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Genomic Organization of the Human FXYD2 Gene Encoding the γ Subunit of the Na,K-ATPase

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Although the γ subunit of the Na,K-ATPase has only 66 or 68 amino acids, its human gene (FXYD2) was found to span 9.2 kb and have seven exons, including two alternatively spliced exons encoding different N-termini. Two candidate promoters with consensus sites for transcription factors Sp1, AP-1, and AP-2 are present, consistent with independent transcription of the splice variants. Multiple ESTs support the transcriptional competence of the identified gene elements. In the FXYD2 gene, there are two closely spaced polyadenylation signals, and both are used. A proposed third splice variant encoding a 31-residue N-terminal extension was not found in the gene, nor was the predicted larger protein found in human kidney Na,K-ATPase. Instead, evidence was found for the origin of the larger cDNA clone in homologous recombination with unrelated DNA from chromosome 2. FXYD2 is on chromosome 11q23 close to a site of tumorigenic chromosomal translocations, and has a number of repeat elements.

Key Words: **sodium potassium ATPase; FXYD gene family; genomic DNA; exon; intron; promoter; alternative splicing; ESTs; human.**

The γ subunit of the Na,K-ATPase is a small, single-span membrane protein that regulates the functional properties of the enzyme (1–4). It is expressed in only certain tissues, most notably the kidney (1, 5), but it is not found in all segments of the nephron (2). In transfected cells, it reduces the Na,K-ATPase's affinity for Na^+ and K^+ (2). Na,K-ATPase in certain renal segments, such as the cortical thick ascending limb and the collecting tubule, has a higher affinity for Na^+ than in proximal tubule or medullary thick ascending limb [references in (2)], and we found γ expressed in proximal tubule and medullary thick ascending limb, where the affinity is low, and not in cortical thick ascending limb and collecting tubule, where the affinity is high (2) (Wetzel and Sweadner, manuscript in preparation). The expression of the protein is thus important for understanding renal physiology, but the structure and regulation of the gene has not been investigated.

The gene for the γ subunit, FXYD2, is part of a gene family with at least seven members in mammals (6). The gene family has a 35 amino acid signature sequence that includes seven invariant amino acids. The short motif PFXYD (Pro-Phe-X-Tyr-Asp) at the beginning of the signature sequence is invariant in all known examples in mammals. Because the various known members of the family have different names (phospholemman, gamma, MAT-8, CHIF, and RIC), and because the physiological roles of most are not known, the gene symbol for the family is based on the signature sequence: FXYD. FXYD2 has been mapped to chromosome 11q23 by radiation hybridization mapping (GenMap '99; www.ncbi.nlm.nih.gov/genemap/; stSG26073 and SGC33973).

MATERIALS AND METHODS

Genomic sequence was obtained from GenBank htgs (high-throughput genomic sequence), GSS (genome survey sequence), and dbSTS (sequence-tagged sites) entries. The principal htgs entries used were AP000834, AP000683, AP000757, and AP001554 (authored by M. Hattori, K. Ishii, A. Toyoda, T. D. Taylor, P. Hong-Seog, A. Fujiyama, T. Yada, Y. Totoki, H. Watanabe, and Y. Sakaki, Genomic Sciences Center, Kitasato, Japan). The sequence found on chromosome 4 was in AC021929 (authored by B. Birren, L. Linten, C. Nusbaum, E. Lander, and 84 other authors, Whitehead Institute, Cambridge, MA). Sequence assembly was performed iteratively after identifying positive htgs clones by BLAST analysis (www.ncbi.nlm.nih.gov:80/blast/) and BLAST2sequence analysis (www.ncbi.nlm.nih.gov/gorf/bl2.html) with full-length cDNA consensus sequences for human FXYD2a and FXYD2b (6). This resulted in identification of coding regions. All htgs entries consisted of unordered pieces, and the final order of gene elements was determined with overlapping sequences. The complete gene, with 6.6 kb of 5′ flanking sequence and 5.6 kb of flanking 3′ sequence is in GenBank Accession No. AF316896.

