Characterization of the Gene Encoding the Human Kidd Blood Group/Urea Transporter Protein

EVIDENCE FOR SPlice SITE MUTATIONS IN Jknull INDIVIDUALS

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The Kidd (JK) blood group is carried by an integral membrane glycoprotein which transports urea through the red cell membrane and is also present on endothelial cells of the vasa recta in the kidney. The exon-intron structure of the human blood group Kidd/urea transporter gene has been determined. It is organized into 11 exons distributed over 30 kilobase pairs. The mature protein is encoded by exons 4–11. The transcription initiation site was identified by 5′-rapid amplification of cDNA ends-polymerase chain reaction at 335 base pairs upstream of the translation start point located in exon 4. The 5′-flanking region, from nucleotide −837 to −536, contains TATA and inverted CAAT boxes as well as GATA-1/SP1 erythroid-specific cis-acting regulatory elements. Analysis of the 3′-untranslated region reveals that the two equally abundant erythroid transcripts of 4.4 and 2.0 kilobase pairs arise from usage of different alternative polyadenylation signals.

No obvious abnormality of the Kidd/urea transporter gene, including the 5′- and 3′-untranslated regions, has been detected by Southern blot analysis of the blood of two unrelated Jknull individuals (B.S. and L.P.), which lacks all Jk antigens and Jk proteins on red cells, but was genotyped as homozygous for a “silent” allele. Further analysis indicated that different splice site mutations occurred in each variant. In the first variant, the mutation affected the invariant G residue of the 3′-acceptor splice site of intron 5 (variant B.S.), while the second mutation affected the invariant G residue of the 5′-donor splice site of intron 7 (variant L.P.). These mutations caused the skipping of exon 6 and 7, respectively, as seen by sequence analysis of the Jknull transcripts present in reticulocytes. Expression studies in Xenopus oocytes demonstrated that the truncated proteins encoded by the spliced transcripts did not mediate a facilitated urea transport compared with the wild type Kidd/urea transporter protein and were not expressed on the oocyte’s plasma membrane. These findings provide a rational explanation for the lack of Kidd/urea transporter protein and defect in urea transport of Jknull cells.

The Kidd blood group system (JK) is defined by two codominant alleles, Jka and Jkb, of similar frequency (0.51 and 0.49, respectively) in the Caucasian population, but showing large differences in other ethnic groups (1, 2). Allantibodies against the Jk antigens may occasionally be involved in severe transfusion incompatibilities and newborn hemolytic disease. There are three common phenotypes Jka(a+b−), Jka(a−b−), and Jka(a+b−) and a rare null phenotype, Jka(a−b−), first described by Pinkerton et al. (3), also called Jknull. The frequency of this phenotype is increased in certain populations (Asian, Polynesian, or Indian extraction). The Jknull phenotype results from two different genetic backgrounds: (i) homozygous inheritance of a “silent” allele Jk at the JK locus and (ii) inheritance of a dominant inhibitor gene In(Jk), unlinked to the JK locus (1, 2). Following immunization by transfusion or pregnancy, Jknull individuals may produce an antibody called anti-Jka (or anti-Jkb), which reacts with all common red cells carrying the Jka and/or Jkb antigens, but is unreactive with Jknull cells themselves.

The discovery that red cells from Jknull individuals exhibited an increased resistance to lysis in aqueous 2 M urea (4) led to the suspicion that the Jk antigens might be related to the urea transporter of the human erythrocytes (for a review, see Moulds (5)). This prediction was fully confirmed by molecular cloning of the human erythroid urea transporter (clone HUT11) (6), by cross-hybridization with a rabbit cDNA transporter (7), and the demonstration that the HUT11 urea transporter and the Kidd blood group are carried by the same protein (8). This is based on the following findings: (i) in coupled transcription-translation assays, the HUT11 cDNA directed the synthesis of a 36-kDa protein, which was immunoprecipitated by a human anti-Jkb antibody; (ii) the anti-Jka immunoprecipitated also a protein material of similar mass from all red cell membranes (after N-glycanase treatment), except those from Jknull cells; (iii) a rabbit antibody against the HUT11-protein reacted on immunoblots with all human erythrocytes except those from Jknull cells; and (iv) the structural gene encoding HUT11 was assigned to chromosome 18q12-q21 by in situ hybridization, like the Kidd blood group locus. More recently, the Kidd blood group Jka/Jkb polymorphism (D280N) was determined and used to demonstrate its lack of association with type 1 diabetes mellitus (9).

