Rat kidney band 3 Cl^-/HCO_3^- exchanger mRNA is transcribed from an alternative promoter

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Kudrycki, Katarzyna E., and Gary E. Shull. Rat kidney band 3 Cl^-/HCO_3^- exchanger mRNA is transcribed from an alternative promoter. *Am. J. Physiol.* 264 (*Renal Fluid Electrolyte Physiol.* 33): F540–F547, 1993.—We have previously shown that the rat kidney band 3 Cl^-/HCO_3^- exchanger mRNA encodes an NH_2-terminal truncated form of band 3 and that its 5′ end differs from that of the erythrocyte band 3 mRNA (K. E. Kudrycki and G. E. Shull. *J. Biol. Chem.* 264: 8185–8192, 1989). To determine the genetic basis for the alternative kidney and erythroid mRNAs, we *1*) isolated and characterized a rat erythroid band 3 cDNA, *2*) isolated the rat band 3 gene and determined the exon/intron organization of sequences corresponding to the alternative 5′ ends of the rat kidney and erythroid mRNAs, and *3*) identified the transcription initiation sites of the two transcripts. The unique sequences at the 5′ end of the rat erythroid mRNA are derived from exons 1–3 and are followed directly by sequences from exon 4 that are common to both mRNAs. In the kidney mRNA, sequences upstream of exon 4 are derived entirely from intron 3. Primer extension and S_1 nuclease protection analyses demonstrate the presence of multiple transcription initiation sites for the rat erythroid band 3 mRNA at the beginning of exon 1, whereas the transcription initiation site for the kidney mRNA is located within intron 3. Thus two distinct promoters, separated by almost 5 kb of genomic sequence, are responsible for the highly tissue-specific transcription of the alternative rat erythroid and kidney band 3 mRNAs.

anion exchange; intercalated cell; collecting duct; bicarbonate transport

ABSORPTION OF BICARBONATE in the collecting duct of the mammalian kidney is mediated by a Cl^-/HCO_3^- exchanger located in the basolateral membranes of acid-secreting intercalated cells. Polyclonal and monoclonal antibodies to the membrane-spanning regions of the erythrocyte band 3 Cl^-/HCO_3^- exchanger, as well as polyclonal antibodies to the cytoplasmic region, react with the basolateral membrane of a subpopulation of intercalated cells in both rabbit (26) and human (29) collecting ducts. Using colloidal gold immunocytochemistry and transmission electron microscopy, Verlander et al. (28) demonstrated that band 3 immunoreactivity occurred in all intercalated cells of the rat outer medullary collecting duct and in type A cells of the cortical collecting duct, whereas type B intercalated cells, which secrete HCO_3^-, exhibited no immunoreactivity. Although the kidney protein identified in these studies was, in most respects, immunologically identical to erythrocyte band 3, it did not react with three monoclonal antibodies directed toward epitopes in the NH_2-terminal cytoplasmic regions (29), indicating that there are structural differences between the kidney and erythrocyte transporters.

High-stringency Northern hybridization analyses with band 3 cDNA probes demonstrated the existence of an alternative band 3 mRNA in mouse kidney, and it was suggested that this mRNA might encode a variant band 3 protein (1). The subsequent cloning and sequence analysis of a rat kidney band 3 cDNA (5, 16) revealed that kidney band 3 is an NH_2-terminal truncated form of the exchanger that lacks the first 79 amino acids present in erythrocyte band 3 (12). Northern blot analysis shows that the cDNA corresponds to a 4.8-kb mRNA that is the major band 3 mRNA in rat kidney. Exons 1, 2, and 3 of the band 3 gene, which are included in the mouse erythrocyte band 3 mRNA, are not present in the 4.8-kb rat kidney mRNA, nor in the mouse kidney band 3 mRNA, which is smaller than that of the rat and corresponds in size to a minor 4.4-kb mRNA in rat kidney (1, 5). The 5′ untranslated region of the 4.8-kb rat kidney mRNA consists of sequences from the 3rd intron, as well as sequences from exons 4 and 5 that encode amino acids 46–79 of erythrocyte band 3. On the basis of the strong correlation between the immunological data and the deduced primary structure of the protein, it seems clear that kidney band 3 corresponds to the Cl^-/HCO_3^- exchanger of the basolateral membrane of acid-secreting intercalated cells.

