# First mutation in the red blood cell-specific promoter of hexokinase combined with a novel missense mutation causes hexokinase deficiency and mild chronic hemolysis

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## ABSTRACT

### Background

Hexokinase is one of the key enzymes of glycolysis and catalyzes the phosphorylation of glucose to glucose-6-phosphate. Red blood cell-specific hexokinase is transcribed from *HK1* by use of an erythroid-specific promoter. The aim of this study was to investigate the molecular basis for hexokinase deficiency in a patient with chronic hemolysis.

### Design and Methods

Functional studies were performed using transient transfection of *HK* promoter constructs in human K562 erythroleukemia cells. The DNA-protein interaction at the promoter of hexokinase was studied using electrophoretic mobility shift assays with nuclear extracts from K562 cells. DNA analysis and reverse transcriptase polymerase chain reaction were performed according to standardized procedures.

### Results

On the paternal allele we identified two novel mutations in *cis* in the erythroid-specific promoter of *HKI*: –373A>C and –193A>G. Transfection of promoter reporter constructs showed that the –193A>G mutation reduced promoter activity to 8%. Hence, –193A>G is the first mutation reported to affect red blood cell-specific hexokinase specific transcription. By electrophoretic mobility shift assays we showed that *in vitro* binding of c-jun to an AP-1 binding site was disrupted by this mutation. Subsequent chromatin-immunoprecipitation assays demonstrated that c-jun binds this region of the promoter *in vivo*. On the maternal allele we identified a novel missense mutation in exon 3: c.278G>A, encoding an arginine to glutamine substitution at residue 93, affecting both hexokinase-1 and red cell specific-hexokinase. In addition, this missense mutation was shown to compromise normal pre-mRNA processing.

### Conclusions

We postulate that reduced erythroid transcription of *HK1* together with aberrant splicing of both hexokinase-1 and red cell specific-hexokinase results in hexokinase deficiency and mild chronic hemolysis.

Key words: hexokinase, erythroid-specific transcriptional regulation, promoter mutation, hemolysis, AP-1.

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## Introduction

Hexokinase (HK; EC 2.7.1.1) is one of the rate-limiting enzymes of glycolysis and catalyzes the phosphorylation of glucose to glucose-6-phosphate.[1] HK-1 is the predominant isozyme in tissues strongly dependent on glucose for their physiological functions, including brain, kidney, erythrocytes, and platelets. Red blood cell hexokinase (HK-R) is transcribed from the same gene as HK-1 and is mainly present in erythroblasts, reticulocytes and young erythrocytes. Its half-life of 10 days is shorter than that of HK-1 (66 days). HK-1 replaces HK-R as the erythrocyte matures[2] and, as a result, mature erythrocytes contain only 2-3% of the HK activity of reticulocytes.[3]

*HK1* (Genbank RefSeq NM_000188), the gene encoding HK-1 and HK-R, is located at chromosome 10q22, has 25 exons, including an erythroid-specific exon (exon R), and spans more than 130 kb.[4-8] HK-R expression is driven by an erythroid-specific promoter.[9]

HK deficiency is very rare and associated with hereditary non-spherocytic hemolytic anemia (HNSHA). It is caused by mutations in *HK1* and approximately 20 cases of HNSHA due to HK-1 deficiency have been described to date.[9] Bianchi *et al.*[10] were the first to demonstrate the molecular defect underlying HK deficiency in a patient with hemolytic anemia. This so-called HK-Melzo variant was due to compound heterozygosity for a 95 bp deletion and a c.1667T>C missense substitution causing a p.Leu529Ser amino acid change. Kanno *et al.*[11] described a homozygous 9490-bp intragenic deletion variant and, more recently, van Wijk *et al.*[12] reported a homozygous missense mutation in exon 15 of HK1 (c.2039C>G, HK Utrecht), in a patient previously diagnosed with HK deficiency. To our knowledge, these three patients are the only ones in which the molecular basis has been studied. In all three cases mutations were located in that part of the gene that encodes both HK-R and HK-1.

