Transcriptional regulation of the *KEL* gene and Kell protein expression in erythroid and non-erythroid cells

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The Kell blood-group antigen was originally reported to be a protein expressed in erythroid tissue only. Transcriptional analysis of the *KEL* promoter activity in human erythroleukaemia K562 and epithelial HeLa cells by electrophoretic mobility-shift and supershift assays, chloramphenicol acetyltransferase assays, co-transfection studies and site-directed mutagenesis provided the following results: (i) the *KEL* promoter exhibits a strong transcriptional activity in K562 cells and, unexpectedly, a basal non-erythroid activity in HeLa cells, (ii) up-regulation of the 5′ distal promoter activity occurs only in the erythroid context, and (iii) two motifs localized in the exon 1 region, which bind the Sp1/Sp3 and the human GATA-1/Ku70/80 factors, were required for down-regulation of the promoter activity, but inhibition of the promoter activity by the repressing factors in HeLa cells was incomplete. *KEL* expression in HeLa cells was performed further by primer-extension analysis, which revealed the presence of a low amount of Kell transcript correlating with basal expression of the Kell protein in these cells, as shown by immunopurification and Western-blot analysis. DNA sequencing of the transcript revealed a sequence identical to that obtained from erythroid tissue. In human tissues, *KEL* expression was investigated by dot-blot analysis and revealed high levels of Kell mRNAs, particularly in brain tissues, testis and lymphoid tissues. Moreover, most tissues analysed exhibited low levels of Kell transcripts. The Kell protein was also detected by immunohistochemistry in the Sertoli cells of the testis and in lymphoid tissues like spleen and tonsil, specifically localized in the follicular dendritic cells. Altogether, the results indicated that *KEL* expression is not restricted to erythroid tissue.

Key words: blood-group antigen, tissue expression, *trans*-acting factor, transcriptional activity.

INTRODUCTION

Most of the membrane proteins carrying blood-group antigens have a large tissue distribution and to date only a few, like those expressing Rh, LW, Kell and MNSs, are still considered as erythroid-specific (for reviews, see [1,2]). The Kell blood-group system is composed of 23 antigens with high and low frequencies [3]. The Kell (K1) antigen is a strong immunogen and can induce feto-maternal immunization [4]. Newborns with haemolytic anaemia caused by anti-Kell antibodies present signs of suppressed erythropoiesis [5,6], suggesting that anti-Kell antibodies may cause fetal anaemia by suppressing erythropoiesis at the progenitor level. This correlates well with recent studies showing expression of the Kell antigens on early erythroid progenitors, well before the glycophorin A erythroid marker [7,8].

The Kell antigens reside on a transmembrane protein of 93 kDa that shares significant homology with a family of zinc-dependent metalloendopeptidases, presumably indicating that this protein might catalyse the inactivation of bioactive peptides in bone marrow and/or peripheral blood [9]. This was confirmed recently by demonstrating the ability of the Kell protein to process *in vitro* big endothelin-3 (ET-3) and less efficiently big ET-1 and ET-2 [10], although it is not known if these molecules are the only substrates. In erythroid cells, Kell is covalently linked to the Kx protein, a putative transporter of 37 kDa (apparent molecular mass), by a disulphide bond between Kell cysteine 72 and Kx cysteine 347 [11,12]. However, no data are yet available on the putative role of the Kell–Kx complex in erythroid cells.

Previously we studied the transcriptional mechanism leading to the erythroid-specific expression of the glycophorin gene family (MNSs markers) using glycophorin B (*GPB*) as a model [13,14]. Here we investigated the molecular basis of *KEL* expression in erythroid and non-erythroid contexts by analysing the *KEL* promoter's transcriptional regulation using the human erythroleukaemic K562 and epithelial HeLa cell lines. Based on the published sequence of the *KEL* promoter [15], we identified *trans*-acting factors which regulate *KEL* gene expression and determined their role on the *KEL* promoter transcriptional activity. During the course of these studies we found that the *KEL* promoter exhibited significant transcriptional activity in HeLa cells. These findings prompted us to investigate the expression pattern of the *KEL* gene further through primer-extension, sequence and dot-blot analyses. Moreover, we were able to partially purify the Kell protein from HeLa cells using a monoclonal antibody, and to demonstrate that although the Kell mRNA level was low in these cells, the protein was still produced. From preliminary immunohistochemical studies performed on human adult tissues we concluded that Kell expression was not restricted to the erythroid tissues.

MATERIALS AND METHODS

Materials

Oligonucleotides and primers came from Eurogentec (Herstal, Belgium). Restriction enzymes came from Appligene (Strasbourg, France). T4 DNA ligase and T4 polynucleotide kinase were from

Abbreviations used: CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; EMSA, electrophoretic mobility-shift assay; *GPB*, glycophorin B; hGATA-1, human GATA-1.

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Biolabs (Northbrook, IL, U.S.A.) and radiolabelled nucleotides from Amersham (Little Chalfont, Bucks., U.K.). The random-priming labelling kit came from Boehringer Mannheim (Mannheim, Germany) and the Quikchange site-directed mutagenesis kit was from Stratagene (La Jolla, CA, U.S.A.).

Plasmids

The sequences introduced in the reporter plasmids are shown in Table 1. For the chloramphenicol acetyltransferase (CAT) assays, the reporter vectors were constructed as described previously [13] by subcloning the wild-type *GPB* promoter (−95 to +43 region) and either the wild-type or the mutated *KEL* promoter (−176 to +114) upstream of the bacterial *CAT* gene in the pBLCAT3 vector [16]. The deleted *KEL* promoter constructs (pBL*KEL1*, pBL*KEL2* and pBL*KEL3*) were obtained by PCR using the following oligonucleotides containing 5′ *Sal*I and 3′ *Bam*HI cloning sites: 5′-GGGTCGACCAAGACAAAAGGAGCAG-ACCAGG-3′ (−176 to −154, coding strand); 5′-GGGTCGA-CAGGAGAAGCCTGGGTGCCCC-3′ (−87 to −68, coding strand); 5′-GGGGATCCCTGCTGCTCTTTCGCCTTGTC-3′ (+1 to −20, non-coding strand); 5′-GGGGATCCATCTGTC-TATCTTCTGTGGCTG-3′ (+114 to +91, non-coding strand).

For the Dual-luciferase assays (Dual-luciferase reporter assay system, Promega, Madison, WI, U.S.A.), the wild-type *GPB* promoter (−95 to +43) and the wild-type *KEL* promoter (−176 to +114) were subcloned in the pGL3 vector upstream of the firefly luciferase reporter gene (Promega). The pGL3 control plasmid, containing the ubiquitous simian virus 40 promoter and enhancer sequences and the promoterless pGL3 Basic plasmid were used as positive and negative controls, respectively.

Cell cultures and transfections

The erythrocytic K562 cell line [17] was grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal calf serum. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies), also supplemented with 10% (v/v) fetal calf serum. For CAT assays, the cells were transiently co-transfected using the Superfect reagent (Qiagen, Hilden, Germany). Before transfection (24 h), 5×10^5 HeLa or 5×10^6 K562 cells were plated in 60 mm-diameter dishes. Reporter plasmid (5 μg) was added to 20 μl of Superfect reagent to 150 μl of medium without fetal calf serum and incubated for 10 min. Rous sarcoma virus (RSV)-luciferase vector (2 μg) was mixed with the plasmid constructs for normalization. The mixture was added to the cells and incubated at 37 °C for 24 h before harvesting. For Dual-luciferase assays, 2×10^6 HeLa cells and 10^7 K562 cells were transiently transfected with 34 μg of the pGL3*KEL*, pGL3*GPB* or control vectors, using the lipofectin procedure (Life Technologies). pRL-TK control vector (0.7 μg) carrying the *Renilla* luciferase gene downstream of the herpes simplex virus thymidine kinase promoter was mixed with each plasmid construct for normalization. Cells were harvested 48 h after transfection.