The Kidd/HUT11 polypeptide is expressed on human red cells as well as on the endothelial cells of vasa recta in the inner and outer medulla of the kidney (10, 11). Rapid urea transport may help to preserve the osmotic stability and deformability of the red cells and to stabilize osmotic gradients in the renal medulla (12, 13). In the kidney, this transport system contributes to the urinary concentrating mechanism (14, 15) involved in water preservation. Recently, a new urea transporter specific for the human kidney (clone HUT2) was cloned (16) and functionally compared with the erythroid transporter (17).
with SacI restriction enzyme according to the supplier (10 units/µg of DNA), resolved by electrophoresis in 0.8% (w/v) agarose gel and transferred to a nylon membrane (Hybond N+, Amersham, UK). The blot was prehybridized in 0.25 M Na2HPO4 (pH 7.2), 7% SDS for 5 min at 65 °C and then hybridized using a full-length [32P]-labeled cDNA probe (exon 1–5) to end exon 11 in the same buffer. Hybridization was carried overnight at 65 °C, and the last washing was in 0.02 M Na2HPO4 (pH 7.2), 3% SDS for 20 min at 65 °C.

**Amplification by Reverse Transcription-PCR of Jk cDNAs**—Five micrograms of total reticulocyte RNA extracted by the acid-phenol-ammomum method (22) were used to produce the first cDNA strands using the Avian Myeloblastosis Virus (AMV) reverse transcriptase kit (Pharmacia, Uppsala, Sweden). One sixth of the cDNA products was used to perform a hemi-nested PCR (94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min 15 s, for 30 cycles) in a first step between primers SP-A (sense primer, position 21–1 to 21) and AS-B (antisense primer, position 1260–1237). The second PCR was performed with 12/25 of the first PCR products in the same conditions, using primers SP-A and AS-C (antisense primer, position 1234–1211). Final PCR products were identified by Southern blot analysis, subcloned, and sequenced on both strands, using an automated Alf-Express sequencer (Pharmacia, Uppsala, Sweden).

**Analysis of Splice Sites**—Direct PCR amplification was carried on genomic DNA (100 ng) under stringent conditions (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, for 30 cycles) between primers designed from intronic sequences flanking each exon. The PCR products were subcloned and sequenced. To avoid PCR artifacts, sequencing was performed on both strands using independent PCR reactions.

**Transcription-Translation and Immunoprecipitation Assays**—Full-length (Jk*) and spliceforms (Jk*(J6) and Jk*(J7)) cDNAs were subcloned into the EcoRI-digested pTTTS plasmid (kindly provided by P. Krieg, Austin, TX) and placed under the control of the T7 promoter. The corresponding proteins were synthesized in vitro in the transcription-translation-coupled reticulocyte lysate system from Promega (Madison, WI) in the presence of [35S]methionine (1.85 Gbq/mmol, Amersham, Bucks, UK) and immunoprecipitated with the human anti-Jk3 antisem antibody obtained from an immunized Jk(a–b–) individual and with an affinity-purified polyclonal antibody raised against the N-terminal region of the Jk co-transporters (88, 92–94). Approximately 106 phages from EMBL3/Sp6/T7 (CLONTECH Laboratories, Inc., Palo Alto, CA) were plated and hybridized under standard procedures with a [32P]-labeled full-length HUT111 cDNA probe (1–1268) using the random primed DNA labeling kit (Boehringer Mannheim, Germany) and Southern blot hybridization using HUT111 and the 5′-end region (see below) as probes. The positive individual gene fragments were subcloned into pUC vector and sequenced. The exons, identified from the HUT111 cDNA sequence, and their flanking regions, were fully sequenced as were some short introns. The introns were sized by PCR using primers flanking regions of the intron. At least, all sizes were confirmed by PCR using human genomic DNA as template and Expand Long Template PCR system.