The erythroid-specific promoter of *HK1*, like other erythroid-specific genes, does not contain a TATA box.[4,13] Functional analysis of the human erythroid-specific promoter of *HK1* has indicated that nts –275 to –229 are critical for erythroid-specific expression of HK-R. Consensus binding motifs for transcription factors Sp-1 and GATA, as well as CCAAT and GGAA sequence motifs are considered to be responsible for this.[4] Recently, PKR-RE1, the CTGTC sequence motif located at position –261 to –257, was shown to be involved in erythroid-specific transcriptional regulation of *HK1*.[14]

In this study we investigated the molecular basis for HK deficiency in a patient with mild chronic hemolysis. We describe the identification and characterization of the first mutation in the erythroid-specific promoter of HK-1 and a missense mutation that causes aberrant splicing of both HK-1 and HK-R mRNA.

## Design and Methods

### Patient

The patient is a 33-year-old Dutch man, who visited the hematology outpatient clinic for evaluation of chronic hemolysis. He was suffering from jaundice, fatigue and inability to concentrate. Laboratory analysis confirmed mild chronic hemolysis (Table 1). An investigation of the patient's family was undertaken. All family members were free of any signs of chronic hemolysis or other clinical symptoms. Informed consent was obtained from all participants.

### Hemocytometry analysis and determination of glycolytic enzyme activity

Hemoglobin concentration, red cell indices and erythrocytes were measured using an automated cell counter (Cell-Dyn 4000, Abbott Diagnostics, Santa Clara, CA, USA). HK activity, glucose-6-phosphate dehydrogenase (G6PD) activity and activity of the red blood cell age-related enzyme pyruvate kinase (PK) were determined according to standardized procedures.[15]

### Polymerase-chain reaction amplification and DNA sequence analysis

DNA was extracted from peripheral blood samples collected into EDTA tubes according to standard protocols. The erythroid-specific promoter, the red blood cell-specific exon 1 and exons 2 to 18 of *HK1* were amplified using primers and thermal cycler conditions described previously.[12] Polymerase chain reaction (PCR)

**Table 1.** Laboratory results of the patient and his parents.

| | Patient | Mother | Father | Reference ranges | |
|---|---|---|---|---|---|
| | | | | Male | Female |
| Hemoglobin (g/dL) | 8.8 | 8.9 | 10.1 | 8.6-10.7 | 7.4-9.6 |
| Erythrocytes ($\times10^{12}$/L) | 4.3 | 4.3 | 5.6 | 4.2-5.5 | 3.7-5.0 |
| Mean corpuscular volume (fL) | 95 | 97 | 87 | 80-97 | |
| Reticulocytes ($\times10^{9}$/L) | 79 | 70 | 74 | 25-120 | |
| Immature reticulocyte fraction | 0.57 | 0.29 | 0.22 | 0.1-0.3 | |
| | | | | Reference ranges | |
| Hexokinase (U/g Hb) | 0.79 | 0.90 | 0.83 | 1.02-1.58 | |
| Pyruvate kinase (U/g Hb) | 7.2 | 9.5 | 9.0 | 6.9-14.5 | |
| G6PD (U/g Hb) | 9.9 | 10.3 | 10.4 | 7.1-11.5 | |
| Total bilirubin (µmol/L) | 73 | 5 | 7 | 3-21 | |
| Unconjugated bilirubin (µmol/L) | 1 | | | 0-5 | |
| Lactate dehydrogenase (U/L) | 561 | | | 300-620 | |
| Aspartate aminotransferase (U/L) | 46 | | | 15-45 | |
| Ferritin (µg/L) | 438 | | | 25-280 | |
| Haptoglobin (g/L) | <0.1 | 1.0 | 1.2 | 0.3-2.0 | |

*G6PD: Glucose-6-phosphate dehydrogenase.*

products were sequenced using a Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Sequencing reactions were all carried out in forward and reverse directions. Samples were analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems). Restriction enzyme digestion with Hpy188I, BspHI, and PstI was used for a *HK1* population survey on the –373A>C, –193A>G and c.278G>A mutations, respectively, in 54 normal Caucasian control subjects.