For co-transfection assays with the Ku70 cDNA, 10^7 K562 cells were electroporated at 200 V and 960 μF in 180 μl of PBS containing 10 mM Hepes, pH 7.4, with 5 μg of pcDNA3 together with 10 μg of the CAT reporter gene vector, and 2 μg of RSV-luciferase construct [13]. Cells were harvested 24 h after transfection.

CAT and luciferase assays

For CAT assays, the transfected cells were harvested, washed twice with PBS and resuspended in 100 μl of lysis buffer [250 mM Tris/HCl, pH 7.8/0.2% (w/v) Triton X-100/5 mM dithiothreitol/10% (w/v) glycerol]. Cell extracts were assayed for both luciferase [18] and CAT activities [19]. For Dual-luciferase assays, K562 and HeLa cells were lysed in 200 μl or 1 ml of passive lysis buffer, respectively. Cell lysate (20 μl) was mixed with 100 μl of luciferase assay reagent and the firefly luminescence, reflecting the activity of the *GPB* or *KEL* promoters, was immediately quantified in a luminometer (Bio-Orbit, Turku, Finland). This reaction was then quenched and the *Renilla* luciferase reaction was simultaneously initiated by adding 100 μl of Stop and Glo reagent to the mix. The measured luminescence was used to normalize the protein amount in each cell lysate. All the activities represented the average of at least three normalized experiments.

Nuclear extracts and electrophoretic mobility-shift assays (EMSAs)

Nuclear extracts were prepared either from HeLa or K562 cell lines as described previously [20]. The protein concentration was measured by the Bradford method [21]. The oligonucleotides used for EMSAs are shown in Table 1. Labelling of oligonucleotides, binding reactions and electrophoresis were performed as described previously [13]. The gels were run for 2 h, dried and autoradiographed overnight. For competition experiments, DNA-binding reactions were allowed to reach the equilibrium and a 250-fold excess of unlabelled specific competitor DNA was added to the binding-reaction mixture. To confirm the presence of specific proteins within the retarded complexes, supershift assays were performed using the following antibodies: monoclonal antibody 162 directed against the Ku70/80 heterodimer (Interchim, Montluçon, France), a polyclonal antibody directed against Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and an irrelevant monoclonal antibody, NBL-1, directed against the red-cell Xg^a membrane protein (a gift from Dr G. Banting, University of Bristol, Bristol, U.K.). Antibodies were added to the EMSA mixture for 10 min at room temperature before loading (15 μg of monoclonal antibodies or 5 μg of polyclonal antiserum).

Primer-extension analysis

For primer-extension analysis, total RNAs (10 μg) from K562 or HeLa cells were annealed to 0.1 pmol of ^{32}P-labelled oligonucleotide probe, 5′-CCATCTGTCTATCTTCTGTGGCTC-3′ (non-coding strand, positions +114 to +91). The pellet was dissolved in 20 μl of 400 mM NaCl/10 mM Pipes, pH 7.5/1 mM EDTA, and denaturated at 90 °C for 5 min. Hybridization was carried out at 50 °C for 6 h. The first cDNA strand was synthesized with murine moloney leukaemia virus reverse transcriptase (Life Technologies) as described previously [22] and sized on a 6% (w/v) polyacrylamide/8% (w/v) urea sequencing gel alongside a sequencing ladder of the same DNA fragment.

Reverse transcriptase PCR and DNA sequencing of the HeLa PCR product

Total HeLa RNAs were extracted using the High Pure RNA Isolation Kit from Roche (Mannheim, Germany). A total of 1 μg was reverse-transcribed for 30 min at 70 °C using C. therm. (*Carboxydothermus hygrogenoformans*) polymerase (Roche) with a *KEL* oligonucleotide (positions 2328–2303, non-coding strand) deduced from the *KEL* cDNA sequence [9]. The synthesized first-strand cDNA (0.5 μg) was used in a first round of PCR with the GC-rich PCR system of Roche under conditions recommended by the manufacturer. Primers were 5′-ATGGAAGGT-GGGCACCAAAGTG-3′, derived from *KEL* exons 1 and 2

Table 1 Sequences introduced into reporter plasmids for CAT assays, and oligonucleotides used in EMSAs

The nucleotides shown in bold correspond to the normal or mutated sequences. Positions of sequences are relative to the transcription start point being at position +1; numbers indicate the positions on the nucleotides.

	Mutation	Sequence
Plasmid		
pBL—95wt*GPB*	Wild type	5′-CATCAGC**T**$^{-75}$**GATA**GGC-3′
pBL*KEL1*	Wild type	5′-ACTGG**G**$^{+34}$**GCGGGG**GGT ... AGA**AG**$^{+103}$**ATAGAC**AGATGGTA-3′
pBL*KEL1*/M10	Mutated WGATAR	5′-ACTGG**G**$^{+34}$**GCGGGG**GGT ... AGA**AG**$^{+103}$**ATAAGC**AGATGGTA-3′
pBL*KEL1*/M19	Mutated Sp1	5′-ACTGG**T**$^{+34}$**TCGGGG**GGT ... AGA**AG**$^{+103}$**ATAGAC**AGATGGTA-3′
pBL*KEL1*/M10-M19	Mutated WGATAR and Sp1	5′-ACTGG**T**$^{+34}$**TCGGGG**GGT ... AGA**AG**$^{+103}$**ATAAGC**AGATGGTA-3′
Oligonucleotide		
+34wt*KEL*	Wild-type Sp1	5′-GGAACTGG**G**$^{+34}$**GCGGG**GGGTGTT-3′
+103wt*KEL*	Wild-type WGATAR	5′-GCCACAGA**AG**$^{+103}$**ATAGAC**AGATGGTA-3′
+103M10*KEL*	Mutated WGATAR*	5′-GCCACAGA**AG**$^{+103}$**ATAAGC**AGATGGTA-3′
−37wt*GPB*	Wild-type WGATAR	5′-TGGGCCTGGA**AG**$^{-37}$**ATAA**CAGCTAGTA-3′
−50wt*GPB*	Wild-type Sp1	5′-ATGGCCGCAGGAA**GGT**$^{-50}$**GGG**CCTGGAAGAT-3′
−75wt*GPB*	Wild-type	5′-ATCATCAGC**T**$^{-75}$**GATAGGC**AGGGGAG-3′
−75M18*GPB*	Mutated WGATAR†	5′-ATCATCAG**AA**$^{-75}$**GACTGGC**AGGGGAG-3′

* Binds GATA-1 only.
† Binds Ku70/80 only.

(positions 124–145), and 5′-TTACCAGAGCTGGCAGCGGC-3′, derived from *KEL* exon 19 (2322–2303). Then, a second round of PCR amplification was performed using 1 μl and 0.2 μl of the first PCR to reamplify the 5′ and the 3′ parts of the *KEL* cDNA respectively. Primers were 5′-CAAAGTGAGGAAGAGCCGAG-3′ (139–158) with 5′-AGTCCCTCTGCTTTAGCAGCA-3′ (1201–1181), and 5′-CAGATAGACCAGCCAGAG-3′ (793–810) with 5′-GCGGCTGGAGGGGTTCAAG-3′ (2307–2289).

5′ and 3′ PCR fragments were subcloned in the pCR II TA cloning vector (Stratagene, San Diego, CA, U.S.A.) and sequenced using M13 forward and reverse primers and *KEL* internal primers. Sequencing was performed with ABI BigDye terminator reagents and an ABI Prism 310 genetic analyser (Applied Biosystems, Foster City, CA, U.S.A.).

