The Major Kidney Band 3 Gene Transcript Predicts an Amino-terminal Truncated Band 3 Polypeptide

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Frank C. Brosius III §§, Seth L. Alper §§, Ana Maria Garcia *, and Harvey F. Lodish [*]

From the §Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, the ¶Divisions of Molecular Medicine and Nephrology, Beth Israel Hospital, Boston, Massachusetts 02215, and the ¶Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

We have characterized multiple transcripts from the band 3 gene expressed in rat and mouse kidney. In each species, the major transcript lacks sequence from the first three exons of the band 3 gene. The murine transcript predicts a kidney band 3 polypeptide with a truncated amino terminus, lacking the first 79 amino acids of erythroid band 3. When expressed in Xenopus oocytes this truncated band 3 functions in anion transport.

Basolateral anion exchange in Type A intercalated cells of mammalian kidney collecting duct appears to be mediated by an activity closely related to erythroid band 3 (EB3)

Experimental Procedures

**cDNA Cloning**—The library from male CD-1 mouse kidney (9) has been described. Two unamplified λgt11 male CD-1 mouse kidney cDNA libraries were loaned from R. Woychik. A λgt11 cDNA library from male Sprague-Dawley rat kidney was the gift of M. Mueckler.

**5'-RACE**—Total RNA was isolated from male CD-1 mouse or male Sprague-Dawley rat tissues (15). Poly(A) RNA was prepared by oligo(dT)-cellulose chromatography (16). Northern blots were probed at high stringency as previously described (9) using probes defined in the figure legends. Transcript length was estimated by comparison to a 1-kb DNA ladder (Bethesda Research Laboratories). S1 nuclease protection was performed (17) with uniformly 32P-labeled single-stranded M13 probes purified by denaturing polyacrylamide gel electrophoresis, electrophoresis, phenol-chloroform extraction, and ethanol precipitation. The probes were hybridized to mouse kidney RNA at 50-60 °C overnight. Digestions were performed after hybridization (17) with 40-250 units of S1 nuclease. The digestion products were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. A dyeoxy sequencing ladder or an end-labeled 1-kb DNA ladder served as size standards.

**5'-RACE**—Transcript mapping with RNase H followed the procedures of Majzoub and colleagues (18, 19). Twenty μg of poly(A) RNA or mouse or rat kidney RNA or 1 μg of poly(A) mouse or rat kidney RNA or 1 μg of poly(A) mouse or rat spleen RNA was hybridized at 37 °C to an oligodeoxyribonucleotide complementary to EB3 (nt 214-195). The mixture was digested with 1.6 units of RNase H (40 units/ml final volume) at 37 °C for 1 h. The digestion products were separated on a 20% agarose gel. 5'-RACE transcript mapping was performed by primer extension for 30-60 min at 37-50 °C with 5-40 units of avian myeloblastosis virus reverse transcriptase or 500 units of mouse mammary leukemia virus reverse transcriptase. The primer extension products were purified from an agarose gel, then incubated in DEPC water for 20 min at 70 °C. After phenol-chloroform extraction and ethanol precipitation, the primer extension products were amplified by the polymerase chain reaction (22) using as one primer oligo(dAT)15-18 and as the other primer one of the specific oligomers complementary to EB3 (nt 214-195). The amplified products were probed with agarose gels and subcloned into M13 vectors for dyeoxy sequencing or oligo-labeled for use as probes in Northern blots.

**Expression in Xenopus laevis Oocytes**—Stage V-VI oocytes were prepared in ND-96 buffer as described (23). Oocytes were injected with 50 nL of water, RNA (10-50 ng) transcribed by T3 RNA polymerase from the linearized plasmid containing L2A (Fig. 1), or size-selected (23) poly(A) RNA from mouse spleen. Injected oocytes were incubated in ND-96 for 1-2 days. Groups of 10 oocytes were placed in borosilicate culture tubes and incubated for 1 h in 180 μL of ND-96 containing 5-50 μM DIDS, with or without 1 μM ruthenium red. Oocytes were then assayed for DIDS-sensitive anion uptake.

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The abbreviations used are: EB3, erythroid band 3; KB3, kidney band 3; nt, nucleotide; kb, kilobase; DIDS, 4,4-diisothiocyanato-2,2'-disulfonic stilbene.


2 M. Mueckler, unpublished data.

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FIG. 1.  a, partial restriction map of EB3 showing location of open reading frame (box) and restriction sites present in the KB3 clones (below). Abbreviations: E, EcoRI; Av, AvaI; P, PstI; X, XhoI; Ac, AccI; Hc, HincII; B, BglII; RV, EcoRV; Ap, ApaI; G, BglII; S, Smal; Sph, SphI; N, NstI; H, HindIII. b, schematic diagram of mouse KB3 cDNA clones. Dark stretches were sequenced. Stars at the 5'-end indicate the unshown presence of unrelated or recombined sequence at the 5'-end of the clone.