**Northern Blot Analysis—Poly(A)+ RNA** FROM HUMAN FETAL LIVER (CLONTECH) or total RNAs isolated from six injected oocytes according to Chomczynski and Sacchi (21), were resolved by electrophoresis on 6% (w/v) formaldehyde, 1% (w/v) agarose gel, and transferred to nylon filters (Zeta-probe GT, Bio-Rad). Hybridization with [32P]-labeled probes was carried at 65 °C in 0.25 M Na2HPO4, 7% (w/v) SDS. Stringent washes were performed in 0.02 M Na2HPO4, 1% (w/v) SDS at 65 °C for 30 min and exposed to Biomax-MR film with intensifying screens at −80°C.

**Southern Blot Analysis**—Total genomic DNA from B lymphoid cell lines (Epstein-Barr virus-transformed) or from leukocytes was digested

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1 The abbreviations used are: PCR, polymerase chain reaction; RACE, 5′-rapid amplification of cDNA ends; UT, untranslated; SP, sense primer; AP, antisense primer; nt, nucleotide; bp, base pair(s); kb, kilobase pair(s).
the 5'-UT part of the gene (see below) and the remaining eight exons the open reading frame. The translation initiation codon was located in exon 4 and the stop codon in exon 11. Thus, exons 4–11 corresponded to amino acids 1–50, 51–113, 114–164, 157–221, 222–270, 271–315, 316–332, and 333–389, respectively (Fig. 1B). The intron sizes determined by PCR, ranged from 0.6 kb to approximately 8.6 kb. After partial sequence of the introns, all exon/intron junctions were found to contain the canonical 5'-donor gt and the 3'-acceptor ag sequences (Fig. 1B). Each class of intron-exon boundary was found in the JK gene. When all exons were sequenced and compared with the published HUT11 cDNA isolated from a human bone marrow library, two differences, one amino acid change (K44E) and one dipeptide deletion were noted in exon 4 (Fig. 2). This was confirmed by Southern blot with an exon 3-specific probe which hybridized with the ~430-bp but not the ~270-bp fragments (not shown). All of this information was used to deduce the structure organization of the three first exons of the JK gene described above. Further studies (reverse transcription-PCR) of the transcripts isolated from normal human reticulocytes indicated the presence of alternatively spliced transcripts lacking sequences encoded by exons 8 and 9, and exons 7–9 (data not shown).

Next, 500 bp upstream of the erythroid transcription initiation site from the Jk2 genomic clone was sequenced and analyzed to identify putative cis-acting regulatory elements. This region (nt −837 to −336) contains the consensus motifs AP-3, NF-ATp, and GATA-1 in reverse orientation at position −529/−522, −586/−581, and −806/−801, respectively. Sequence deviating by one nucleotide from the consensus motifs Ets-1, AP-2, reverse Ets-1, CTCF, and a reverse SP-1 were identified between positions −587 and −794 (Fig. 2). A typical TATA box was also present at nt −362/−358, which is in the expected range of 25 to 30 nucleotides upstream of the erythroid transcription initiation site (nt −335). Although two inverted CAAT boxes (ATTG) were identified at nt −195 and −220, only the closest of transcription initiation site might be functional.

Characterization of the 3'-UT Region of the Jk Transcripts—The transcription start site of the JK gene is short (335 bp upstream of the translation initiation site) and cannot account for the size difference between the two major transcripts (~2.0 and ~4.4 kb) detected in erythroid tissues by Northern blot (16). Therefore, we examined whether these differences arose from the 3' end of the JK transcripts. At first, two consensus polyadenylation signals (AATTAAA and TATAAA) and four consensus sites (AATAAA) have been located by sequence analysis of 3'-RACE-PCR amplifications performed with reticulocyte transcripts (see "Materials and Methods" and by sequence
analysis of the 3'-end from the Jk12 genomic clone (Fig. 3).