Exon–intron junctions were identified by comparison with the consensus cDNA sequences and verified with geneid (www1.imim.es/geneid.html) scoring of splice consensus sites. Promoter elements were detected with the ProScan utility of BIMAS, the BioInformatics and Molecular Analysis Section of the NIH (bimas.cit.nih.gov/molbio/

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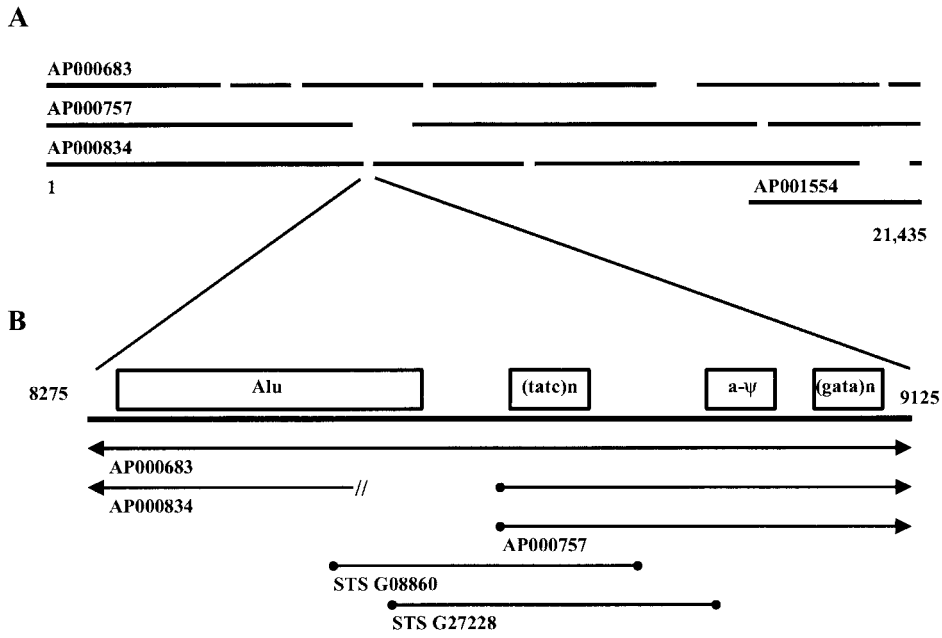

FIG. 1. Assembly of the FXYD2 gene sequence. (A) The sequence was assembled from overlap between htgs entries AP000683, AP000757, AP000834, and (in the 3′ end only) AP001554. The numbering of the sequence is from the numbering of the deposited FXYD2 gene sequence. Overlap at all positions was 2–4 sequences except for one short gap where only one htgs clone provided sequence. This portion lies between the γ-b and γ-a exons. (B) The region where only one htgs entry provided sequence is expanded. Above the line are boxes representing an Alu repeat, two inverse tetranucleotide repeats, and a pseudo-exon derived from the FXYD2 gene. Htgs sequences that end in arrowheads continue in the indicated direction. Those that end in balls terminate at that point. The one that ends in double-slash (//) continues as a chimera with a region with several concatenated Alu repeats. To check the AP000683 sequence that is not included in the other two htgs entries, we located two STS entries (G08860 and G27228) that did overlap it, as shown. Consequently the entire sequence was verified.

proscan/). GrailEXP analysis (grail.lsd.ornl.gov/grailexp/), of the Oak Ridge National Laboratory, confirmed the deduced gene structure, including that of the splice variant. Transcripts of both splice variants were identified in the human EST (expressed sequence tag) database of NCBI. To identify adjacent expressed genes, flanking 5′ and 3′ gene sequence was also examined for ESTs.

