** *A,* schematic representation of the promoter proximal region of the *HCK* gene. GC-rich elements are represented by *boxes*, the site of transcription initiation is marked by an arrow. The sequence of the GC-rich regions (*wt*) and mutants (*M1* and *M2*) are shown. Identical nucleotides are marked by *dashes*. *B,* CAT activity of *HCK* mutants. CAT constructs containing mutations within the distal (*M1*) or proximal (*M2*) GC box were transiently transfected in RAW264 and NIH3T3 cells. CAT activity was normalized for transfection efficiency with a co-transfected LacZ expression plasmid. The values represent mean and standard deviation of at least five independent transfections, and are presented as the CAT activity of wt or mutants relative to that of the promoterless pCAT-Basic vector.

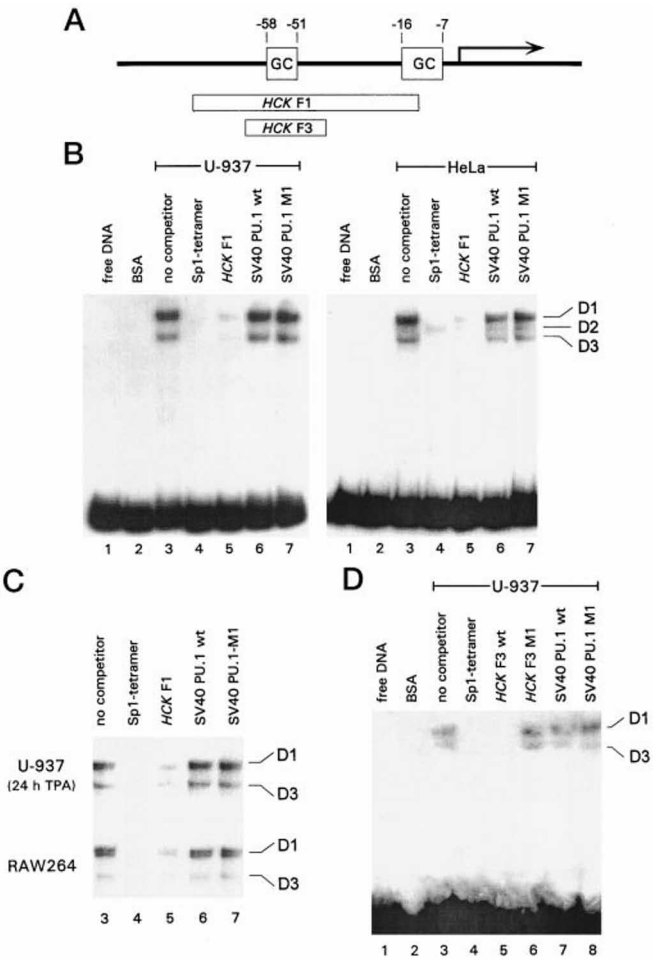

FIG. 3. **Binding of nuclear factors to the distal GC box of the human *HCK* promoter.** A, schematic representation of the DNA fragments *HCK* F1 (−77 to −10) and *HCK* F3 (−66 to −44) used in EMSA. The *HCK* promoter region is drawn as a *line*, the two GC-rich elements are marked by *boxes*. The transcriptional start site is indicated by an *arrow*. The probe fragments are shown as *bars below*. *B,* binding of nuclear factors to the *HCK* F1 DNA fragment. A ^{32}P-labeled F1 DNA fragment (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 either from U-937 (*left*) or HeLa (*right*) cells (*lanes 3–7*). The following unlabeled double stranded competitor oligos were added at 100-fold molar excess: *lane 4*, Sp1 tetramer, a tetramerized binding site for the transcription factor Sp1 (36); *lane 5*, *HCK* F1; *lane 6*, SV40 PU.1 wt, an oligonucleotide containing the binding site for the myeloid transcription factor PU.1 derived from simian virus 40 (37); *lane 7*, SV40 PU.1 M1, mutant of the SV40 PU.1 sequence incapable of transcription factor binding (68). *D1* and *D3* refer to the DNA-protein complexes formed with nuclear extracts from U-937 and HeLa cells, *D2* refers to the HeLa cells specific complex. *C,* binding of nuclear factors obtained from 12-*O*-decanoylphorbol-13-acetate-induced U-937 cells (*upper panel*) or RAW 264 cells (*lower panel*) to the *HCK* F1 DNA fragment. For lanes description see *B*. In *D*, a ^{32}P-labeled *HCK* F3 DNA fragment (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 cells (*lanes 3–8*). The following unlabeled double stranded competitor oligos were added at 100-fold molar excess: *lane 4*, Sp1 tetramer; *lane 5*, *HCK* F3 wt; *lane 6*, *HCK* F3 mutant M1; *lane 7*, SV40 PU.1 oligonucleotide; *lane 8*, SV40 PU.1 M1 mutant. *D1* and *D3* refer to the DNA-protein complexes formed with nuclear extracts from U-937 cells.