Then, Northern blot analysis using the HUT11 probe as well as probes p1, p2, and p3 located in the 3'-UT region of the JK gene clearly indicated that the two erythroid transcripts of 4.4 and 2.0 kb arose from differential usage of two distinct polyadenylation signals which are separated by 2.0 kb. Indeed, the HUT11 probe located 5' to the proximal signal AATTAAA hybridized both with the 4.4- and 2.2-kb transcripts, while probes p1, p2, and p3, located downstream this signal hybridize only with the 4.0-kb species (Fig. 3). The colinearity of the full 3'-end region (exon 11) was confirmed by hemi-nested reverse transcription-PCR and hybridization of the 2.7-kb reaction product with the HUT11 probe (see Fig. 3).

The JK Gene Is Not Rearranged in Jk null Cells—In preliminary investigations, we confirmed by Western blot analysis with the affinity-purified anti-HUT11 antibody (nonglycosylation dependent) (10), that the Jk null cells under study (B.S. and L.P.) lack the Jk erythrocyte membrane protein (45–69 kDa), as seen in Fig. 4A, as well as the Jka and Jkb antigens (data not shown). Next, we demonstrated by Southern blot hybridization of genomic DNA an identical SacI digestion pattern of the Jk null samples as compared with common Jk(a1b2) and Jk(a2b1) phenotypes (Fig. 4B), indicating that a gross rearrangement of JK gene did not occur in these variants. The five detected bands (ranging from 2 to 7 kb) are fully concordant with the known restriction sites in the JK gene (see Fig. 1) and altogether contain all 11 exons (Fig. 4A). Moreover, restriction analysis with a number of other enzymes showed a simple pattern consistent with a single copy gene (data not shown). Further analysis by DNA genotyping (9) indicated that both Jk null individuals were homozygous for a silent Jkb allele (data not shown).

Molecular Analysis of the Jk null Mutations—To determine the molecular basis of the Jk null phenotypes, total reticulocyte RNA from donors B.S. and L.P. were prepared and used to amplify full-length Jk transcripts. Three amplification products of different size (not shown) were obtained from the B.S. sample, and several independent clones of each size were se-
quenced. We identified spliceforms that resulted from the alternative splicing of exon 6 (Jk\(^{-}\Delta 6\)) and exons 6, 8, and 9 (Jk\(^{-}\Delta 6, 8,\) and \(9\)) and Jkb\(^{-}\Delta 7\)) transcripts that affected the invariant G residue of the 5′-donor splice site of intron 7 (Fig. 4D). The protein isoform encoded by the Jk\(^{-}\Delta 7\) transcript would lack amino acids 157–221 and include new C-ter residues generated by a frameshift and premature termination. In all instances, the protein isoforms potentially encoded by the spliced transcripts identified in B.S and L.P. were not detected on red cells by Western blot analysis with the anti-HUT11 antibody (see above).

Expression and Functional Analysis of Jk\(^{-}\Delta 6\) and Jk\(^{-}\Delta 7\) Protein Isoforms—To understand why Jk\(_{null}\) red cells lack Jk polypeptides and exhibit a defective urea transport activity, we examined whether the Jk\(^{-}\Delta 6\) and Jk\(^{-}\Delta 7\) transcripts characteristic of these cells could be expressed in vivo and in vitro.

In the cell-free transcription-translation coupled system, the plasmids pT7TS-Jk\(^{-}\), -Jk\(^{-}\Delta 6\), and -Jk\(^{-}\Delta 7\) directed the synthesis of 36-, 31-, and 17-kDa protein bands, respectively, which were immunoprecipitated with the affinity-purified anti-HUT11 antibody and with the human anti-Jk\(^{-}\)antibody (Fig. 6). No radioactive material in these regions could be immunoprecipitated from transcription-translation of the luciferase peptide control vector.

To test whether these Jk\(^{-}\Delta 6\) and Jk\(^{-}\Delta 7\) proteins were expressed as functional urea transporters, cRNA transcripts were synthesized and injected into Xenopus oocytes. Three days later, we found that [\(^{14}\)C]urea uptake of Jk\(^{-}\Delta 6\) and Jk\(^{-}\Delta 7\) cRNA-injected oocytes was not different from that of water-injected control oocytes, whereas the [\(^{14}\)C]urea uptake of Jk\(^{-}\)cRNA-injected oocytes reached 38 pmol/oocyte after 3 min (Fig. 7A). Northern blot analysis also revealed that all cRNA...
**Kidd / Urea Transporter Gene and Jknull Phenotypes**