Dot-blot analysis

A Multiple Tissue Expression Array, containing dotted mRNAs from a panel of 83 different normal human tissues, cell lines and controls, was purchased from Clontech (Palo Alto, CA, U.S.A.). Poly(A)$^+$ RNA samples were normalized to the mRNA expression levels of eight different housekeeping genes. The hybridization was performed according to the manufacturer's instructions, using a 0.75 kb *KEL* cDNA probe (positions 450–1200 of the complete *KEL* cDNA) obtained by PCR. The amplified cDNA was labelled by the random-priming method with a specific activity of about 10^9 c.p.m./μg.

Immunopurification of the Kell protein and Western-blot analysis

Washed HeLa cells (2×10^8) were broken in a Dounce homogenizer until complete lysis in 20 ml of hypotonic buffer (200 mM sucrose/20 mM Tris/HCl, pH 7.4/2 mM EDTA) containing a cocktail of protease inhibitors (Roche). The homogenate was loaded on top of an 80 ml cushion (400 mM sucrose in the same buffer), and centrifuged for 15 min at 2500 ***g*** to discard nuclei and cell debris. Triton X-100 (1 %, v/v), 0.2 % (w/v) deoxycholic acid and protease inhibitors were added to the upper layer and the mixture was incubated for 1 h at 4 °C with vigourous stirring. The extract was then centrifuged for 1 h at 200000 ***g***. The clarified supernatant was loaded on to an immunoaffinity column containing the immobilized mouse monoclonal antibody 5A11 directed against the Kell glycoprotein [23], previously equilibrated with PBS containing 1 % Triton X-100 and 0.2 % deoxycholic acid. The column was further rinsed with equilibrium buffer, then with PBS containing 0.1 % Triton X-100. Finally, the purified Kell protein was eluted with 0.1 mM glycine buffer, pH 2.8, and fractions were immediately neutralized to pH 7.5 with 1 mM Tris/HCl, pH 12.

SDS/PAGE was performed using a Novex apparatus (San Francisco, CA, U.S.A.) on a 10 % (w/v) polyacrylamide gel [24]. Samples were then either stained by silver nitrate or transferred on to nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.) and incubated with appropriate dilutions of 5A11 or anti-Kx antibodies. Secondary antibodies were peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Biosys, Compiègne, France), respectively. Immunoblots were finally stained with the ECL chemiluminescence reagent (Amersham) and exposed to X-ray films (Biomax MR, Kodak, Rochester, NY, U.S.A.).

Immunohistochemical studies

Immunohistochemistry was performed on 4 μm-thick sections of frozen samples with the purified monoclonal antibody 5A11. The staining was amplified and revealed by a goat anti-mouse peroxidase antibody (Dako, Copenhagen, Denmark). Negative controls were obtained by either omission of the first antibody or its replacement by an isotypic irrelevant mouse monoclonal antibody. An internal positive control was obtained by the staining of follicular dendritic cells using appropriate antibodies.

RESULTS

CAT and luciferase activities of the *KEL* promoter

K562 and HeLa cells were chosen as models of erythroid and non-erythroid cell lines, respectively. The proximal *KEL* promoter contains several putative *cis*-regulatory elements [15],

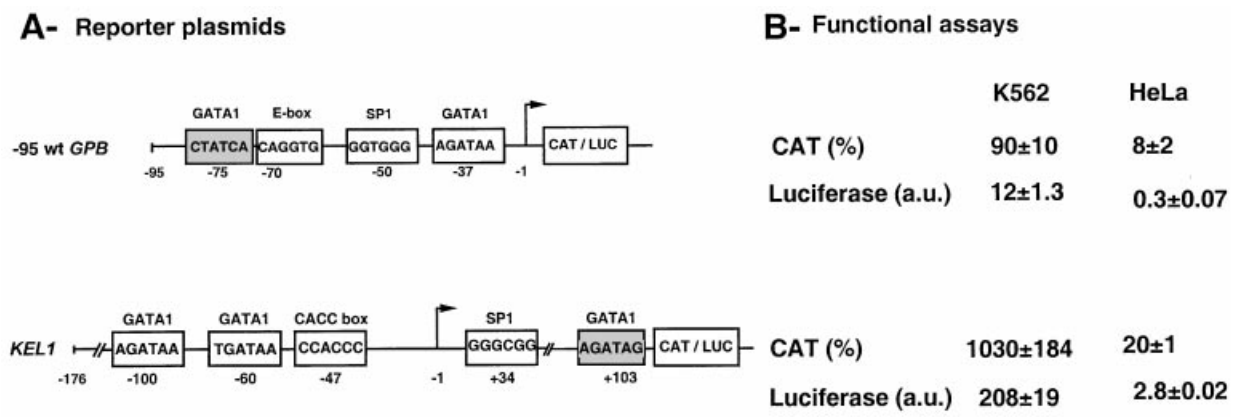

Figure 1 Functional activity of the *KEL* and *GPB* promoters

(**A**) Schematic representation of reporter plasmids used for functional assays. The well-characterized −95wt*GPB* plasmid was used in each assay as a control and reference [14]. (**B**) K562 and HeLa cells were transfected with the −95wt*GPB* and *KEL1* promoter fragments inserted into the pBLCAT3 or pGL3 reporter plasmids and assayed for CAT or luciferase activities, respectively. Transfection efficiency was normalized using a luciferase assay [13]. CAT activities were quantified using the −95wt*GPB* construct as reference (90% in K562 cells and a background of 8% in HeLa cells, as described previously [14]). Luciferase activities were expressed as arbitrary units (a.u.). The amount of protein in each cell lysate was normalized from *Renilla* luciferase activity obtained by co-transfection with the pRL-TK vector.

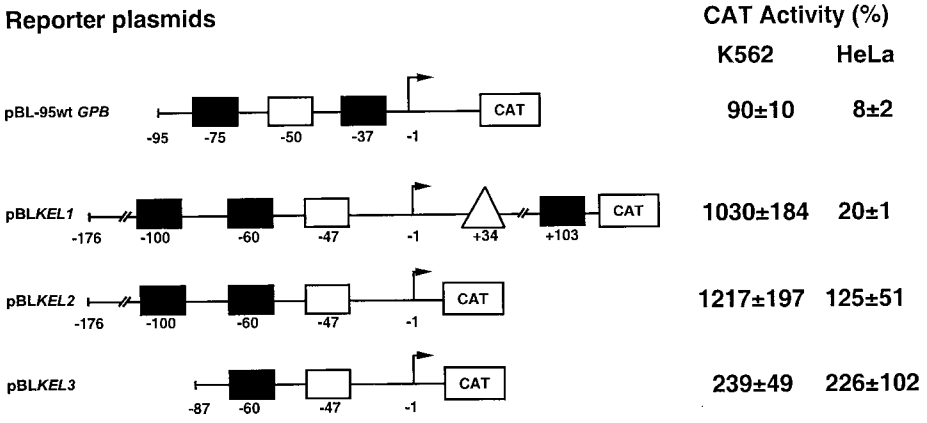

Figure 2 Deletion analysis of *KEL* promoter activity in K562 and HeLa cells

Left-hand panel: schematic representation of CAT reporter plasmids used to transfect K562 and HeLa cells. Deleted constructs were obtained by PCR (see the Materials and methods section). Symbols are as follows: black rectangle, GATA box; white rectangle, CACCC box; white triangle, Sp1 box. Right-hand panel: CAT activity, as in Figure 1.

particularly three GATA-1 motifs at positions −100, −60 and +103 (+1 refers to the transcription start point), one CACC box at position −47 and one Sp1 element at position +34 (Figure 1A). Since *cis*-regulating elements are also present in exon 1, transient transfections in K562 and HeLa cell lines were performed with the *KEL* gene region from position −176 to +114 (*KEL1* construct).