Na,K-ATPase-enriched membranes were obtained from kidneys from rats and a human cadaver (cause of death: trauma) by differential centrifugation and detergent extraction (2). Gel electrophoresis was with the Tricine–SDS gel system that separates low-molecular-weight proteins (7) and immunoblotting was performed as described elsewhere (2). The antibody was RCT-G1, prepared against a C-terminal sequence of rat γ (2).

RESULTS

Features of the Gene

The sequence was assembled principally from four htgs entries containing unordered fragments of clones from chromosome 11 (Fig. 1A). Overlap between source sequences was very extensive, and gaps in any given htgs entry were covered by one or more other entries except in one location. Its sequence had a high concentration of repeat elements (an Alu repeat, and two inverse tetranucleotide repeats, mostly TATC/GATA, of 22–26 units) (Fig. 1B). The existence of the repeats had complicated assembly of the unordered pieces, since one of the unordered pieces (shown ending with //) proved to be an assembly chimera formed with a region of concatenated Alu's in a different unordered piece, and two others terminated in a tetranucleotide repeat. We were able to verify the sequence in question, however, with the aid of two overlapping STS (sequence tagged site) entries. As a consequence, the deposited sequence, AF316896, is contiguous and has a high level of confidence for 21.4 kb, including the 9.2 kb of FXYD2.

Figure 2A shows the deduced amino acid sequences of the two splice variants of the human Na,K-ATPase γ subunit. Figure 2B shows the organization of the human FXYD2 gene. Each exon is labeled with its content, to compare with Fig. 2A. Sizes of exons and introns are indicated above and below the line, respectively. Figure 2C shows the splicing pattern deduced from ESTs and from analysis of splice junctions. Canonical splice junctions were found for the constructs diagrammed (Table 1). In addition, a 3′ splice acceptor site was found at the beginning of the γ-a exon, but it is not certain whether this is used since no γ-a transcripts were found with additional 5′ sequence. The predicted transcript sizes are 0.55 and 0.56 kb, consistent with the single 0.7 kb transcript reported for Northern blots of human tissues (8).

Common promoter elements were detected (Fig. 2B). No TATA or CCAAT sequences were observed for the