### Promoter constructs

The wild-type *HK1* promoter reporter construct containing 562 nts of the immediate 5' upstream region (pGL3-HKWT) was produced as described.[14] The double mutant *HK1* promoter construct, containing the –373A>C and –193A>G substitution (pGL3-HK373C/193G), was amplified from the patient's DNA. Two single-mutant promoter reporter constructs were obtained by site-directed mutagenesis using double-stranded plasmid DNA templates.[16] The –373A>C mutant promoter reporter vector was produced from the pGL3-HK373C/193G promoter construct by use of primer HK-193G>A sense: 5'-CAGTTAGGCAGTCATGACTCAGTGTTACTTATC-3' (nts –209 to –177) and its complementary primer HK-193G>A antisense (pGL3-HK373C). The –193A>G mutant promoter reporter vector was produced from the pGL3-HK373C/193G promoter construct by use of primer HK-373C>A sense: 5'- CCTTAGCAGGGATCTAAGAGCTATGCAAGAGC-3' (nts –388 to –357) and its complementary primer HK-373C>A antisense (pGL3-HK193G). All plasmids were verified by DNA sequence analysis.

### Cell culture and DNA transfections

Human erythroleukemic K562 cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin, and 100 μg streptomycin (Invitrogen) per mL. Cells were grown at 37°C in a humidified atmosphere containing 5% $CO_2$. K562 cells were transiently transfected using Superfect (Qiagen, Valencia, CA, USA), as previously described.[14] Forty-eight hours after transfection, luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA), using a Veritas™ Microplate Luminometer (Promega). Firefly luciferase activities were corrected for transfection efficiency by using Renilla luciferase activity measurements. The corrected activity (Firefly luciferase divided by Renilla luciferase activity) was compared to the pGL3-HKWT corrected promoter activity (results expressed as percentages). The pGL3-control vector and the promoterless pGL3-Basic Luciferase Reporter Vector (Promega) were used as positive and negative controls, respectively.

### Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) with K562 nuclear extract was performed as described previously[14] using the following wild-type and mutant oligonucleotide probes (sense, mutation in bold): HKWTs, 5'-GGCAGTCATGACTCAGTGTTA -3'; HK193G, 5'- GGCAGTCATG**G**CTCAGTGTTA -3'. Pre-incubation for 10 min with 1 μg poly(dI-dC) (Roche Diagnostics, Mannheim, Germany) prevented non-specific binding. For supershift assay, 1 μg of anti-NF-E2 p18 (sc-16276X), 1 μg of anti-c-Jun H-79 (sc-1694X) or 1 μg of anti-c-Fos 4 (sc-52X) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was incubated with nuclear extract for 30 min at room temperature prior to addition of the probe.

### Chromatin immunoprecipitation assay

To investigate *in vivo* binding of c-jun to the erythroid-specific promoter of *HK1* we performed chromatin immunoprecipitation (ChIP) assays, using the ChIP-IT™ Express Enzymatic kit (Active Motif, Carlsbad, CA, USA) and the ChIP-validated antibody c-jun pAB (39309, Active Motif), as described.[17] Briefly, 7.5 μL of immunoprecipitated DNA were PCR-amplified using primers flanking the AP-1 binding site: primers HK1 ChIP forward 5'-AACAGAGAGGGGACACAGCA-3' and HK1 ChIP reverse 5'-TGGTAGAGTTTTGAGCCAGG-3'. The length of the amplified HK1 PCR product was 227 nts.

### In vitro *production of human (pro)erythroblasts and reverse transcription-polymerase chain reaction*

Nucleated erythroid cells from the patient and a healthy control subject were produced using $CD34^+$ cells from peripheral blood as described elsewhere.[18] Total RNA was isolated from these nucleated erythroid cells using RNABee reagent (Campro Scientific, Veenendaal, The Netherlands), according to the manufacturer's instructions. Reverse transcription (RT)-PCR was performed using the GeneAmp RNA PCR Core Kit (Roche, Branchburg, NJ, USA). Briefly, 1 μg total RNA was reverse transcribed using random hexamers as primers. A region encompassing exons 1-4 was amplified using 30 pmol of primers HK-C1F: 5'-AGCACAGCCTGAGTTTGCC-3' (erythroid-specific exon 1, nts +10-+29) and HK-C4R: 5'-AATCCCACAGGTAACTTCTTGTCC-3' (exon 4, c.429_452). Samples were heated for 5 min at 95°C and subjected to 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min), followed by an elongation step of 10 min after the last cycle. A control without RNA and one in which the reverse transcription step was omitted were included. Sequence analysis was performed according to standard procedures using primers HK-C1F and HK-C4R.