The genetic basis for the alternative kidney band 3 mRNA and the mechanisms controlling its expression are of considerable interest. Since the kidney band 3 promoter seems to be active only in a specific subset of kidney cells, it may be controlled by kidney-specific regulatory elements. Very few promoters that act exclusively in kidney have been described so far (17, 23), and regulatory elements that control kidney-specific expression have not been identified. Also, induction of kidney band 3 mRNA levels observed during respiratory acidosis (27) may be the result of transcriptional regulation in response to changes in acid-base balance. The mechanisms by which this occurs are not understood at the molecular level. Characterization of the kidney band 3 promoter is a necessary prerequisite for a detailed analysis of its genetic regulation. In this study we have obtained data showing that the rat kidney band 3 mRNA is transcribed from an alternative promoter located in the 3rd intron of the band 3 gene.

MATERIALS AND METHODS

Construction of rat spleen cDNA library. Total and poly(A)$^+$ RNA from whole CD rat spleen was isolated as described previously (15). cDNA was synthesized by the ribonuclease (RNase) H procedure (9), and *Bst* XI linkers (Invitrogen) were added by blunt-end ligation. The cDNA was size-fractionated by agarose gel electrophoresis, and the 3.3–8.5 kb cDNA fraction was ligated into the *Bst* XI site of the plasmid vector pcDNA II (Invitrogen). *Escherichia coli* MAX Efficiency DH5αF′IQ transformation-competent cells (Bethesda Research Laboratories) were transformed with the cDNA and plated at 5,000 colonies/plate on 22 agar plates containing ampicillin.

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Replica filters were prepared using Zeta Bind (AMF CUNO) membranes.

Isolation and characterization of erythroid band 3 cDNA clones. Replica filters were prehybridized for 36 h at 65°C in 500 ml of 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml of denatured salmon sperm DNA (see Ref. 20 for composition of SSC and Denhardt's solutions). The filters were hybridized for 3 days at 60°C in 200 ml of the same solution containing 0.25 μg of an *Xba* I-*Xba* I restriction fragment (nucleotides 211-3125) of the kidney band 3 cDNA (15). The probe was labeled with [α-^{32}P]dCTP to a specific activity of 1×10^9 cpm/μg using the random primer oligolabeling kit from Pharmacia LKB Biotechnology. The replica filters were washed two times in 3× SSC and 0.1% SDS for 10 min each at room temperature, one time in 3× SSC and 0.1% SDS for 60 min at 60°C, one time in 1× SSC and 0.1% SDS for 120 min at 60°C, and one time in 1× SSC and 0.1% SDS for 90 min at 65°C. The filters were then examined by autoradiography. Twenty cDNAs that gave positive hybridization signals were colony purified and analyzed by restriction endonuclease mapping and Southern blot hybridization. cDNAs containing sequences from the extreme 5′ end of the erythrocyte band 3 mRNA were identified by hybridization with a ^{32}P-labeled oligonucleotide probe complementary to nucleotides −123 to −69 of the mouse band 3 gene (14). DNA sequence analysis was performed by the dideoxy chain termination method (25).

Isolation and characterization of rat band 3 genomic clones. Forty replica filters of a rat genomic library containing 2×10^5 recombinants of rat spleen DNA in the cosmid vector pWE15 (Stratagene) were screened with a ^{32}P-labeled *Xmn* I-*Xmn* I restriction endonuclease fragment (nucleotides −393 to 65) from the rat kidney band 3 cDNA. The replica filters were hybridized and washed under essentially the same conditions as those used in screening the cDNA library except that the temperature was raised to 65°C. Two positive clones were identified by autoradiography, colony purified, and analyzed by Southern blot hybridization using various ^{32}P-labeled restriction fragments of the kidney band 3 cDNA and the oligonucleotide complementary to nucleotides −123 to −69 of the mouse band 3 gene.