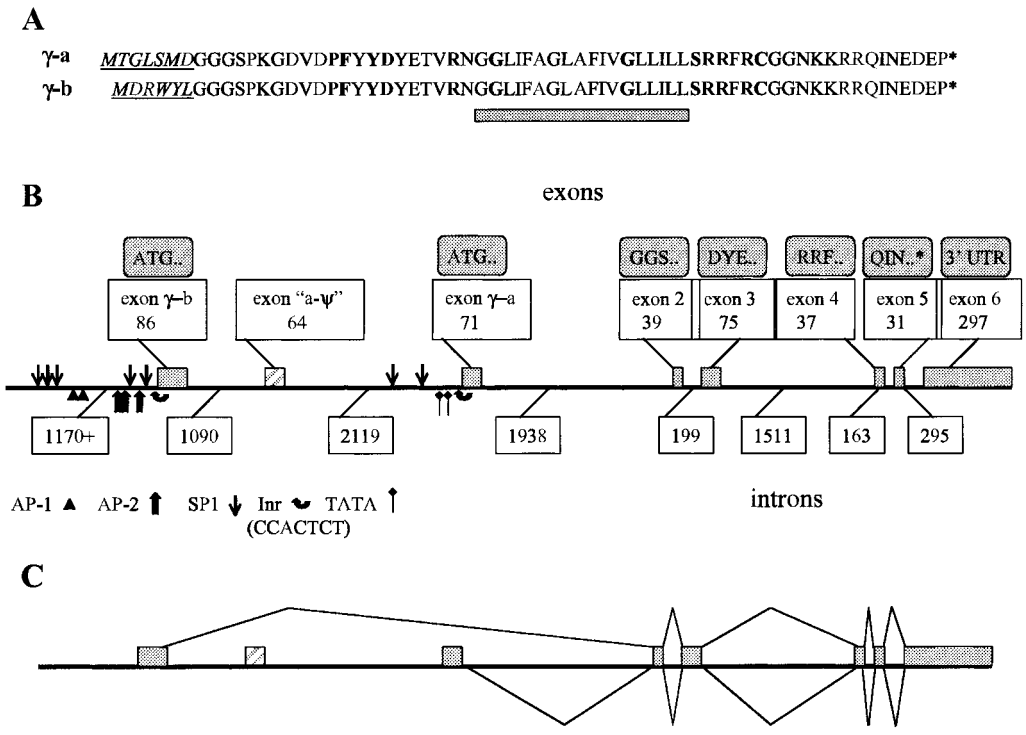

FIG. 2. Exon organization of the human FXYD2 gene. (A) Amino acid sequences of the human γ subunit splice variants. The bar marks the hydrophobic membrane span, and residues in boldface are invariant in the signature sequence that defines the FXYD gene family. (B) Human FXYD2 gene. Exons and introns are indicated along with their lengths. Exons γ-a and γ-b encode the γ-a and γ-b alternative N-termini. Promoter regions in front of exons γ-b and γ-a are indicated, with predicted transcription factor binding sites. Evidence for promoter elements in front of the γ-a exon is weaker than for the γ-b exon. There are two functional polyadenylation sites near the end of exon 6. (C) The splicing patterns of the cDNAs for γ-a and γ-b are shown.

γ-b exon, but there was a typical initiation site (Inr) sequence (CCACTCT). Its adenine was 9 bases upstream of the longest ESTs, and it is the probable transcription initiation site. ESTs began either at CCAGGCCCCAGG or at the second CCAGG. A promoter region was detected from −1180 to −100 bases from the exon γ-b transcription start site. This contained five consensus sequences for Sp1, two for AP-1, and three for AP-2.

No promoter region was detected 5′ to exon γ-a by the ProScan program. Inspection, however, revealed a TAAATA and two TAAT sequences at −88 to −48, as well as a possible Inr consensus (ACACTCT) at −18, and possible Sp1 sites at −283 and −650 (Fig. 2B). Alternative transcription initiation at exon γ-a is a credible hypothesis, but the density of common promoter elements was much lower than for exon γ-b, and the sequences might be of only evolutionary significance. GrailEXP, however, scores the transcript beginning with exon γ-a as the principal gene product.

An unusual copy of the γ-a exon was found in the FXYD2 gene (Fig. 1B). In the first intron, an Alu repeat 456 bases after the end of the γ-b exon was closely followed by the inverted pair of tetranucleotide repeats, and between them was the copy of the γ-a exon. The copy had the same coding sequence as the regular γ-a exon further downstream, but the sequence that corresponds to the 5′UTR had a number of replacements, and no ESTs containing these replacements were detected in the EST database. The copy had an intact splice site at the beginning, but was truncated by the tetranucleotide repeat that followed it and had

TABLE 1

Exon–Intron Junctions in FXYD2

Splice	5′ splice donor	3′ splice acceptor
γ-b/2	ACCTGG **gt**gagt	tcct**ag** GCGGCA
?/γ-a		aagc**ag** AGACAG
γ-a/2	ACGGTG **gt**gagt	tcct**ag** GCGGCA
2/3	ACTATG **gt**aagc	ccgg**ag** ACTATG
3/4	TCCTCA **gt**aagt	ttca**ag** GCAGAA
4/5	GCGGAG **gt**gagc	cacc**ag** GCAAAT
5/6	CAGCAG **gt**atgc	tctc**ag** CCTCGG
6	AGCCGG **gt**ctca	

Note. Splice donor and acceptor sites observed in human FXYD2 are listed. Although the γ-a exon has promoter elements that might make it transcriptionally competent, it also has a 3′ acceptor site. Other than the beginning of the first exon, γ-b, all exons are bordered by cannonical donor and acceptor sites.

no splice site at the end. This is marked as a pseudo-exon in Fig. 1B (exon a-ψ). It is possible that the a-ψ exon and its flanking sequence could form a stem-loop structure, but the inverse repeats are somewhat degraded.