FIG. 2.  A, Northern blot of poly(A+) RNA from mouse spleen (1 µg, lanes 1 and 3) and mouse kidney (20 µg, lanes 2 and 4) probed with murine EB3 cDNA fragments encoding exons 10–12 (nt 937–1202) or exons 1–3 (nt –126–137). B, Northern blot of poly(A+) RNA from rat spleen (2 µg; lanes 1 and 3) and rat kidney (20 µg; lanes 2 and 4) probed with murine EB3 cDNA fragments encoding exons 12–19 (nt 1203–2623) or exons 1–3 (nt –126–137). C, Northern blot of poly(A+) RNA from mouse spleen (1 µg; lane 1) and mouse kidney (20 µg; lane 2) probed with a genomic fragment encoding exon 2, intron 2, exon 3, and intron 3 (from nt –150 of intron 1 to nt 907 of intron 3) (6).

FIG. 3.  S1 nuclease protection analyses of total mouse kidney RNA (250 µg), mouse spleen poly(A+) RNA (0.4 µg), and tRNA (250 µg). A, probe encoding portions of exons 1–5 (nt –126–306) plus a short M13 sequence. B, probe encoding portions of exons 1–3 (nt –126–137) plus a short M13 sequence.
The radioactive medium was removed and the oocytes washed in 30 ml of chloride-free buffer. Individual oocytes were placed in scintillation vials and counted in ACS (Amersham Corp.). Additional groups of 20 microinjected oocytes were incubated for 16–24 h in ND-96 containing 1–1.5 mCi/ml [35S]methionine. After removal of the labeling medium, oocyte membranes were prepared (24), solubilized, and immunoprecipitated (24) with a polyclonal antiserum prepared against the C-terminal dodecapeptide of EB3 (25).

RESULTS AND DISCUSSION

A total of eight positive mouse KB3 clones and five rat KB3 clones was purified from three mouse kidney and one rat kidney cDNA libraries. Four of the mouse clones are diagrammed in Fig. 1, which shows that the detailed restriction map and the partial sequence of murine KB3 cDNAs are identical to that of EB3 from nt 176 in exon 4 (nt 137–210) of the EB3 sequence to the poly(A) tail. The 5'-end of the 5'-most rat clone corresponds to nt 161 of murine EB3.

An EB3 probe encoding exons 10–12 (Fig. 2A, lane 2) or exons 12–19 (not shown) detected the expected (7) 4.2-kb transcript in mouse kidney as well as the 4.5-kb EB3 mRNA in spleen (lane 1). An EB3 probe encoding exons 12–19 detected two minor transcripts in rat kidney at approximately 4.5 and 4.7 kb and a major transcript of 5.1 kb (lane B2). In contrast, an exon 1–3 probe detected neither the 4.2-kb transcript in mouse (lane A4) nor the 4.5- and 5.1-kb transcripts in rat (lane B4). The exon 1–3 probe did detect a faint mouse transcript at 4.5 kb (evident on longer exposure of the blot, not shown) and the 4.7-kb transcript in rat (lane B4). A band 3 genomic probe encompassing sequences from the 3'-end of intron 1 through the 3'-end of intron 3 also did not detect the major mouse KB3 transcript (Fig. 2C, lane 2). These data show that the major mouse KB3 transcript lacks sequence encoded by band 3 gene exons 1–3 and introns 2 and 3 and that two of the three rat kidney band 3 transcripts also lack sequences corresponding to murine exons 1–3.

The results in mouse were confirmed by S1 nuclease protection assays (Fig. 3). These showed that the entire EB3 portion of a single-stranded cDNA probe encompassing EB3 exons 1–5 was protected by spleen RNA (Fig. 3A). In contrast, only 170 nt were protected by kidney RNA, and no portion of an exon 1–3 probe was protected by kidney RNA (Fig. 3B), although the entirety of the exon 1–3 probe was protected by spleen RNA (Fig. 3B). From the sizes of the protected fragments, we conclude that mouse kidney RNA contains transcripts which include sequences from all of exons 4 and 5.