## Results

### Routine laboratory analysis and glycolytic enzyme activities

Routine laboratory analysis of the patient's blood showed elevated total bilirubin with normal unconjugated bilirubin levels, undetectable haptoglobin, and elevated ferritin levels (Table 1). Furthermore, the

patient's spleen was enlarged, suggesting a condition of chronic hemolysis. The TA-repeat polymorphism in the *UGT1A1* gene (Gilbert's syndrome) and *HFE* gene mutations, associated with hereditary hemochromatosis, were absent. Red cell osmotic resistance was normal, suggesting absence of red cell membrane disorders. However, determination of red blood cell enzyme activities showed normal activities for G6PD and PK whereas HK activity was decreased (Table 1). These results were indicative of HK deficiency. Other causes of chronic hemolysis (paroxysmal nocturnal hemoglobinuria, thalassemias and hemoglobinopathies) were excluded. Subsequently, red cell enzyme activity measurements were extended to other family members (Figure 1).

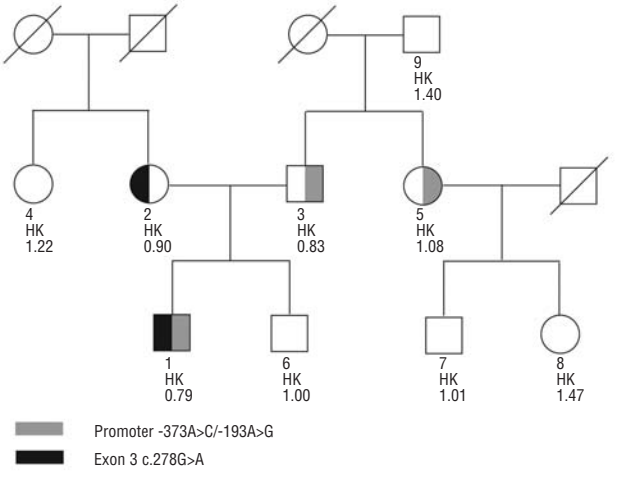


**Figure 1.** Pedigree of the family described in this study. ***HK1*** mutations and HK enzyme activities are indicated. Individual 1 represents the propositus. Squares denote males, circles females.

### *DNA sequence analysis of* HK1

DNA sequence analysis of the patient's paternal allele revealed two novel mutations in *cis* in the erythroid-specific promoter of *HK1*: –373A>C and –193A>G (relative to the start codon) (Figure 1). On the maternal allele we identified a novel missense mutation in exon 3 (c.278G>A), encoding an arginine to glutamine change at residue 93. No other mutations were detected in the coding region or flanking intronic sequences. None of the identified mutations was detected in a normal Caucasian control population (n=108 alleles). Subsequently, other family members were screened for the presence of these mutations (Figure 1).

### *Functional characterization of the –373A>C and –193A>G promoter mutations*

To determine the functional consequences of the two *HK1* promoter mutations we transfected constructs pGL3-HKWT (wild type), pGL3-HK373C (nt –373A>C), pGL3-HK193G (nt –193A>G) and pGL3-HK373C/193G (nt –373A>C and nt –193A>G) in K562 cells and compared their relative luciferase activities. The –373A>C *HK1* promoter mutation did not affect luciferase activity compared to the wild-type *HK1* construct. Luciferase activity of the –193A>G HK1 promoter reporter construct, however, was only 8% of that of the wild-type *HK1* promoter reporter construct. The concomitant presence of the –373A>C mutation did not show an additional effect on promoter activity. We, therefore, concluded that the –373A>C mutation is non-functional (Figure 2). In contrast, the –193A>G mutation strongly reduces promoter activity.