Several other repeat elements were found within the gene. An Alu repeat was found 950 bases upstream of the first promoter region (at −2125 from the γ-b exon). A direct repeat of 89 and 84 bases was found in the fourth intron (between exons 3 and 4), followed by a third Alu repeat after another 270 bases. Additional repeats were found at distant positions in the flanking sequence.

At the 3′ end of the transcript, in exon 6, there were two polyadenylation signals. The one at −102 from the end of the exon, ATAATAAA, was used in 19 ESTs, while the other one at −14 from the end of the exon, ACAAATA, was used in 4 ESTs. The predicted difference in transcript size is very small and is unlikely to be resolved with standard procedures.

Flanking DNA Sequence

We examined 6.6 kb of upstream sequence and 5.6 kb of downstream sequence for additional exons and for adjacent transcriptionally active genes. No other exons or genes scored well with GrailEXP or geneid programs. We used the 5′ and 3′ sequences to BLAST the human EST database to detect any additional transcription activity. There were three positive locations quite far from the FXYD2 gene. Two transcribed sequences in the 5′ end (−7425 and −5364 bases from the γ-b exon transcription start) were single ESTs (AI376999 and AI015894) that did not encode any known protein. One transcribed sequence right at the 3′ end (+5035 from the end of exon 6) (AA868641) was also a single unidentified EST. It appears that there are no genes close to FXYD2. There is other evidence that FXYD6 (a paralog expressed in the nervous system) is linked to FXYD2 on chromosome 11q23 (manuscript in preparation), but it is clearly not in the 21 kb stretch of DNA containing the FXYD2 gene.

Chimeric cDNA

Among the human ESTs for FXYD2, at this time there are 16 for γ-a, 7 for γ-b, and 22 others that contain only shared parts of the transcript. There is a report that human γ has a 31 amino acid N-terminal extension (9) that would presumably be encoded by an additional exon. The cDNA clone that is the source of the sequence (GenBank Accession No. X86400) was obtained in a screen for genes down-regulated in Wilm's tumor, a kidney tumor disease of children (10). Although it does encode the γ subunit of the Na,K-ATPase, the clone has a larger open reading frame in the 3′ strand, and a nonsense protein translation is given in the GenBank and EMBL entries. This is apparently an unintended consequence of EMBL's automatic generation of translations instead of accepting investigator-determined translations. The correct amino acid sequence was published by Minor *et al.* (9), who noted the 31 amino acid extension. Because X86400 is the longest human cDNA for FXYD2 in GenBank, it has been used as the representative clone for computer-generated analyses such as UniGene 19520 in GenBank and THC's (tentative human consensus) in TIGR, and in GeneMap'99.

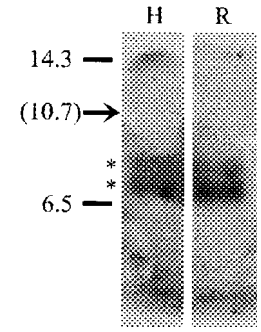

FIG. 3. Identical gel mobilities for human and rat γ subunits. The rat γ subunit migrates as a doublet, and it has been demonstrated by mass spectroscopy analysis that the upper band is γ-a and the lower band γ-b (13). The mobilities, in comparison to molecular weight markers, are 7.9 and 6.9 kDa on Tricine gels. The blot, which was stained with an anti-γ antibody that binds at the C-terminal end of the protein, shows that human renal γ subunit migrates at identical mobilities. A protein of predicted weight of 10.7 kDa (arrow) could have easily been resolved. The positions of molecular weight markers are indicated. Only the bottom portion of the gel's blot is shown because the top portion was stained for Na,K-ATPase α subunit (not shown).