When the −95wt*GPB* promoter was used as a control and reference in CAT assays, we found 90±10% of CAT activity in K562 cells and a background of 8±2% in HeLa cells, as described before [13]. In K562 cells, the CAT activity of the pBL*KEL1* construct was more than 10-fold higher compared with the pBL−95wt*GPB* construct (1030 versus 90%), indicating that the *KEL* promoter was stronger than the *GPB* promoter in erythroid cells (Figure 1B). In HeLa cells, pBL*KEL1* exhibited a 2.5-fold higher level than the pBL−95wt*GPB* (20 versus 8%), suggesting that this promoter may present a small but reproducible activity in non-erythroid cells (Figure 1B). This is supported further by previous studies showing that 24% CAT activity of a *GPB* promoter mutant (at position −70) compared with the 8% background level of the wt*GPB* in HeLa cells is relevant for positive CAT activity [25]. All these results were confirmed using the Dual-luciferase reporter assay system, as the promoter activity in K562 cells was 17-fold higher for the pGL3*KEL1* than for the pGL3*GPB* construct. Importantly, pGL3*GPB* exhibited a background luciferase activity in HeLa cells (similar to that of the promoterless pGL3 basic plasmid, results not shown), whereas the luciferase activity of pGL3*KEL1* was 9-fold higher (Figure 1B). This finding confirms the CAT studies and indicates unambiguously that the *KEL* promoter exhibits a weak but positive activity in HeLa cells.

Deletion analysis of the *KEL* promoter region

To determine whether the weak CAT/luciferase activity of the *KEL* promoter in non-erythroid versus erythroid cells could

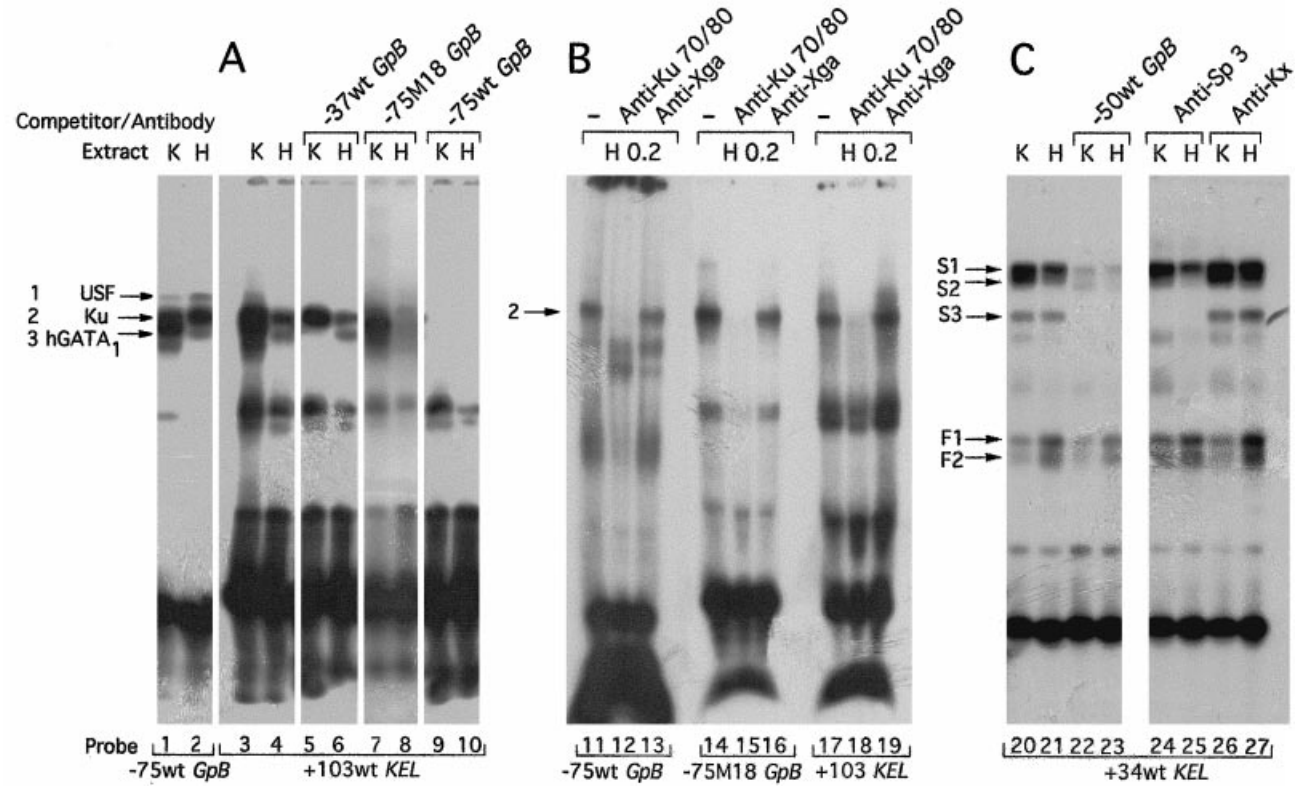

Figure 3 EMSA using the +34 and +103 *KEL* oligonucleotide probes

EMSAs were performed with nuclear extracts prepared from erythroid K562 (K) and non-erythroid HeLa (H) cells. Supershifts assays with the anti-Ku antibody were performed with a HeLa nuclear extract purified on heparin–Sepharose (H 0.2 fraction). (**A**) The −75wt*GPB* and the +103wt*KEL* 5′ end-labelled oligonucleotide probes were used in lanes 1 and 2 and 3–10, respectively, as noted below the autoradiographs. Positions of the retarded complexes are indicated on the left. A 250-fold excess of unlabelled −37wt*GPB* oligonucleotide efficiently competed for complex 3 formation (lane 5), whereas addition of a 250-fold excess of unlabelled −75M18*GPB* oligonucleotide competed for complex 2 formation (lanes 7 and 8). A 250-fold excess of unlabelled −75wt*GPB* oligonucleotide resulted in a complete inhibition of complex 2 and 3 formation (lanes 9 and 10). (**B**) Bandshift and supershift assays. Oligonucleotide probes −75wt*GPB*, −75M18*GPB* and +103wt*KEL* were used in lanes 11–13, 14–16 and 17–19, respectively. Normal bandshifts were performed without antibody (lanes 11, 14 and 17). Anti-Ku antibody was added to the EMSA mixture in lanes 12, 15 and 18. Lanes 13, 16 and 19 refer to assays performed with an irrelevant antibody against the red-cell Xg^a membrane protein. The position of complex 2 is indicated. (**C**) EMSA performed with the +34 5′-end-labelled *KEL* oligonucleotide (lanes 20–27). Slow- (S) and fast- (F) migrating complexes are indicated. A 250-fold excess of unlabelled −50wt*GPB* oligonucleotide efficiently competed for the formation of slow-migrating complexes (lanes 22 and 23). The addition of anti-Sp3 antibody resulted in a complete disappearance of the S3 complex (lanes 24 and 25), whereas the irrelevant anti-Kx antibody had no effect (lanes 26 and 27).

result from the absence of human GATA-1 (hGATA-1) alone, we first performed 5′ and 3′ deletions of the *KEL* promoter, which sequentially removed DNA-binding consensus sequences. In the pBL*KEL2* construct (−176 to −1) the exon 1 region was removed, whereas the 5′ distal promoter region was also deleted in the pBL*KEL3* plasmid (−87 to −1; Figure 2, left-hand panel).

In K562 cells, the pBL*KEL1* and pBL*KEL2* constructs displayed nearly equal CAT activities, whereas pBL*KEL3*, which lacks the −176 to −87 region, showed a 5-fold decrease in the CAT activity level. This suggests that the region −176 to −87 contains *cis*-acting sequences involved in the activation of *KEL* transcription in erythroid cells, presumably the GATA box in position −100 [15].