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. 2*B*).

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. 3*A*) 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. 3*B*). 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 unlabeled F1 fragment (Fig. 3*B*), 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. 3*C*).

To delimit the promoter region involved in protein-DNA interaction more precisely, a 22-bp oligonucleotide (F3) centered at the distal GC box (positions −66 to −44) was synthesized and used as a probe in EMSA experiments. Similarly, an oli-

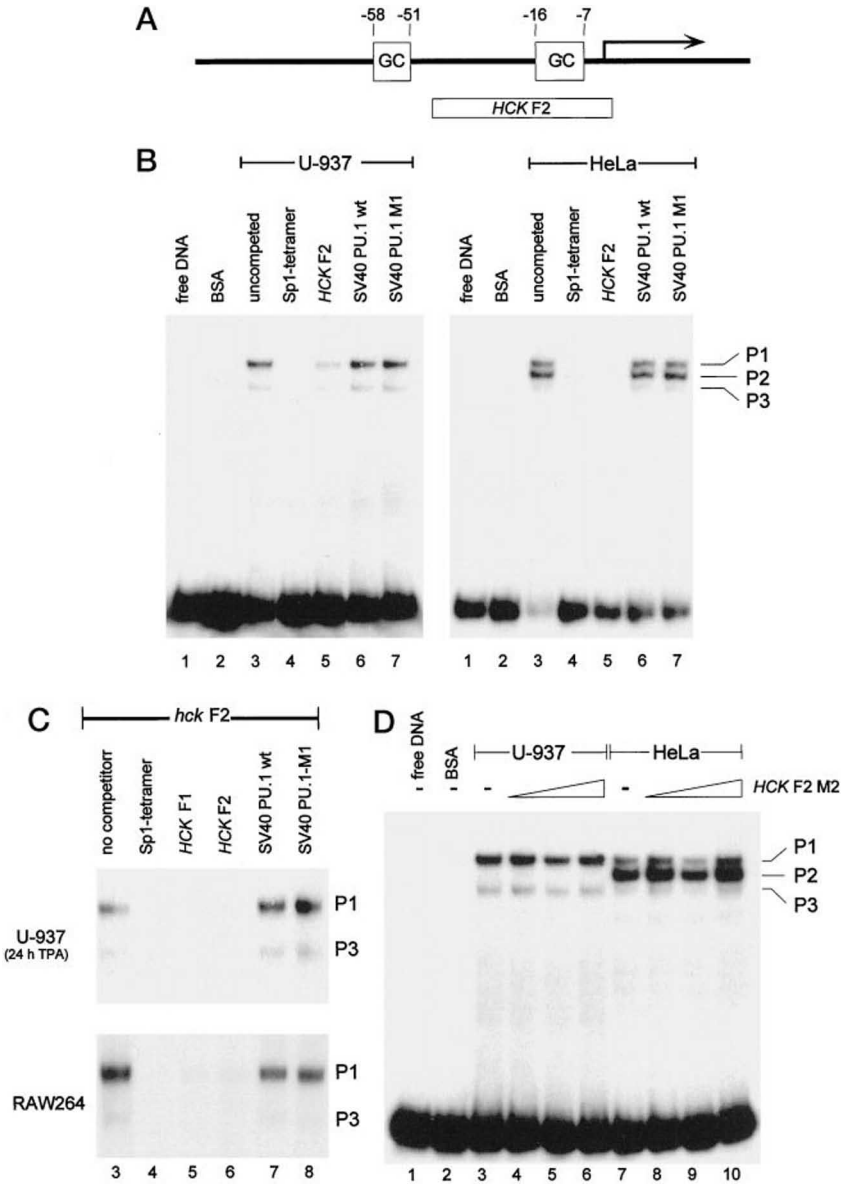

FIG. 4. **Binding of nuclear factors to the proximal GC box of the human *HCK* promoter.** *A,* schematic representation of the *HCK* F2 oligonucleotide (−44 to +3) used for EMSA. The *HCK* promoter region is drawn as a *line*, GC-rich elements are marked by *boxes*. The transcriptional start point is 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 ^{32}P-labeled F2 oligonucloetide (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 ^{32}P-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.

gonucleotide containing a disrupted distal box (GACTAGAG; F3-M1) was used as a control. Complexes D1 and D3 were clearly visible when F3 was incubated with nuclear extracts derived from U937 cells (Fig. 3*D*). The complex was competed with a 100-fold molar excess of the Sp1 tetramer or with a 100-fold excess of the unlabeled probe fragment, but not by a 100-fold molar excess of F3-M1, clearly showing that the mutated nucleotides are essential for binding. Similarly to the observations made with the F1 fragment, no competition was observed with an excess of oligonucleotides containing either a wild type or a mutated PU.1-binding site.