The first indication that the 31 amino acid extension was not transcribed was that it could not be found in the EST database for human FXYD2, and nothing comparable was found for mouse or rat FXYD2 (6). The sequence was also not present in any of the chromosome 11 clones used to compile the FXYD2 gene sequence. When we compared the gel mobilities of rat kidney γ-a and γ-b with that of the human kidney γ doublet (Fig. 3), the mobilities were essentially identical. An extension that increases the size of the protein by almost 50% (10,747 Da instead of 7,341 Da) could have easily been detected because these gels resolve peptides well. Another oddity was that in GeneMap'99, the mapping of the X86400 clone resulted in conflicting assignments to chromosomes 11 and 2. We can now explain the problem (Fig. 4). The sequence following the extension is found on chromosome 11 as described above. The sequence containing the extension, however, was found in two STS entries that mapped to the long arm of chromosome 2. When the longest of these entries was aligned with the cDNA consensus sequence for human FXYD2, there was a 32-base exact overlap with the 5′ UTR of the FXYD2 exon γ-a transcript, GCAGGAAGAGGGGAGTGGAGGCAGCCCATTCA.

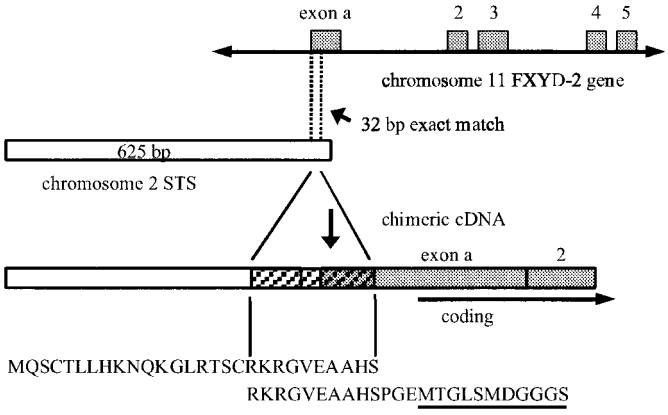

FIG. 4. Cloning origin of a chimeric cDNA. On the top of the diagram, the chromosome 11 genomic sequence of human FXYD2 is diagrammed, covering the portion just before and in the γ-a exon. On the bottom is a 625 base STS sequence (G26731) that maps to chromosome 2. The overlap indicated between them is a 32-nucleotide stretch of identical residues that apparently permitted recombination. The resulting cDNA, X86400, is chimeric.

Apparently the X86400 clone was inadvertently derived by a homologous recombination event. These conclusions can be verified by BLAST search of the htgs database: the chromosome 2 portion of the sequence appears in two htgs entries so far, AC021781 and AC079122.

DISCUSSION

Structure and Transcription of FXYD2

Although it may seem surprising that such a small protein is encoded by such a large gene (9.2 kb) with so many exons, a similar gene organization was determined for another member of the FXYD gene family, mouse FXYD1 or phospholemman (GenBank Accession No. AF091390, work of R. C. Bogaev, Y. M. Kobayashi, J. P. Mounsey, J. R. Moorman, L. R. Jones, and A. L. Tucker, published only in GenBank). In that case, there were a total of 8 exons spanning 4.1 kb of genomic DNA, encoding a protein of 92 amino acids. Many of the exon–intron boundaries are similar in FXYD2 and FXYD1. In contrast, another small membrane protein that regulates the SERCA2a Ca^{2+}-ATPase of cardiac tissue, phospholamban, has no sequence homology to the FXYD family. It is encoded by a single exon and has just one intron in the 5′ UTR sequence (11).