The pBL*KEL3* construct was as active in HeLa as in K562 cells (Figure 2, right-hand panel). This suggests that the minimal promoter contains *cis*-acting elements (Sp1/CACC and GATA-1) that potentially bind ubiquitous and/or tissue-specific transcription factors promoting basal initiation of transcription in non-erythroid as well as erythroid cells. The pBL*KEL2* construct exhibited about the same CAT activity in non-erythroid cells as the pBL*KEL3* plasmid, indicating that there is no important regulatory element for non-erythroid expression in the 5′ distal region of the promoter. In contrast, pBL*KEL1* showed a 6-fold lower CAT activity compared with pBL*KEL2*. This result strongly suggests that the exon 1 region (−1 to +114) is involved in negative regulation of the promoter in non-erythroid cells.

Characterization of the *trans*-regulating factors by EMSAs

To characterize *trans*-acting factors that bind to the exon 1 region and negatively regulate the *KEL* promoter activity in non-erythroid cells, we performed EMSAs, using the +103 GATA-1 and the +34 Sp1 *cis*-elements as probes (see Table 1). The oligonucleotide corresponding to the +103wt*KEL* region formed two distinct complexes with K562 and HeLa cell extracts (Figure 3A, lanes 3 and 4). When compared with the pattern obtained previously with the −75wt*GPB* oligonucleotide [13,14], we found that one complex corresponded to the binding of the heterodimeric Ku70/80 factor in both cells (complex 2 in Figure 3A), whereas the other complex corresponded to hGATA-1 binding in K562 cells (complex 3 in Figure 3A, lanes 1 and 3). A 250-fold

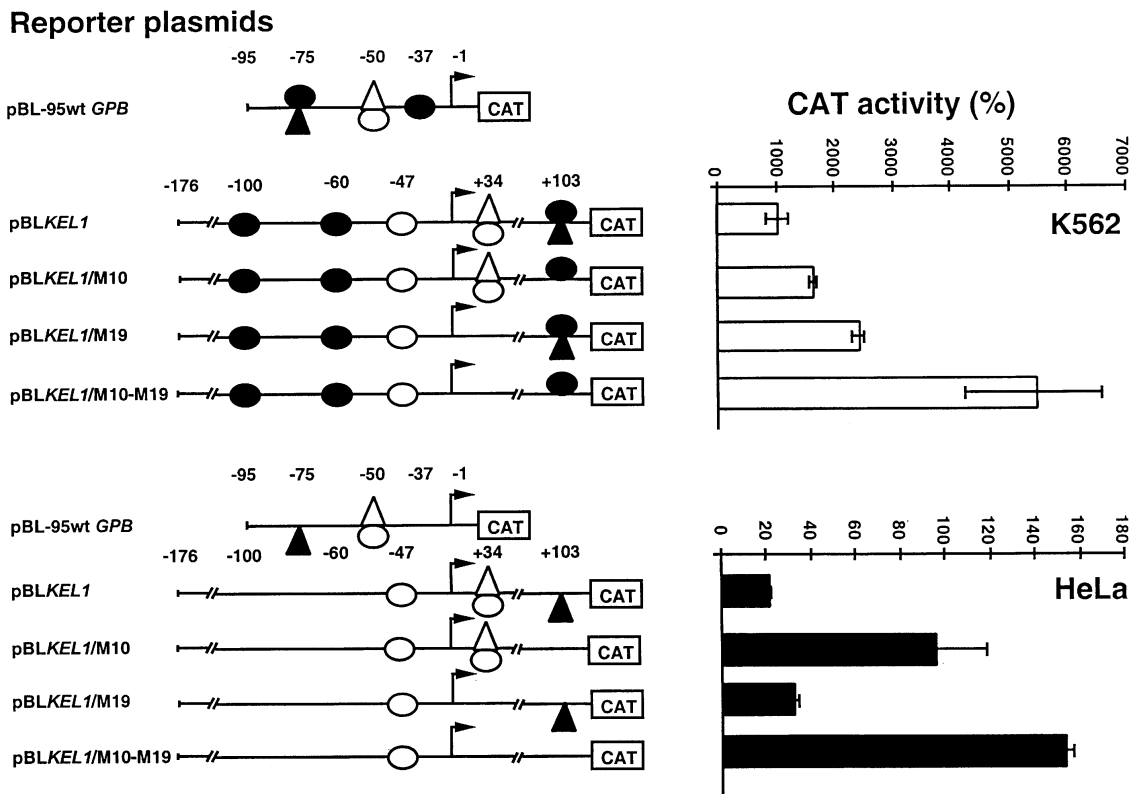

Figure 4 Effect of point mutations on the *KEL* promoter activity

Mutations altering the binding of *trans*-acting factors to *cis*-acting motifs centred at positions +34 and +103 were investigated. Left-hand panels: schematic representation of reporter plasmids used for transfection of K562 and HeLa cells. S.E.M. of CAT activity values are indicated. Right-hand panels: CAT assays performed on cells transfected with wild-type or mutant pBL*KEL1* (results expressed as in Figure 1). *Trans*-acting factors binding to promoter regions are symbolized as follows: black oval, hGATA-1; white oval, Sp1; white triangle, Sp3; black triangle, Ku70/80.

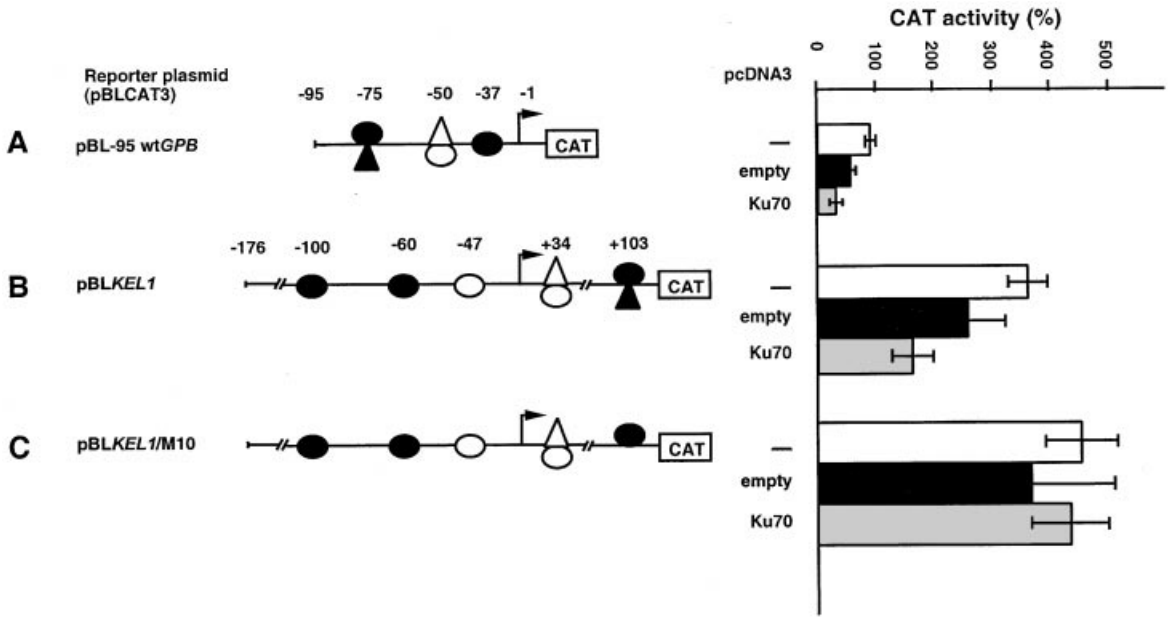

Figure 5 Co-transfection assays in K562 cells with the Ku70 cDNA plamid

CAT assays performed on K562 cells transfected by electroporation with pBL—95wt*GPB* (**A**), pBL*KEL1* construct (**B**) and pBL*KEL1*/M10 (**C**) constructs. CAT activities are expressed as in Figure 1. Symbols are as follows: white bar, reporter construct alone; black bar, empty pcDNA3 plasmid; grey bar, co-transfection with pcDNA3 Ku70. Co-transfections with the plasmid encoding Ku70 resulted in an approx. 50% reduction in CAT activities (compared with the co-transfections with the empty plasmid) of the pBL—95wt*GPB* and the pBL*KEL1* constructs. No effect was detected on the CAT activity of the pBL*KEL1*/M10 mutant, for which Ku70/80 DNA binding was impaired.