Three protein-DNA complexes were observed with nuclear extracts derived from HeLa cells (Fig. 3*B*). Two of them, D1 and D3, showed identical migration characteristics as the bands previously described. A novel HeLa specific band, designated D2, appeared between complexes D1 and D3. Addition of a 100-fold molar excess of a Sp1 tetramer competes all three bands, albeit with different efficiencies. While complexes D1 and D3 were competed with equal efficiencies, complex D2 was still visible in the presence of 100-fold molar excess of Sp1 tetramer (Fig. 3*B*). All complexes were equally competed with a 100-fold molar excess of unlabeled F1-fragment, while no competition was observed with SV40 PU.1-wt or SV40 PU.1-M1 derived oligonucleotides.

Protein binding to the proximal GC-rich element within the *HCK* promoter was analyzed in similar experiments. A fragment from −44 to +3 (*HCK* F2), centered at the GC box proximal to the transcriptional start site (Fig. 4*A*), was used as probe for EMSA. When extracts from U-937 cells were used, two protein-DNA complexes were detected (P1 and P3 in Fig. 4*B*). Binding to the F2 fragment was competed with an 100-fold molar excess of the tetramerized Sp1-binding site as well as with an 100-fold excess of the unlabeled probe. No competition was observed with the SV40-PU.1 wt and SV40-PU.1 M1 oligonucleotides. Similarly binding analysis of HeLa nuclear extracts with fragment *HCK* F2 as a probe revealed again two bands (P1 and P3) displaying identical migration characteristics as the complexes obtained with the U-937 extracts. However, an additional HeLa specific DNA-protein complex (P2) appeared between P1 and P3. Again protein binding to the labeled DNA was competed by Sp1-binding sites and by the unlabeled probe at 100-fold molar excess, but not by an 100-fold excess of unrelated oligonucleotides. Similar results were obtained with nuclear extracts prepared from U-937 cells induced