S_1 nuclease protection analysis. Two oligonucleotide primers (see Fig. 6) were used to prepare probes for S_1 nuclease protection analysis of the 5′ end of the kidney band 3 mRNA. The primers were 5′-end-labeled with [α-^{32}P]ATP and T4 polynucleotide kinase, annealed to a single-stranded M13mp18 template containing a 2.5-kb *Pst* I restriction fragment spanning the region containing the kidney transcription initiation site, extended with the Klenow fragment of *E. coli* DNA polymerase I, and the resulting double-stranded products were cut with *Pst* I. The single-stranded probes, ~1.5 kb in length, were fractionated on a 1.2% alkaline agarose gel, electroeluted, precipitated with ethanol, and dissolved in a solution containing 85% formamide and 15% 10 mM tris(hydroxymethyl)aminomethane (Tris) and 1 mM EDTA, pH 8.0. Kidney poly(A)$^+$ RNA was precipitated with ethanol, washed with 70% ethanol, and resuspended in hybridization buffer [80% formamide, 0.05% SDS, 1 mM EDTA, 0.4 M NaCl, and 10 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES), pH 6.4]. An amount of 5×10^4 cpm of each probe and 10 μg poly(A)$^+$ RNA were heated separately at 65°C for 15 min, combined and incubated at 65°C for 1 h, and then incubated overnight at 37°C. After annealing, the samples were digested with 150 U of S_1 nuclease in 300 μl of a solution containing 200 mM NaCl, 30 mM sodium acetate, pH 4.5, and 3 mM $ZnSO_4$ at 30°C for 1 h. The reaction products were precipitated with ethanol, dissolved in loading dye, and analyzed on a 6% polyacrylamide sequencing gel. Control reactions were performed using 10 μg of tRNA rather than poly(A)$^+$ RNA. Two oligonucleotide primers (see Fig. 4), were used to prepare probes for S_1 nuclease protection analysis of the erythrocyte band 3 mRNA. The primers were annealed to a single-stranded M13mp19 template containing a 1.25-kb *Sph* I restriction fragment spanning the region containing the erythrocyte transcription start sites and extended with the Klenow fragment of *E. coli* DNA polymerase I. The resulting double-stranded products were cut with *Sph* I. The single-stranded probes were fractionated by electrophoresis in a 5% denaturing polyacrylamide gel and eluted from the polyacrylamide. The probes were annealed with 10 μg of rat spleen poly(A)$^+$ RNA, digested with S_1 nuclease, and analyzed as described above. The sequencing ladders used as markers in both S_1 nuclease protection and primer extension analyses were prepared by the dideoxy chain termination method (25) using the same single-stranded templates and primers used in each analysis.

Primer extension analysis. Primer extension analysis of the erythroid transcription start sites was performed using the same primers that were used to prepare probes for S_1 nuclease protection analysis. The 5′-end-labeled primers, $1\text{-}3 \times 10^5$ cpm of each, were precipitated with 10 μg of rat spleen poly(A)$^+$ RNA. The pellets were resuspended in hybridization buffer, heated for 10 min at 80°C, incubated at 30°C overnight, and then precipitated with ethanol. The pellets were resuspended in reverse-transcription buffer, and the primers were extended with 200 U of RNase H$^-$ MMLV reverse transcriptase (Bethesda Research Laboratories). The RNA was then digested for 30 min with RNase A at a concentration of 40 μg/ml. Control reactions containing 10 μg of tRNA were performed using the same primers. Extension products were precipitated with ethanol, dissolved in loading dye, and analyzed on a 6% polyacrylamide sequencing gel.

Polymerase chain reaction (PCR) analysis. The upstream primer used for polymerase chain reaction (PCR) analysis of the 4.4-kb kidney band 3 mRNA corresponded to nucleotides 14-33 in Fig. 6 (5′ AGGCTGGCGGCTTGGCTAAG 3′); the downstream primer (5′ CACTCCCTCCAGATCCTTTAGG 3′) was complementary to nucleotides 303-324 of the kidney band 3 cDNA (16). Approximately 5 ng of first strand cDNA, synthesized by random priming of male CD rat kidney poly(A)$^+$ RNA using Superscript reverse transcriptase (GIBCO, Bethesda Research Laboratories), was amplified for 30 cycles using Hot Tub DNA polymerase (Amersham) under the following conditions: denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. Sizes of the PCR products were analyzed by agarose gel electrophoresis. PCR products were subcloned into pBluescript II (Stratagene) and DNA sequence analysis was performed by the chain termination method (25).

RESULTS

Isolation and characterization of rat erythrocyte band 3 cDNA. To establish the identity of rat erythrocyte band 3 and to obtain sequence information necessary for analysis of the gene, a rat spleen cDNA library was constructed and screened with a kidney band 3 cDNA probe. Over 100 colonies that gave strong hybridization signals were identified, and cDNAs corresponding to 20 of these signals were colony purified. The cDNAs were examined by both restriction endonuclease mapping and by Southern blot hybridization using an oligonucleotide probe corresponding to sequences from exon 1 of the murine band 3 gene (14). Two cDNAs were identified that contained exon 1, and one of these clones, a 3.9-kb cDNA designated RSp19-7 (Fig. 1), was examined by additional restriction endonuclease mapping and partial DNA sequence analysis.