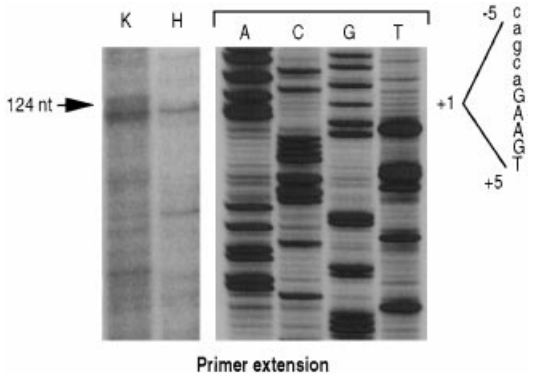

Figure 6 Primer-extension analysis

The first *KEL* cDNA strand synthesized was sized on a 6% acrylamide/8% urea sequencing gel alongside a sequencing ladder of the same DNA fragment. A major primer-extension product of 124 nt was revealed in HeLa (H) as well as in K562 (K) cells.

excess of an unlabelled oligonucleotide containing a specific hGATA-1 sequence (the −37wt*GPB* oligonucleotide) efficiently competed with complex 3 formation in erythroid cells (Figure 3A, lane 5). The addition of a 250-fold excess of the −75M18*GPB* mutant oligonucleotide, which only recognized the heterodimeric form of the ubiquitous factor Ku70/80, competed with complex 2 formation (Figure 3A, lanes 7 and 8). Further evidence that the same complexes corresponding to Ku70/80 and hGATA-1 were formed with the +103wt*KEL* and −75wt*GPB* oligonucleotides was obtained by showing that the addition of a 250-fold excess of the −75wt*GPB* probe impaired the formation of both complexes formed with the +103wt*KEL* probe (Figure 3A, lanes 9 and 10).

To confirm the specific binding of the heterodimeric form of the Ku antigen (Ku70/80) to the +103wt*KEL* oligonucleotide, supershift assays were performed with a partially purified HeLa nuclear-protein extract (heparin–Sepharose H0.2 fraction), as described in [13], using the anti-Ku70/80 antibody (murine monoclonal clone 162) and the anti-Xg^a antibody (NBL-1 clone) as an irrelevant control. As shown in Figure 3(B), when clone 162 was used with the −75wt*GPB* oligonucleotide (lane 12), the −75M18*GPB* mutant (lane 15) or the +103wt*KEL* oligonucleotide (lane 18), a complete disappearance of complex 2 was obtained, as compared with the patterns obtained without antibodies (Figure 3B, lanes 11, 14 and 17, respectively). As a control, complex 2 formation was not affected by the presence of the irrelevant anti-Xg^a antibody (Figure 3B, lanes 13, 16 and 19). Taken together, the results of both bandshift and supershift assays strongly suggest that complex 2 formation corresponds to the binding of the Ku70/80 heterodimer to the +113 region of the *KEL* promoter.

The oligonucleotide corresponding to the +34 Sp1 region (GGGCGG *cis*-element) of the *KEL* promoter [15] formed three slow-migrating complexes (named S1, S2 and S3) and two fast-migrating complexes (named F1 and F2), as shown in Figure 3(C) (lanes 20 and 21). Competition experiments were performed using the well-characterized −50wt*GPB* oligonucleotide, which binds Sp1 [14]. The addition of a 250-fold excess of this unlabelled oligonucleotide completely inhibited S3 complex formation and almost completely inhibited the S1 and S2 complexes, without affecting F1 and F2 (Figure 3C, lanes 22 and 23). This is in agreement with studies showing that the binding of the Sp1 factor generates a doublet (S1/S2) in bandshift assays [14,26]. As the Sp3 binding factor was identified as a negative regulatory factor that recognizes some GC-rich *cis*-elements [27,28], we investigated the ability of anti-Sp3 antibodies to affect the mobility of one or the other complex. The addition of rabbit polyclonal antibodies to the Sp3 factor clearly abolished S3 complex formation (Figure 3C, lanes 24 and 25), whereas an irrelevant anti-Kx antiserum had no effect (Figure 3B, lanes 26 and 27). These results indicate that the Sp1 and Sp3 factors bind to the +34 region of the *KEL* promoter. Altogether, the results show that the exon 1 region of the *KEL* gene binds the two ubiquitous factors Ku70/80 and Sp3.

Effect of point mutations on the activity of the *KEL* promoter

In order to evaluate the biological significance of the binding proteins revealed by EMSA, we performed mutagenesis on the +34 and/or +103 binding sites and functional assays were achieved with the altered regulatory region(s). We have reported previously that a WGATAR motif was involved in the erythroid-specific expression of the *GPB* gene, by competition-binding experiments between Ku70/80 and hGATA-1 [13]. We first carried out mutagenesis that selectively impaired Ku70/80 binding to the +103 *KEL* region but preserved hGATA-1 binding by changing AGATA**G** to AGATA**A** [14]. The resulting pBL*KEL1*/M10 plasmid (positions −176 to +114) exhibited a 1.5-fold increase in CAT activity in K562 cells and a 4-fold increase in HeLa cells compared with the wild-type pBL*KEL1* plasmid (Figure 4). These data indicate that Ku70/80 acts as a negative regulatory factor of the *KEL* promoter in both erythroid and non-erythroid cells, but with higher efficiency in the latter.

We also performed mutagenesis on the +34 region, by changing G**GG**CGGGG to G**TT**CGGGG, which abolished the binding of Sp1 and Sp3 [29]. The CAT activity of the mutant construct pBL*KEL1*/M19 was 2.5-fold and 1.5-fold increased compared with the CAT activity obtained with the pBL*KEL1* construct in K562 and HeLa cells, respectively. These results show that the GC-rich *cis*-element also negatively regulates the *KEL* promoter in erythroid and non-erythroid cells.

We finally performed a combination of the two mutageneses, destroying the binding sites of both Ku70/80 and Sp1/Sp3. The pBL*KEL1*/M10-M19 mutant showed a high increase in the CAT activity in K562 (5-fold) and HeLa (7-fold) cells compared with the wild type, corresponding to the additional effect of each mutation. We conclude that two *cis*-acting elements in exon 1 bind repressing factors (Sp3 and Ku70/80) that down-regulate *KEL* gene expression in both erythroid and non-erythroid cells.

Co-transfection of CAT constructs with Ku70 cDNA

As Ku70 is the DNA-binding subunit of the Ku70/80 heterodimeric factor [30], we performed co-transfection assays of CAT reporter constructs into the K562 cells together with the pcDNA3 plasmid expressing Ku70. We analysed the overexpression of Ku70 towards the pBL*KEL1* construct (wild type) and the pBL*KEL1*/M10 construct (mutated WGATAR sequence so that the binding for Ku was abolished), and the pBL−95wt*GPB* construct was used as a reference. As controls, the CAT reporter constructs were co-transfected with the pcDNA3 parental vector. As shown in Figure 5, overexpression of the Ku70 subunit decreased the *KEL1* and *GPB* promoter activities by 50%, whereas no effect was observed with the *KEL1*/M10 mutant. Altogether, these results indicated that the +103 *KEL* region is a specific DNA target for the repressing factor Ku in mammalian cells.