5'-RNase H mapping experiments were performed by cleaving band 3 mRNA at the binding site of oligomer 3, complementary to EB3 nt 214–195 (bridging exons 4 and 5). This produced a 3'-fragment from each band 3 transcript of approximately 4 kb in length and a 5'-fragment between the oligomer-binding site and the transcript cap site. Northern blots of these digests (Fig. 4) show that the 5'-fragment for mouse and rat EB3 (as measured in mRNA isolated from spleen) was between 300 and 350 nt in length (lanes 1 and 3), which agrees with previous 5'-end mapping of mouse spleen EB3 mRNA (26). The 5'-fragment for mouse kidney was approximately 150 nt in length (lane 2). Therefore, the cap site of the major murine KB3 lies approximately 93 nt 5' to the start of sequence encoded by exon 4. These 93 nt are encoded by alternative exonic sequences but not by DNA between the 3'-end of intron 1 and intron 3, inclusive, of the EB3 gene (Fig. 2C). Oligomer 3 (see "Experimental Procedures") yielded a primer extension product extending 83 nt 5'-ward of the start of exon 4 (data not shown), in general agreement with the RNase H mapping. Oligomer 1 suggested a 5'-extension of 185 nt and oligomer 2, a 145-nt extension.
After polymerase chain reaction amplification of the extension product of oligomer 1, DNA sequencing and Northern blotting revealed it not to be a copy of KB3 mRNA. Attempts at amplifying the other two primer extension products have been unsuccessful.

Fig. 4 also shows that the 5'-end of the major rat KB3 transcript extends approximately 400 nt upstream of oligomer 3 binding site (nt 214-195), a conclusion sustained by primer extension analysis (not shown). Since this transcript (probably the 5.1-kb transcript of Fig. 2B) lacks sequences corresponding to murine exons 1-3, approximately 340 nt at its 5'-end must be derived from alternative exonic sequences. Kudrycki and Shull found that the major rat KB3 mRNA has a 377-nt extension 5' of the start of the rat sequence corresponding to murine exon 4. At least the 349 of those nucleotides present in the cDNA clone are transcribed from the rat equivalent of the 3'-end of murine intron 3, which Fig. 2C shows is absent in mouse KB3 mRNA.

The 5'-most in-frame ATG triplets in the available mouse KB3 cDNA sequence begin at nt 235 and nt 238 of the mouse KB3 cDNA sequence (in exon 5 and approximately 200 nt 3' from the KB3 transcript cap site). The second of these two ATGs lies in a favorable consensus sequence (27) for transcription initiation, ATGATGGAC. A KB3 polypeptide initiating at this codon would lack the first 79 amino acids of EB3 but would be otherwise identical. We microinjected in vitro-transcribed mRNA encoding this truncated KB3 polypeptide into X. laevis oocytes. Injected oocytes synthesized an 80-kDa KB3 polypeptide, shorter than the 95-kDa EB3 polypeptide (Fig. 5, inset). These oocytes carried out isotopic chloride uptake at 6.6 times the rate of water-injected oocytes and at 1.5 times the rate of oocytes injected with size-selected spleen RNA (Fig. 5). The entire increase in chloride uptake was blocked by 1 μM DIDS. Therefore, this putative KB3 polypeptide translocates anions with pharmacologic sensitivity characteristic of EB3.

Kudrycki and Shull have cloned a rat KB3 cDNA which encodes a polypeptide which initiates at the methionine residue corresponding to murine EB3 residue 80 but otherwise is identical to EB3. The corresponding rat KB3 mRNA lacks the rat equivalent of murine exons 1-3. Thus rat and mouse KB3 polypeptides would be homologous along their entire length. The major difference between the mouse and rat KB3 mRNAs resides in the site of transcriptional initiation. In rat, the major KB3 transcript initiates at or before nt -475, corresponding to nt 608 of the 924-nt murine intron 3 (6). In mouse, the major KB3 transcript initiates in an alternative first exon which does not lie within intron 3 (Fig. 2C). Our attempts to localize it elsewhere within introns 1 or 2 or within the 5'-flanking 2.0 kb of the murine band 3 gene (6) have been unsuccessful.

Our findings also suggest the presence of an additional much less abundant mouse KB3 transcript with an apparent mobility of 4.5 kb, which encodes exons 1-3 and likely corresponds to the KB3 transcript mapped by Kopito et al. (26). One of the three detected rat KB3 transcripts (at 4.7 kb) also encodes the rat equivalent of murine exons 1-3. Our major rat KB3 transcript of 5.1 kb likely corresponds to the 4.5-kb transcript of Kudrycki and Shull. The differences in the apparent lengths of rat KB3 mRNAs reported by us and by Kudrycki and Shull probably arise from the use of different size standards and different RNA denaturants in Northern gels.

The use of alternative transcription initiation sites for KB3 mRNA suggests the presence of promoters different from those that regulate EB3 mRNA expression. The alternative promoters could regulate the tissue specificity (or developmental specificity) of expression of the kidney and erythroid forms of band 3. The functional consequences of the amino-terminal truncation of KB3 remain to be determined. The several possibilities include a change in subplasmalemmal compartmentation of enzymes in the Embden-Meyerhof pathway (28, 29), a change in cytoskeletal interactions with the plasma membrane (30, 31), and a change affecting the targeting of KB3 to the basolateral membrane of intercalated cells in kidney (1-4).

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