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The erythrocyte (spleen) band 3 cDNA was identical to the previously characterized kidney band 3 cDNA (16) except for differences at the 5′ and 3′ ends. The first 345 nucleotides of the 5′ end of the rat erythroid cDNA (corresponding to nucleotides −58 to 287 shown in Fig. 4), which were not present in the kidney cDNA, are 88% identical to the nucleotide sequence of exons 1–3 of the murine band 3 gene (12, 14). The rest of the cDNA, beginning with sequences from exon 4, is identical to the rat kidney band 3 cDNA except that it terminated at nucleotide 3479 of kidney band 3. Thus it is missing the last 547 bases of the 3′-untranslated region, most likely as a result of internal priming of reverse transcriptase during construction of the library. The position of the initiation methionine codon in the rat erythrocyte band 3 cDNA exactly matches that of its murine homologue (12), and the first 79 amino acids, which are not present in kidney band 3, are 85% identical to those of mouse erythrocyte band 3. This confirms that rat erythrocyte band 3 is similar to that of other mammalian species and that the 5′ end of its mRNA differs from that of the kidney band 3 mRNA.

Intron/exon organization of the 5′ end of rat band 3 gene. To determine the mechanisms responsible for generating the alternative erythrocyte and kidney mRNAs, it was necessary to isolate the rat band 3 gene and analyze the regions corresponding to the 5′ ends of each mRNA. A rat cosmid library was screened with a ^{32}P-labeled restriction fragment from the 5′ untranslated region of the rat kidney band 3 cDNA, and two clones were identified and colony purified. One of these cosmids, clone AE37-1, which had an insert of ~17 kb, was analyzed by restriction endonuclease mapping and Southern blot hybridization. These analyses demonstrated that clone AE37-1 included regions upstream of exons 1 and 4, where promoter elements for the erythrocyte and kidney band 3 mRNAs were likely to be present. The 5′ end of the insert extended ~6 kb upstream of exon 1, and the 3′ end included exon 14 but did not contain exon 20. The exact location of the 3′ end of the insert was not determined. Restriction fragments from clone AE37-1 that included the first 5 exons were subcloned and used to determine the exon/intron organization of the 5′ end of the gene. Detailed restriction endonuclease mapping and partial DNA sequence analysis demonstrated that the organization of this region of the rat band 3 gene (Fig. 2) was the same as that of the murine band 3 gene (13).

Identification of transcription initiation sites of rat erythrocyte band 3 mRNA and analysis of 5′-flanking se-

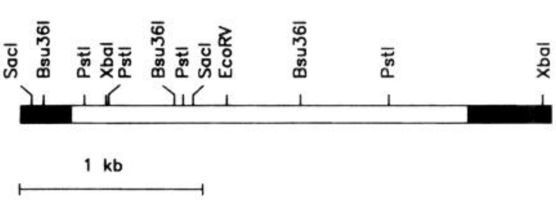

Fig. 1. Restriction map of erythrocyte band 3 cDNA from rat spleen. Solid area, untranslated sequences; open area, coding region.

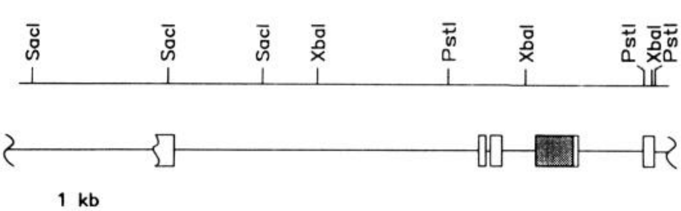

Fig. 2. Restriction map (*top*) and exon/intron organization of the 5′ end of rat band 3 gene (*bottom*). Open rectangles, exons 1–5 of erythrocyte transcription unit. Shaded rectangle, part of 3rd intron of erythrocyte transcription unit, which forms part of 1st exon of kidney transcription unit (see text).