The sequence of the γ-a splice variant was the first to be obtained in several labs (AF129400, for rat, is the most accurate GenBank entry). We first detected splice variants of the Na,K-ATPase as abundant ESTs (12), and verified the authenticity of the rat γ-b splice variant by cloning and sequencing it (GenBank Accession No. AF233060). Independently, Küster *et al.* obtained two N-terminal sequences from mass spectroscopy of purified rat renal γ subunit (13). The N-terminal sequences were identical to those deduced from the ESTs except that rat γ-a protein lacked the initial methionine, presumably cleaved during biosynthesis. The splice variants can account for the doublet of bands seen on SDS gels, although there is also evidence for post-translational modification that alters electrophoretic mobility (2, 5, 13). In recent experiments entailing *in vitro* synthesis of rat γ-a and γ-b, we have observed both intrinsic gel mobility differences and posttranslational modification, and in stable transfectants the structural variants had different functional consequences in the regulation of Na,K-ATPase properties (14). The genetic control of expression of the splice variants thus may be informative.

There are already indications of the importance of the Na,K-ATPase γ subunit in human disease. The initial observation that the Na,K-ATPase γ subunit was down-regulated in Wilms' tumor (10) is logical because many proteins that carry out tissue-specific functions are lost in tumor cells. Very recently, a dominant negative mutation in the human γ subunit has been linked to a human renal disease, hypomagnesemia (15, 16). The mutation is thought to disturb Na,K-ATPase biosynthesis. It is known that γ is also expressed in mouse preimplantation embryos (17). Na,K-ATPase γ subunit expression in rat kidney has been observed to decrease in SHR hypertensive rats, in concert with Na,K-ATPase α subunit (18). The structure of the gene will facilitate future investigation of these roles.

GenBank Entries with Apparently Conflicting Information

In addition to the chimeric cDNA described above, certain other older GenBank entries for Na,K-ATPase γ subunits can cause confusion. A human cDNA for FXYD2 (U50743) did not contain the chimera's upstream sequence, but its authors postulated an unusual translation start based not on methionine but on methionine substitution at a valine codon, resulting in the translated sequence MAAAK*GDVDPFYYDY*. . . (8). This sequence entry, however, was marred by a compression artifact that affected the reading frame, and as noted above the true sequence is MTGLSMDGGGSPK*GDVDPFYYDY*. . . . Similar truncated and out-of-frame sequences were reported for rat and mouse (GenBank Accession Nos. X70062 and X70060), showing that a compression was a common technical problem at this point in the sequence. The corresponding rodent sequences proved to be MTELSANHGGSAK for rat (9, 13, 19), and MAGEISDLSANSGGSAK for mouse (6), rather than MVAVQ.

A final observation is that the GenBank htgs database contains a single clone from chromosome 4 that has copies of exons b, a, 4, 5, and 6 (Accession No.

AC021929). Alignment of their sequences with the chromosome 11 gene and with the EST-derived consensus cDNA sequence reveals some scattered base differences, but on the whole the correspondence is very strong. It is likely that the missing exons are present in the clone, but not yet sequenced, and that some of the sequence differences are insignificant errors due to the preliminary nature of the data. The question is whether the clone is authentically from chromosome 4, because that would suggest that there is a second copy of the FXYD2 gene, either an active copy, or a pseudogene containing introns and without much accumulation of errors. However, no firm evidence was found that the DNA in AC021929 was actually from chromosome 4. When used to BLAST the htgs database, only chromosome 11 hits were found, and two STS sequences within it (G22746 and G24898) were also linked to chromosome 11. A possible explanation is that this region of chromosome 11 is prone to translocations. A locus for (4;11)(q21;q23) chromosome translocations that are common in acute myelogenous and lymphoblastic leukemias is the MLL (HRX) gene, which maps 1.6 Mbp telomeric of FXYD2 (ENTREZ map view, FXYD2 and MLL). Also nearby, but more centromeric, is the locus for BRCA3, site of (11;22)(q23; q11) chromosome translocations. While only particular translocations result in leukemias, a high incidence of benign translocations in the vicinity could have produced an aberrant chromosome 4 BAC clone.

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