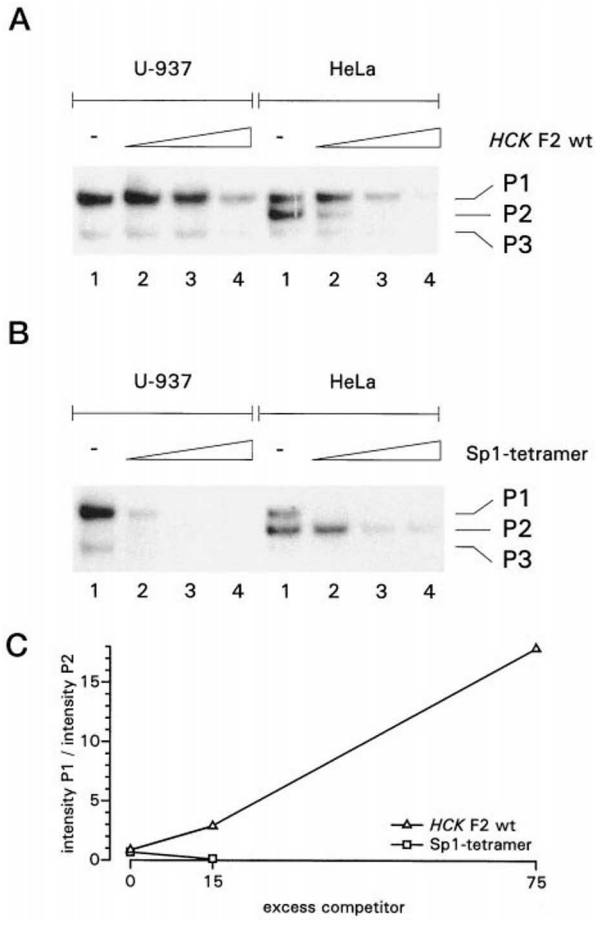

FIG. 5. **Nuclear factors compete for binding to the proximal GC box.** A ^{32}P-labeled F2 oligonucleotide was used as a probe in EMSA experiments with nuclear extracts of U-937 or HeLa cells as indicated *above* the panels. *A,* EMSA reactions containing 1.25 fmol of ^{32}P-labeled probe and 10 μg 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.

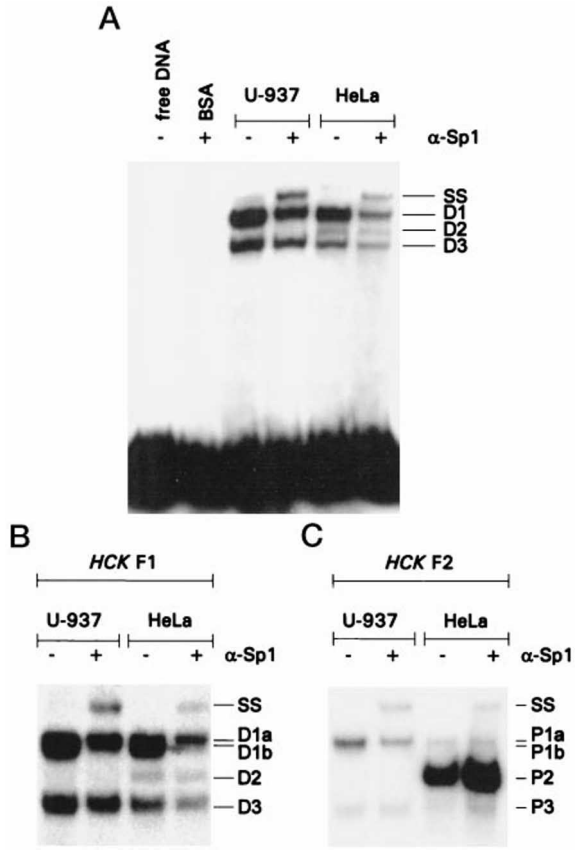

FIG. 6. **Identification of Sp1 as an *HCK* promoter binding protein.** *A,* 1.25 fmol of ^{32}P-labeled *HCK* F1 probe was incubated with 10 μg of nuclear extracts of U-937 or HeLa cells in the presence or absence of 2 μg of a monoclonal anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as 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 ^{32}P-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.

with 160 nM 12-*O*-decanoylphorbol-13-acetate for 24 h and from RAW264 cells (Fig. 4*C*). 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 ^{32}P-labeled *HCK* F2 oligonucleotide was incubated with either U-937 or HeLa nuclear extracts in the presence of increasing molar amounts of the *HCK* F2-wt oligonucleotide (Fig. 5*A*) or the Sp1 tetramer (Fig. 5*B*). While complex P2 was efficiently competed with a 75-fold molar excess of unlabeled F2-wt oligonucleotide, complex P1 was not fully competed even at a 125-fold molar excess of F2-wt (Fig. 5*A*). A densitometic analysis of the EMSA bands showed that the ratio between the intensities of bands P1 and P3 remained constant, while the ratio P1/P2 increased constantly (Fig. 5*C*), suggesting that the factor(s) involved in complex P2 have higher binding affinity for the F2 oligonucleotide than the factor(s) involved in complex 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. 5*B*), 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. 6*A*), 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. 6*B*). 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. 6*C*).