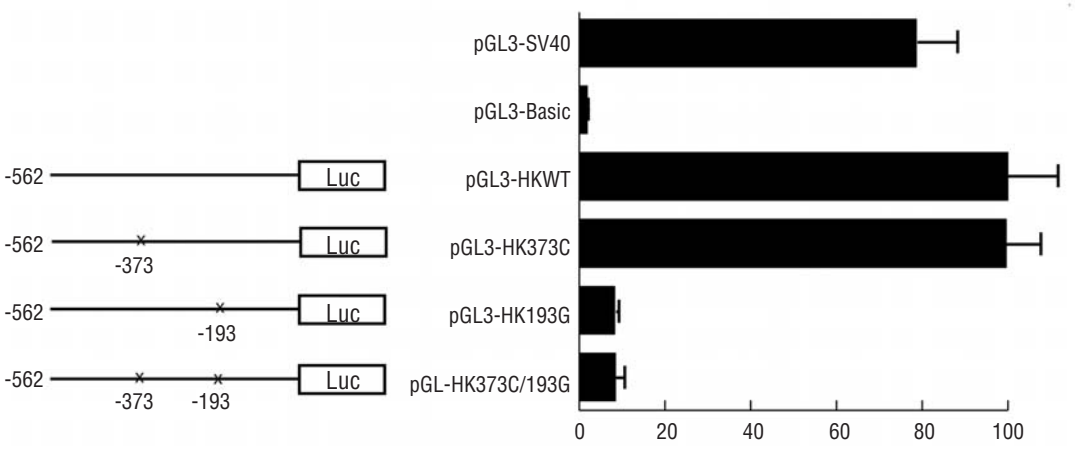


**Figure 2.** The –193A>G mutation in the erythroid-specific promoter of ***HK1*** strongly down-regulates promoter activity *in vitro*. ***HK1*** pGl3 promoter reporter gene constructs (spanning nts –562 to –1) without (pGL3-HKWT) and with the promoter mutations (pGL3-HK373C, pGL3-HK193G, pGL3-HK373C/193G) were transiently transfected in human erythroleukemic K562 cells. Luciferase activities were calculated relative to the wild-type pGL3-HKWT promoter reporter construct. pGL3-SV40 and pGL3-Basic were included as positive and negative (promoterless) controls. Results are the average of four independent experiments with each sample assayed in duplicate.

### *c-Jun mediates transcription of red blood cell-specific hexokinase*

The DNA sequence of the erythroid-specific promoter of *HK1* was subjected to analysis by Transcription Element Search Software (TESS[19]) in order to identify potential transcription factor binding sites. In line with the transfection results, the –373A>C mutation did not affect a putative transcription factor binding site. Analysis of the region surrounding nt –193 showed a putative binding site for transcription factors NF-E2 and AP-1 (–190 to –193). This transcription factor binding site was disrupted by the –193A>G mutation. Subsequent EMSA, using K562 nuclear extract and a wild-type radiolabeled *HK1* oligonucleotide probe, revealed two slow-migrating complexes A and B (Figure 3, panel A, lane 1, arrows) and a group of fast-migrating complexes. Competition experiments with non-radiolabeled wild-type and mutant oligonucleotide probes showed that only complex A and B were specific (Figure 3, lanes 2 and 3), in contrast to the slower migrating complexes. Addition of anti-c-jun antibody caused a supershift of the specific oligonucleotide/protein complexes (complex C), resulting in disappearance of complex B and reduction of complex A (Figure 3, panel B, lanes 4, 6 and 8). This was not seen when using NF-E2 and c-fos antibodies (Figure 3, panel B, lane 5 and 7). As a control, EMSA was performed using a labeled oligonucleotide harboring the –193A>G mutation. No specific oligonucleotide/protein complexes were observed (Figure 3, panel A, lanes 5-8 and panel B, lane 9). We concluded that the –193A>G mutation disturbed binding of c-jun to the putative AP-1 binding site in the erythroid-specific promoter of *HK1 in vitro*.

To evaluate *in vivo* binding of c-jun to the AP-1 site in the erythroid-specific promoter of *HK1* we performed ChIP assays in K562 cells. Specific c-jun/DNA complexes were immunoprecipitated using a ChIP-validated c-jun antibody. Additional amplification of recovered sheared chromatin with primers annealing to nts flanking the AP-1 binding sites, revealed a 227 bp-PCR product specific for *HK1* (Figure 4). This product was absent