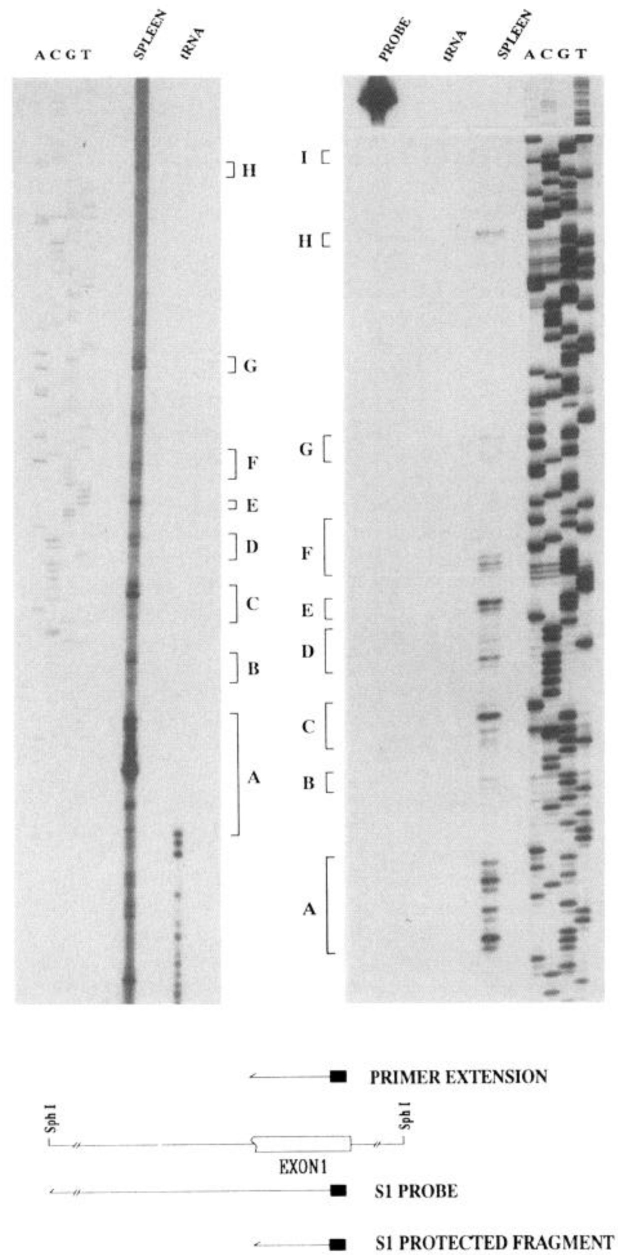

Fig. 3. Primer extension and S_1 nuclease protection analyses of erythrocyte band 3 mRNA. Spleen poly(A)$^+$RNA (10 μg) was analyzed by primer extension using *primer 1* (*left*) or by S_1 nuclease protection analysis using a probe prepared by extension of *primer 2* (*right*) as described under MATERIALS AND METHODS. Clusters of extension products or protected fragments are indicated by lettered brackets (*A–I*). Lanes containing undigested S_1 probe and tRNA controls for both analyses are shown. Sequencing ladders (A, C, G, T) are labeled according to the nucleotides on sense strand. Strategies for primer extension and S_1 nuclease protection analyses are drawn schematically at *bottom*.

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quence. The transcription initiation sites for the rat erythrocyte band 3 mRNA were mapped by primer extension and S_1 nuclease protection analyses of spleen poly(A)$^+$ RNA. Two oligonucleotide primers (complementary to the sequences underlined in Fig. 4) were used for primer extension analysis, and the results of the experiment using *primer 1* are shown in Fig. 3 (*left*). Multiple clusters of extension products, designated *A-H* and spanning about 120 nucleotides, were observed with both primers. Although high background was observed, the major initiation sites detected with both primers appeared to be within *cluster A*, with the strongest site located ~151 nucleotides upstream of the translation initiation site (excluding intron sequences). These results are in close agreement with the results seen in primer extension analysis of the mouse erythrocyte band 3 mRNA (14).

To confirm that the clusters of extension products represent initiation sites for band 3 mRNAs, S_1 nuclease protection analysis was performed using two single-stranded probes constructed by extension of each primer on a template spanning the putative promoter region of the gene. The results of the experiment using the probe constructed with *primer 2* are shown in Fig. 3, *right.* Eight clusters of protected fragments between position 10 and −112, which matched *clusters A-H* seen in primer extension analysis, were observed with both probes. An additional site at position −134 to −132, *cluster I*, was also detected with both probes. *Cluster A*, as noted above for the primer extension experiments, also appeared to be the major site of transcription initiation identified by nuclease protection.