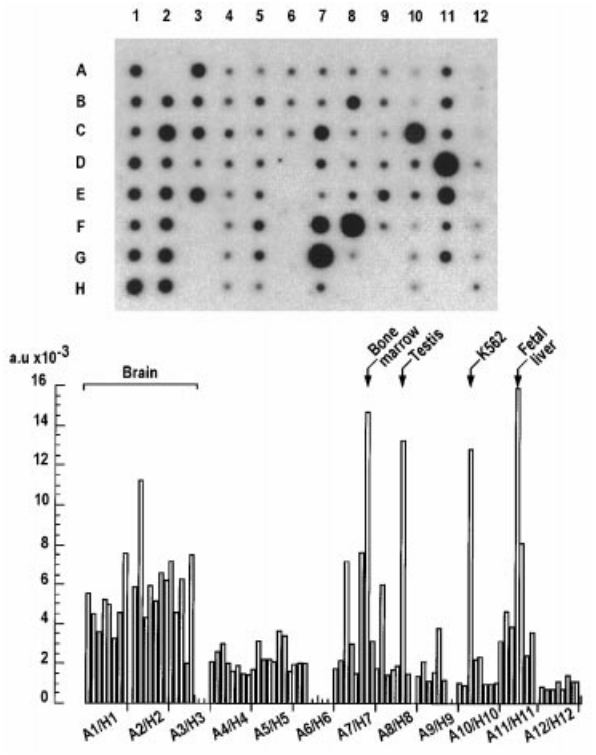

Figure 7 Dot blot analysis

(**A**) A dot blot containing 75 dots of mRNAs from normal human tissues and cell lines, and eight DNA or RNA controls was hybridized with the ^{32}P-labelled *KEL* cDNA probe. Positions of the samples are as follows: A1–H1, whole brain, cerebral cortex, frontal cortex, parietal cortex, occipital cortex, temporal cortex, paracentral gyrus of cerebral cortex and pons; B2–H2, right cerebellum, corpus callosum, amygdala, caudate nucleus, hippocampus, medulla oblongata and putamen; A3–E3, subsantia nigra, nucleus accumbens, thalamus, pituitary gland and spinal cord; A4–H4, heart, aorta, left atrium, right atrium, left ventricle, right ventricle, inter-ventricular septum and apex of the heart; A5–H5: oesophagus, stomach, duodenum, jejunum, ileum, ileocecum, appendix and ascending colon; A6–C6, transverse colon, descending colon and rectum; A7–H7, kidney, skeletal muscle, spleen, thymus, peripheral blood leucocyte, lymph node, bone marrow and trachea; A8–G8, lung, placenta, bladder, uterus, prostate, testis and ovary; A9–F9, liver, pancreas, adrenal gland, thyroid gland, salivary gland and mammary gland; A10–H10, leukaemia HL60, HeLa S3, leukaemia K562, leukaemia MOLT-4, Burkitts' lymphoma Raji, Burkitts' lymphoma Daudi, colorectal carcinoma SW480 and lung carcinoma A546; A11–G11, fetal brain, fetal heart, fetal kidney, fetal liver, fetal spleen, fetal thymus and fetal lung; A12–H12, yeast total RNA, yeast tRNA, *Escherichia coli* tRNA, *E. coli* DNA, poly r(A), human C_0t-1 DNA, human DNA (100 ng) and human DNA (500 ng). (**B**) Histogram of the dot blot. Relative densitometry values (*y* axis) are in arbitrary units (a.u.) obtained with the NIH Image 1.60/ppc program.

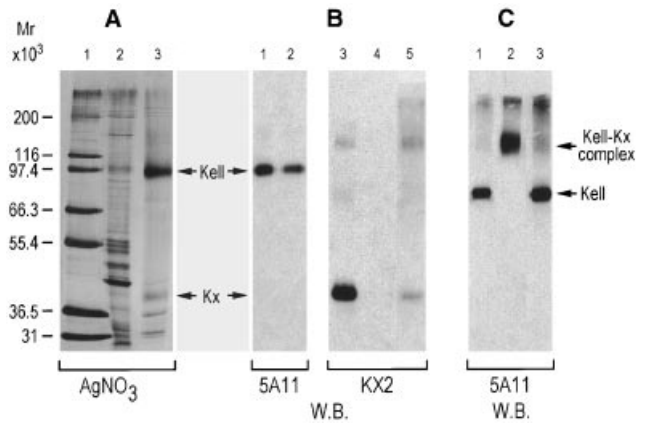

Figure 8 SDS/PAGE analysis of HeLa cell extracts after chromatography on an immobilized anti-Kell antibody

(**A**) and (**B**) show reducing conditions, whereas (**C**) shows non-reducing conditions. The gels were stained by silver nitrate (**A**) or revealed by Western blotting (**B**, **C**) using the indicated antibodies. (**A**) Lane 1, Novex molecular-mass standards; lane 2, aliquot of material eluted by a glycine buffer, pH 2.8, from immobilized 5A11 antibody column loaded with the HeLa cell extract; lane 3, purified Kell–Kx complex from human red cells (93 and 37 kDa, respectively). (**B**) Lanes 1, 4 and 5, purified material from HeLa cells; lanes 2 and 3, purified Kell–Kx complex from erythrocytes. Lanes 1–4, exposure time of 1 min; lane 5, exposure time was increased to 20 min. (**C**) Positions of monomeric Kell protein (93 kDa) and of Kell–Kx complex (130 kDa) are indicated. Lane 1, purified material from HeLa cells; lane 2, purified Kell–Kx complex; lane 3, as for lane 1 but a longer exposure time (30 min) was used.

Primer-extension and dot-blot analyses

The presence of Kell transcripts was investigated by primer-extension analysis in K562 cells and HeLa cells, in which the *KEL* gene is transcriptionally active (see above and [15]). Using a non-coding-strand oligonucleotide (nt +114 to +91) as a primer, a specific extension product of 124 nt, migrating to the same position on the gel for both cell types, was detected as a strong signal in K562 and a weaker signal in HeLa cells, indicating the presence of a Kell transcript in both cells (Figure 6).

Next, we asked whether the low amount of Kell mRNAs found in HeLa cells might reflect a basal ubiquitous expression of the *KEL* gene in normal human tissues. This was addressed by dot-blot analysis of mRNAs from a panel of 83 different normal human tissues, cell lines and controls. As shown in Figure 7, many tissues showed Kell mRNA expression, compared with the controls (dots A12–H12). High expression was noticed in brain tissues, spleen, lymph node, bone marrow, testis and K562 cells. All fetal tissues showed *KEL* expression. The S3 subclone of HeLa cells showed no expression, but the clone used for transcriptional analysis and primer extension was different.

We conclude that Kell transcripts are present in variable amounts in different cells and tissues, from very low to intermediate or high levels.

HeLa *KEL* sequence and Kell protein expression in non-erythroid tissues

The HeLa *KEL* cDNA sequence was analysed on fragments obtained by nested PCR and DNA sequencing. All the sequence was identical to that obtained previously from bone marrow [9]. In addition to the complete cDNA, two isoforms were detected, one corresponding to the splicing of exon 3 and the other to the splicing of exon 15 (results not shown).

In order to correlate the presence of Kell transcripts detected in non-erythroid tissues with Kell protein expression, immunopurification of the Kell protein from HeLa cell extracts on one hand, and immunohistochemical analysis of testis and lymphoid tissues on the other hand, were performed with the monoclonal antibody 5A11, which reacts with the Kell glycoprotein.