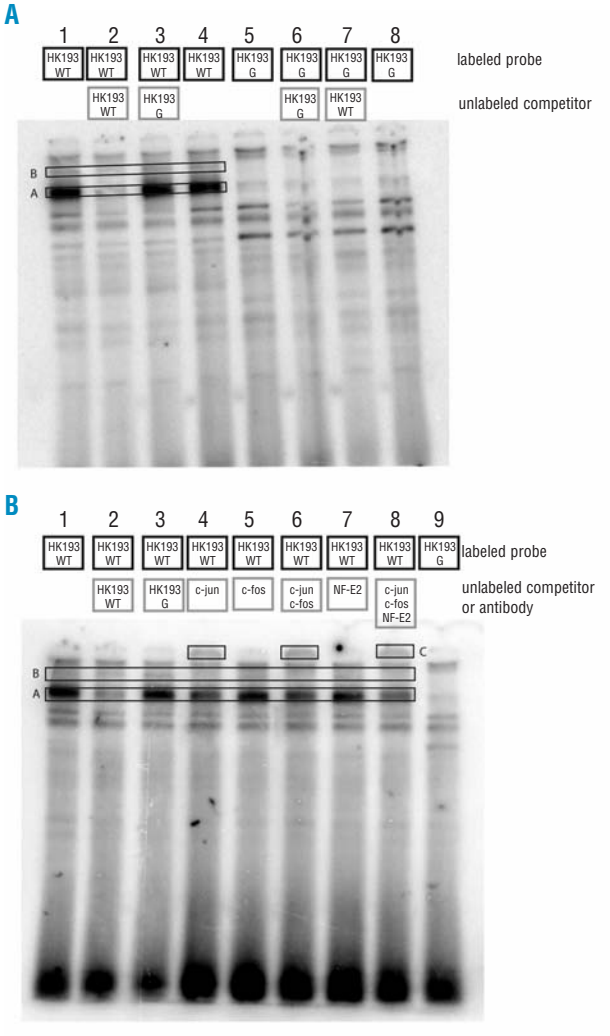


**Figure 3.** c-Jun mediates transcription of red blood cell-specific HK. Electrophoretic mobility shift assays were performed with K562 nuclear extract and labeled wild-type and mutant (–193A>G) oligonucleotide probes. The presence of 500 fmol of unlabeled competitors is indicated, as well as the addition of 1 μg of anti-NF-E2, anti-c-fos and anti-c-jun. Upon incubation with labeled HKWT (lane 1) two slow-migrating complexes (A and B) and a group of fast-migrating complexes were detected. Competition experiments with non-radiolabeled wild-type and mutant oligonucleotide probes showed that only complex A and B were specific (panel A, lanes 2 and 3) in contrast to the slower migrating complexes. Addition of anti-c-jun antibody caused a supershift of the specific oligonucleotide/protein complexes (complex C), resulting in disappearance of complex B and reduction of complex A (panel B, lanes 4, 6 and 8). This was not seen when using NF-E2 and c-fos antibodies (panel B, lanes 5 and 7). As a control, EMSA was performed using a labeled oligonucleotide harboring the –193A>G mutation. No specific oligonucleotide/protein complexes were observed (panel A, lanes 5-8 and panel B, lane 9).

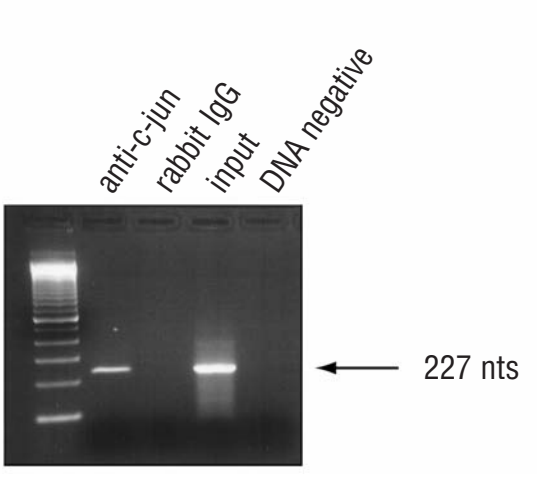


**Figure 4.** c-jun binds to the AP-1 binding motif in the erythroid-specific promoter of *HK1 in vivo*. Chromatin from K562 cells was immunoprecipitated with a ChIP validated c-jun antibody (anti-c-jun) and PCR-amplified using primers flanking the AP-1 binding site in the erythroid-specific promoter of *HK1* (PCR product of 227 nts). Amplification of input chromatin (input) prior to immunoprecipitation served as a positive control for chromatin extraction and PCR amplification. Chromatin immunoprecipitation using non-specific antibody (rabbit IgG) served as a negative control. In addition, PCR in the absence of DNA (DNA–negative control) was performed to exclude DNA contamination of the reaction mixture.

when using normal rabbit IgG. From these results we concluded that c-jun interacts directly with the AP-1 binding site in the erythroid-specific promoter of *HK1* in K562 cells.