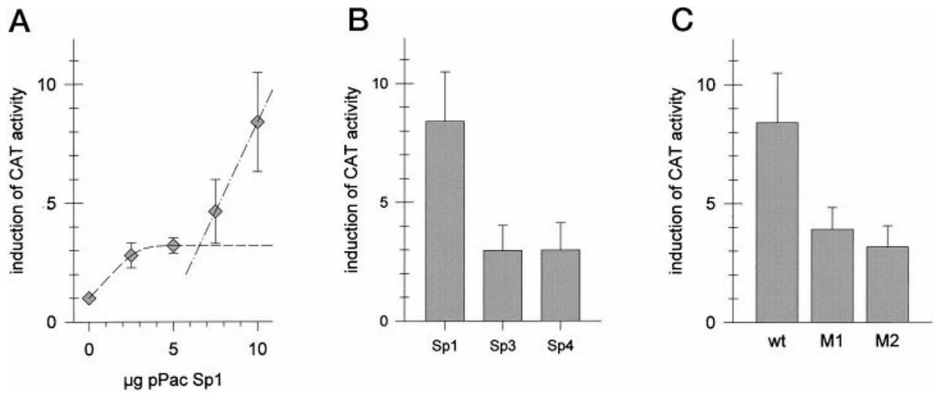

FIG. 7. **The *HCK* promotor is activated by Sp1 in *Drosophila* Schneider cells.** *A,* the *HCK* reporter gene construct p(−101)CAT was transfected in *Drosophila* Schneider cells together with increasing amounts of the Sp1 expression plasmid pPacSp1. 48 h after transfection cells were harvested and CAT activity was determined in samples containing equal amount of protein. CAT activity is shown in relationship to the activity of reporter CAT plasmid in absence of coexpressed Sp1. Shown are mean values of at least five independent experiments. Standard deviation of the mean is indicated by the error bars. *B,* the reporter plasmid p(−101)CAT was co-transfected with 10 μg of pPac-Sp1, pPac-Sp3, or pPac-Sp4. CAT assays were normalized for protein content and plotted in relation to the activity measured without coexpressed transcription factors. The values are mean of at least five independent transfections. *Error bars* indicating standard deviation. *C,* mutations within the distal or the proximal GC boxes reduced Sp1-mediated transactivation of a reporter gene construct. 10 μg of the wild type reporter gene construct p(−101)CAT or constructs containing a mutated distal (p(−101) M1 CAT) or proximal (p(−101) M2 CAT) GC-rich element were transfected together with 10 μg of pPac-Sp1 into *Drosophila* Schneider cells (SL2). CAT activity in the cell extract was measured 48 h after transfection and normalized against protein content. Induction of CAT activity denotes the CAT activity of the wild type or mutant *HCK* reporter constructs in the presence of co-transfected Sp1 *versus* the CAT activity obtained in the absence of coexpressed Sp1. Depicted are mean and standard deviation of at least four independent transfections.

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. 7*A*, 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. 7*A*). 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. 7*B*). 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. 7*C*). 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 promotor 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 promotor activity of reporter gene constructs containing the first 662 bp of the the *HCK* promotor 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 (AGGACGAGAAA). However, competition analysis with a PU.1 oligonucleotide derived from the SV40 early promoter region (AAAGAGGAAC) 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 lipopolysaccarides 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 upregulated 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* promotor 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.

Acknowledgments—We thank Renate Siebenhaar for the isolation of the genomic clones. We are extremely grateful to Guntram Suske (University of Marburg) for generously providing SL2 cells and expression plasmids for transcription factors of the Sp1 family.

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