A protein of 93 kDa, with the same mobility as the Kell protein purified from human red cells, was eluted from the immobilized 5A11 antibody column loaded with the HeLa extract (Figure 8A, lane 2). On the Western blot, the 93 kDa component reacted with the 5A11 antibody (Figure 8B, lane 1) and its size was reduced by about 10 kDa when digested with N-glycosidase (results not shown), as expected for the Kell glycoprotein [23]. When the blot from reduced samples was stained with the anti-Kx antibody (KX2, Figure 8B, lanes 3–5), a faint band of

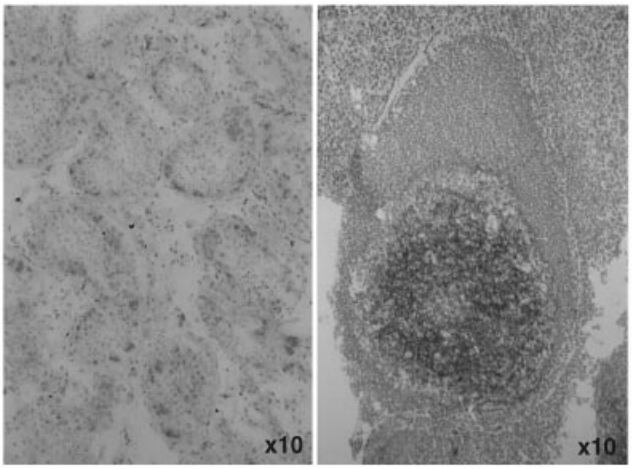

Figure 9 Immunoperoxidase staining of Kell protein in human tissues

Cryostat sections (4 μm) were stained with the 5A11 monoclonal antibody directed against Kell protein, as described in the Materials and methods section. Left-hand panel: (testis specimen) Sertoli cells, recognized on their typical morphological characteristics, express the Kell blood-group protein contrary to the spermatogenic epithelium. Right-hand panel: (tonsil specimen) follicular dendritic cells are strongly positive in the germinal centre of the lymphoid follicle, showing a typical network pattern.

37 kDa, detectable only after a long autoradiographic exposure, was seen with the purified material from HeLa cells (Figure 8B, lane 5). This band migrated to the same position as the Kx protein purified from erythrocytes (Figure 8B, lane 3). This suggests that in HeLa cells only minute amounts of Kx were produced and linked to Kell, in contrast to red cells where most, if not all, of the Kell protein is linked to Kx by a disulphide bond [11,12]. Under non-reducing conditions, nearly all Kell proteins migrated to the same position (93 kDa), as in reduced samples (Figure 8C, lane 1), whereas Kell immunoreactivity of the product purified from red cells was found at the position corresponding to the Kell–Kx complex (130 kDa; Figure 8C, lane 2). This result indicates that, in HeLa cells, the majority of the Kell protein is neither homodimerized nor linked to any other protein through disulphide bonds. A very tiny amount of Kell protein, however, was seen after a long exposure at the position of the Kell–Kx complex (Figure 8C, lane 3), in keeping with the fact that some Kx protein can be seen in gels processed under reducing conditions.

By immunohistochemical analysis, Kell expression was revealed in testis tissue, more precisely in Sertoli cells (Figure 9, left-hand panel). In lymphoid tissues, the tonsils were strongly stained (Figure 9, right-hand panel) as were lymph node, spleen and appendix (results not shown). The Kell protein was detected specifically in the follicular dendritic cells. A high level of Kell expression thus may be restricted to specific cells of a tissue.

DISCUSSION

Transcriptional regulation of the *KEL* gene in erythroid and non-erythroid cell lines was investigated using erythroleukaemic K562 cells and epithelial HeLa cells as models. These studies revealed that the *KEL* promoter exhibited a strong erythroid and, unexpectedly, a basal non-erythroid transcriptional activity. The *trans*-acting factors involved have been identified.

By EMSAs, supershift assays and competition experiments, we demonstrated that the erythroid transcription factor hGATA-1 and the ubiquitous factor Ku70/80 bound to the +103 region, whereas the Sp1 and Sp3 factors bound to the +34 region of the *KEL* promoter. Site-directed mutagenesis performed on the +34 GC-rich box that impaired the binding of Sp1 and Sp3 *trans*-acting factors showed that this region was recognized by a negative regulatory factor, active in erythroid as well as non-erythroid cells. Transfection experiments of mammalian cells have shown that Sp1 is a transcriptional activator, whereas Sp3 represses Sp1-mediated transcription [27–29]. More recently, it has been reported that the *SP3* gene encodes multiple proteins that differ in their capacity to stimulate or repress transcription [31,32]. We suggest that Sp3 could be the GC-binding factor that negatively regulates the *KEL* promoter.

We also demonstrated by co-transfection studies that the Ku70/80 heterodimeric protein acts as a negative regulatory factor of the *KEL* gene expression. The Ku factor is a nuclear protein known to bind to DNA either specifically or independently of the nucleotide sequence [30]. We have shown previously that Ku binds specifically to a TGATAGGC motif overlapping the GATA box of the −75 *GPB* promoter region. We demonstrated here by EMSA that the Ku factor binds to the AGATAGAC motif (also overlapping a GATA box) at position +103 of the *KEL* sequence in a specific manner. The binding of Ku resulted in the repression of the *KEL* promoter activity in erythroid and non-erythroid cells, and is presumably released in erythroid cells when hGATA-1 binds to the overlapping GATA box.

We conclude that the absence of hGATA-1 in HeLa cells is not sufficient to inactivate the *KEL* promoter, and that the presence of the repressing factors Sp3 and Ku70/80, which bind to the exon 1 region, is not sufficient to completely abolish *KEL* promoter activity in HeLa cells. Two other experiments supported basal *KEL* promoter activity in HeLa cells: (i) the Kell transcript was detected by primer-extension analysis in the HeLa clone used for CAT functional assays, amplified by PCR and sequenced, and (ii) the Kell protein could be immunopurified from a partially purified HeLa extract by monoclonal antibody 5A11 directed to the Kell glycoprotein.

Further analyses revealed that the Kell transcript has a large tissue distribution. Previous studies by Northern blotting revealed that the *KEL* gene expressed a transcript of 2.5 kb in adult bone marrow and fetal liver but not in adult brain, kidney, lung and liver [33]. Using poly(A)$^+$ mRNAs in Northern-blotting analysis, we found the Kell transcripts in high amounts in testis and detected weak expression in lymph node, tonsil, spleen, appendix and thyroid (C. Rahuel, V. Verkarre and J. P. Cartron, unpublished work). However, by dot-blot analysis, we found unambigously that the brain tissues expressed a Kell transcript. The higher levels of Kell mRNAs in non-erythroid tissues were found in testis and lymph node. Additionally, all fetal tissues gave a positive signal. Weak signals were detected in other tissues, such as the kidney and lung.

We demonstrated further by immunohistochemistry analysis that the monoclonal antibody 5A11 positively stained lymph node, spleen, appendix and tonsil, more precisely in the follicular dendritic cells of the lymphoid tissue. A strong reactivity was also found in the Sertoli cells of the testis. For detailed understanding of *KEL* gene transcriptional regulation and expression in Sertoli and follicular dendritic cells, further investigation will be needed.

Our results indicating a non-erythroid expression of the *KEL* gene correlate well with computer-search results, using the coding sequence of the human *KEL* cDNA to screen the EST database for identical or homologous sequences using the Blast N.1.4.11 program [34]. Interestingly, we found that Kell or homologous transcripts were detected in various tissues, including human

testis (GenBank accession numbers AI147411, AI142412 and AA860253), rat spleen (AI179459) and mouse lymph node (AA185103). Moreover, when this paper was being peer-reviewed, non-erythroid expression of the Kell protein, primarily in testis, was reported independently [35]. Additionally, the Kell antigen was also found on myeloid progenitor cells, including megakaryocyte progenitors by analysing the inhibitory effect of anti-Kell antibodies on haematopoietic cell cultures [36,37], but curiously this was not found in the initial study [5]. These investigations indicate that only a few blood-group antigens (Rh, MNSs and LW) may be still considered erythroid-specific [1,2]. However, this should be still regarded with caution, as extensive immunohistochemical analysis of human tissues and/or molecular-biology approaches have not been performed in all instances.

Whether the Kell protein plays some role related to its metalloproteinase function in these tissues is currently unknown. Additionally, whether or not it is associated with a Kx-like protein, as in erythroid cells [11,12], remains undetermined, but such studies along this line might help to understand the biological role of these proteins.

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