### *The c.278G>A missense mutation affects normal pre-mRNA processing*

The c.278G>A missense mutation in exon 3 of *HK1* encodes for an arginine to glutamine change at residue 93. To predict the effect of this substitution on the protein's structure and function we analyzed the p.Arg93Gln amino acid change using two on-line prediction tools: SIFT[20] and PolyPhen.[21] Both programs predicted that the glutamine could be well tolerated at residue 93 in HK. To further investigate the effect of the c.278G>A mutation we performed RT-PCR analysis on the patient's and a control's erythroid RNA as obtained from cultured (pro)erythroblasts. HK-R-specific RNA was amplified as described and rendered one RT-PCR product with the expected size of 442 bp in the case of the control (Figure 5, band A). The same product was produced from the patient's RNA but an additional four products could be detected (Figure 5). DNA sequence analysis of all RT-PCR products revealed that the 442-bp product reflected normally processed *HK1* RNA. RT-PCR products B and C in the patient reflected alternative processing of *HK1* pre-mRNA; one transcript lacking exon 3 (Figure 5, C, Δ3) and one transcript lacking 56 bp of the 5'-end of exon 3 due to processing at a cryptic donor site at nt c.279 in exon 3 (Figure 5, band B). The remaining two fragments represented heteroduplexes of the different RT-PCR products (Figure 5, indicated by arrows).

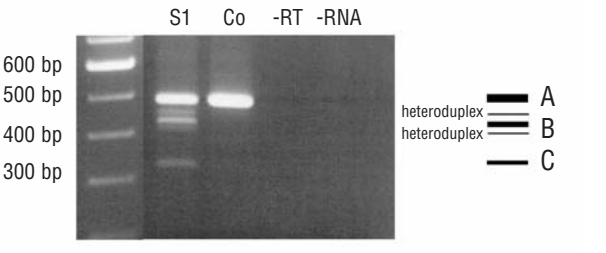


**Figure 5. Association of the exon 3 c.278G>A mutation with aberrant erythroid-specific *HK1* transcripts. Exons 2 and 3 were amplified from total RNA of *ex vivo* cultured erythroblasts of the patient, with primers spanning erythroid-specific exon 1 and exon 4 (S1). With regard to the patient, five RT-PCR fragments could be detected, whereas only one RT-PCR fragment was present in the control (Co). DNA sequence analysis was performed on each RT-PCR fragment. Fragment A corresponds to normally processed *HK1* RNA, fragment B lacks 56 bp of the 5'-end of exon 3, whereas fragment C lacks exon 3 completely. The two other fragments are heteroduplexes of the different RT-PCR products. –RT: control in which the reverse transcription step was omitted. -RNA: control without RNA.**

## Discussion

We investigated the molecular basis for HK deficiency in a patient with mild chronic hemolysis. We identified two in *cis* mutations: –373A>C and –193A>G in the erythroid-specific promoter of *HK1*. These mutations are the first mutations identified in the erythroid-specific regulatory region of *HK1*. The –193A>G mutation strongly down-regulated promoter activity whereas the –373A>C mutation was non-functional (Figure 2). Using EMSA, we showed that binding of a *trans*-acting protein, or *trans*-acting protein complex, to the erythroid-specific promoter of *HK1* was disrupted by the –193A>G mutation (Figure 3). A study of the erythroid-specific promoter of *HK1*, using Transcription Element Search Software (TESS),[19] revealed a putative binding site for the transcription factor complex AP-1. This AP-1 binding site is fully conserved in the mouse (NCBI Ensembl database) and predicted to be disrupted by the –193A>G mutation. AP-1 consists of a diverse group of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun, Fos, Maf and ATF sub-families. They recognize either 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements (5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3').[22] By use of supershift experiments and ChIP assays we show here that c-jun is one of the components of the AP-1/DNA complex specifically binding to the *HK* promoter, both *in vitro* and *in vivo* (Figures 3 and 4).