**Fig. 5. Partial sequences of PCR-products from Jk+ and Jk− transcripts.** Nucleotide sequence of the Jk transcripts isolated from a control donor (Jk+) and from Jknull individuals in the regions of exon/intron junctions 5/6, 6/7, and 7/8. The display of the sequence diagram is from the Alf-Express DNA sequencer. Top, 5/6, 6/7, and 7/8 exon junctions found in normal Jk+ individuals (control). Below, the 6/7 exon junction found in the Jk− (Δ6) spliceoform from Jknull B.S. and the 6/8 exon junction found in the Jk− (Δ7) spliceoform from Jknull L.P.

**Fig. 6. Expression studies in transcription-translation-coupled reticulocyte lysate system.** Autoradiogram of [35S]methionine-labeled proteins immunoprecipitated with the human anti-Jk3 and with the affinity-purified rabbit antibody raised against the N-terminus of the Kidd/urea transporter protein (HUT11) and analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 5, immunoprecipitates from pT7TS-Jk−; lanes 2 and 6, immunoprecipitates from pT7TS-Jk−(Δ6); lanes 3 and 7, immunoprecipitates from pT7TS-Jk−(Δ7); lanes 4 and 8, immunoprecipitates from luciferase control (Ctrl). Band intensity is proportional to the number of [35S]methionine/polypeptide (the Jk+; Jk−(Δ6), and Jk−(Δ7) contain 20, 17, and 8 methionines, respectively). Arrows on the left refer to product size (kDa).

Injected oocytes showed a stable specific signal between day 0 and 3 after injection (see Fig. 7B). Protein expression in oocyte plasma membranes was also analyzed by Western blot analysis with the affinity-purified anti-HUT11 antibody. We found that a strongly reactive band of 46–69 kDa was present in control oocytes expressing the functional Jk+ protein, the size of which was reduced to 36 kDa after N-glycosidase F treatment (Fig. 7C). However, using oocytes injected with the Jk−(Δ6) and Jk−(Δ7) cRNAs, no signal could be detected by Western blotting, neither in the total cell lysates (not shown) nor in the plasma membrane fraction (Fig. 7C).

**DISCUSSION**

The studies reported here define the structural organization of the JK gene which encodes the human Kidd blood group/urea transporter protein. This gene spans 30 kb of DNA and consisted of 11 exons, of which exons 4–11 contained all the coding information for the mature protein. Exons 4–11 appear as being distributed along the gene into two groups of two times two exons separated by a large intronic sequence (E4, E5 and E6, E7, then E8, E9 and E10, E11), evocative of an internal gene duplication. Indeed, this may parallel the topology of the Jk polypeptide which can be subdivided into two homologous hydrophobic parts, each carrying a LP box (LPXTGXF) encoded by exons 7 and 11, respectively, and previously reported to be an internal duplicated signature sequence of urea transporters (26).

The erythroid transcription initiation site of the JK gene was identified 335 bp upstream from the transcription initiation site. Examination of nucleotide sequences that are immediately upstream revealed a typical TATA box, one inverted CAAT box at the expected position, and several putative cis-regulatory elements that may bind a variety of transcription factors (27), among which are those involved in erythroid/megakaryocytic expression (28, 29). The presence of a potential binding site for a NF-ATp factor which regulates the inducible expression of several cytokine genes is intriguing (30, 31). However, functional analysis will be required to determine which elements and which factors bind to this promoter in tissues were the Kidd/urea transporter is expressed.

We have also shown that the 4.4- and 2.0-kb erythroid Kidd/urea transport mRNAs detected by Northern blot (16) arise from usage of two different polyadenylation signals, indicating that the two equally abundant transcripts encode the same 45-kDa polypeptide. This is at the opposite to UT1 and UT2 urea transport transcripts from rat kidney (4.0 and 2.9 kb, respectively), which are alternative splice products derived from a single gene by differential utilization of alternative 5′-exon groups (19). The rat homologue of HUT11, called UT3 (20), is encoded by a single 3.8-kb transcript, which is translated into a protein of 384 amino acids sharing 80% identity with HUT11. It is possible that the first polyadenylation signal used in the human primary transcript is absent or not used in the rat. The role of large 3′-UT sequences in some transcripts is not well understood, although some may play a role in regulation of expression (32, 33).