AP-1 sites are present in the HS 2 element of the locus control region of the human β-globin gene cluster,[23] the chicken β-globin enhancer,[24] and the human porphobilinogen deaminase gene promoter.[25,26] In each of these genes, disruption of AP-1 sites resulted in a dramatic reduction of erythroid-specific promoter activity.[23,24,26,27] We show in this study that AP-1 also mediates expression of HK-R, by interacting with the AP-1 element in the erythroid-specific promoter of *HK1*.

Jarman *et al.*[28] have discussed the role of the ubiquitous transcription factor AP-1 in erythroid-specific transcription. They suggested that other erythroid-specific transcription factors (such as GATA-1) could provide tissue specificity; their binding would prime the element, thus allowing the ubiquitous AP-1 proteins to access their cognate sequences in response to differentiation or induction signals. The erythroid-specific AP-1-like transcription factor NF-E2 would be another putative candidate in mediating erythroid-specific expression of HK-R. However, we were unable to demonstrate interactions of NF-E2 with the AP-1 binding site in the erythroid-specific promoter of *HK1* by supershift experiments with NF-E2 antibody (Figure 3).

Apart from the two promoter variations we identified a novel missense mutation on the maternal allele in our patient. This mutation consisted of a G>A change at cDNA nt 278 in exon 3, encoding an arginine to glutamine change at residue 93 that affects both the HK-1 and HK-R isoforms. Programs that predict the effect of amino acid substitutions on a protein's structure and function showed that the p.Arg93Gln change may be relatively well tolerated in HK. Because of increasing evidence that missense mutations may also exert their effects at other levels, in particular RNA processing,[29] we investigated the effect of the c.278G>A at the level of RNA and demonstrated aberrant splicing as a result

of this mutation (Figure 5). Skipping of exon 3 causes a shift in the reading frame that, because of a premature termination of translation, most likely results in inactive and unstable HK-1 and HK-R polypeptides of, respectively, 80 and 79 amino acids. Similarly, aberrant processing at the cryptic donor site in exon 3 is predicted to result in severely truncated HK-1 and HK-R molecules of, respectively, 77 and 76 amino acids. Taking these facts together, we hypothesize that, due to aberrant splicing, less HK-1 and HK-R is produced from the maternal allele.

Glycolytic enzymes decay gradually during the 120 days of the life span of the red blood cell.[13] HK, on the other hand, decays in a biphasic manner, a rapid decay of HK-R and a slow decay of HK-1.[2] In the youngest reticulocytes, HK-R represents more than 90% of the total HK activity whereas in old red blood cells HK-R is lacking. In a normally aged red blood cell HK-R and HK-1 contribute equally to total HK activity.[2] We, therefore, hypothesize that HK activity in the patient's erythroblasts and immature red blood cells may be more strongly decreased as a result of the HK-R promoter mutation. In agreement with this, the HK deficiency in the patient described in this study is relatively mild; 77% with respect to the lower reference limit. Notably, the similarly decreased HK activity does not produce clinical symptoms in the father. Therefore, despite the fact that our *in vitro* data are unambiguous, we cannot completely rule out the presence of additional phenotypic modifiers in the patient.

In conclusion, in this study we investigated the molecular basis for HK deficiency in a patient with mild chronic hemolysis. We report the –193A>G mutation in the erythroid-specific promoter of *HK1* as the first mutation known to specifically affect HK-R-specific expression. This mutation together with the novel exon 3 c.278G>A missense mutation which causes aberrant splicing of both the HK-R and HK-1 isoforms resulted in hexokinase deficiency and mild chronic hemolysis, as observed in the patient here described.

## Authorship and Disclosures

KMKdV: designed and performed research, collected, analyzed and interpreted data, wrote the manuscript, approved final version to be published; WWvS: designed research, interpreted data, wrote the manuscript, approved final version to be published; ACvW: designed and performed research, contributed vital analytical tools, collected, analyzed and interpreted data, revised article, approved final version to be published; SK: designed and performed research, collected data, wrote the manuscript, approved final version to be published; RvW: designed and performed research, interpreted data, wrote the manuscript, approved final version to be published.

None of the authors have any potential conflicts of interest.

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