We next examined blood from two rare unrelated Jknull individuals, one Caucasian (L.P.) and another of Chinese (B.S.)
origin, that lacked Jk antigens and Jk protein expression on red cells. Genomic DNA analysis indicated that both donors had an allele that exhibited no alteration to the Kidd/HUT11 transcript (6, 8). Six individual oocytes were counted for each incubation. Mean and S.E. from two different experiments are shown. A, Northern blot analysis of cRNA encoding Jk\(^{+}\), Jk\(^{\Delta6}\), and Jk\(^{\Delta7}\) in oocytes. Total oocyte RNAs (15 \(\mu\)g/lane) at the day of injection and 3 days after were separated and hybridized with the \(^{32}\)P-labeled HUT11 cDNA probe, as described under "Materials and Methods." Equal loading and absence of degradation were checked by staining with ethidium bromide. Autoradiography was for 1 h at \(-80^\circ\)C. C, immunoblot analysis of fraction enriched for plasma membranes of oocytes prepared 3 days after injection of water (control) or cRNA encoding for Jknull. Untreated and N-glycosidase F-treated plasma membranes equivalent of six oocytes were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with the rabbit antibody against the N-terminus of the coding sequence. Therefore, although the gene further revealed that B.S. and L.P. indicated that alternatively spliced transcripts lacking at least exon 6 and exon 7, respectively, were present. Examination of exon/intron junctions of the Jk gene further revealed that B.S. and L.P. were homozygous for point mutations at conserved 3' acceptor (ag \(\rightarrow\) aa) and 5' donor (gt \(\rightarrow\) tt) splice sites of introns 5 and 7, respectively. Splice site mutations lead to exon skipping and are well known to abolish or reduce normal splicing (see Maquat (34) and references therein). Since the Kidd/urea transporter protein is absent from Jknull cells, it is likely that the spliced transcripts are either unstable and not translated, or the corresponding truncated proteins are misrouted. At first, we found that the Jk\(^{\Delta6}\) and Jk\(^{\Delta7}\) transcripts typical of Jknull cells could be translated into polypeptides of 31 and 17 kDa, respectively, as seen by immunoprecipitation with specific antibodies in a cell-free transcription-translation coupled system (Fig. 6). Next, we found that when expressed in Xenopus oocytes these truncated proteins did not mediate a facilitated urea transport, in contrast to the full-length wild type Jk\(^{+}\) protein, although all injected cRNAs had a similar stability on a 3 days period, as seen by Northern blot analysis. Further analysis revealed that the plasma membrane fraction from oocytes expressing the functional urea transporter (encoded by the Jk\(^{+}\) cRNA) carried a 46–69-kDa glycoprotein component, which could be deglycosylated into a 36-kDa protein, as expected for the Kidd/urea transporter protein (6, 8). On the contrary, the Jk\(^{\Delta6}\) and Jk\(^{\Delta7}\) polypeptides were neither detected in total cell lysates nor in the enriched plasma membrane fraction from the oocytes, which could be explained by a rapid intracellular degradation. Indeed, the predicted truncated proteins are most likely misfolded by lack the transmembrane domains 3 and 4, and transmembrane domain 5, including the hydrophilic loop carrying the N-glycosylation site at Asn\(^{242}\), respectively. Therefore, these findings provide a rationale explanation for the lack of Kidd/urea transporter protein and defect in urea transport of Jknull cells.

As the Kidd/HUT11 transcript is distributed widely in various organs (16), it is surprising that Jknull individuals who have a urea transport deficiency (35) did not suffer a clinical syndrome, except for a reduced capability to concentrate urine (36), as was the unexpected finding that donors of the blood group Colton null phenotype, who lack the water channel aquaporin-1, did not produce a severe or lethal phenotype (37, 38). It is postulated that mechanisms which compensate or reduplicate the function of the missing protein may exist. This should stimulate more studies of these transporters in red cell membranes and various organs. In addition, further investigations of other Jknull individuals from different ethnic groups (5), from both physiological and fundamental aspects, may provide new information for addressing these issues.

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