The nucleotide sequences flanking the rat erythroid transcription initiation sites are shown in Fig. 4. The clusters of transcription initiation sites correspond well with similar initiation sites observed in the mouse (14). There are remarkable similarities in the nucleotide sequences of the two species, which exhibit almost 90% identity. The 5′-flanking region of the rat gene, like that of the mouse, lacks most of the common regulatory elements, such as TATA (4), CCAAT (7), and Sp1 (8) binding sequences. Two G-rich sequence elements containing GGTGGG imperfect tandem repeats, which were noted previously in the mouse 5′-flanking region (14), are also present in the rat (Fig. 4). A perfect AP-1 binding site (3) and a sequence that is identical at seven of eight positions to the AP-2 binding site (10) are located immediately upstream of the transcription initiation *clusters E* and *B*, respectively. These two sites are also conserved between the species. Other potential regulatory elements include two CACCC factor binding sites (7), one of which (at position −127) is also present in the mouse promoter, and one sequence that corresponds to a half site for thyroid hormone receptor binding (21).

Identification of transcription initiation site of rat kidney band 3 mRNA and analysis of 5′-flanking sequence.

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GGGGGCCAAGAATGCAGGCACTCTCTGCCTAGGGGCCTGGGCTGGGCTGCCACAGAGGAGCCAGTGTGCCCAGACATTGGTGCTTTGTGAGAAGAGGCTTGTGTGGGGACAGTTGTTTTC  -618

TTGACCTGACATTGTTTCAGGTTGACTGTCAGTGTCCTAGACCCTAGAGGCCAACTCTCAGGAGCCAGCAGGGTCTTTGGGAGAAGAGAATGGAAGCCTCCTAGTCAGGGTCAAGAAGTA  -498

GAGAGGACTGATGGGGTCCCATGTGCACCAGGCAGGGCCCCTGGAAGGCTGCCTAGAAAAAGCCAGTCTTAAGGGAAAACAAGGGCAGGAGAGAGAAGATGTCTTGAAACTTGGAGCCAG  -378
             THR

AGAAGACCCGGACAGAGTGGAATGGACAGAGGCCAGGTAGACGACGTGTCCACGAGCCAGCACCCTCCTGCCAGAGATCTGGAAATTCTAAGAACTCAGTAGTCAAGCCTCAGTCCACAG  -258
                                                            CACCC

AGGCAGAGCCAGAGGACTCTCTAGGGCCAGAAAGGCCCAAAGGTCTCAGGGACTGGCCAGGTTGGGTGGGGTGGGCCTGATGCACAGCTGTCCAGATGTGGGTGAGACATAACTCTGTTT  -138

     I                  H                                         G                F      E     D    C
ATGGGGCACCCGGTCGGAGCGGGCCAAGGGGGTGGGGTGGGAAGGGGCTCCCGGCTGTGGGACGGCAGATTAGGAGGCAAGGGCATGAGTCAGGGGTTTGGGAGCCTCCCCCAGGGCAC  -18
      CACCC                                                                            AP-1                AP-2 87%

  B       A                                                                              Exon1 ▼ Exon2
CTGCCGGTGCTGTTAGAGCAGTTGGGAGCTCGGCCAGTCTCAGAGGGCACCGGCGGCAGCAGGCGGGACTTCACAAAGGACCCCCGAGATTCCTGAGCAGGGACCTGAGGTGCAGGTTAT  103
                                                            primer 2              primer 1

                                                Exon2 ▼ Exon3
GCTGGGGGCTCAGAGAGCAGAGGACAACTGGACACCGAGGACAGCATCATGGGGGACATGCAGGACCACGAGAAAGTGCTGGAGATCCCAGACCGTGACAGTGAAGAGGAACTGGAACAT  223
                                                MetGlyAspMetGlnAspHisGluLysValLeuGluIleProAspArgAspSerGluGluGluLeuGluHis
                                                                          10                            20

                                                 Exon3 ▼ Exon 4
GTAATAGAACAGATAGCATACAGAGACCTAGACATCCCTGTGACAGAAATGCAGGAGTCAGAAGCTCTTCCCACAGAGCAAACAGCCACAGACTACATCCCCACCAGTACCTCCACATCG  343
ValIleGluGlnIleAlaTyrArgAspLeuAspIleProValThrGluMetGlnGluSerGluAlaLeuProThrGluGlnThrAlaThrAspTyrIleProThrSerThrSerThrSer
            30                           40                            40                           50
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Fig. 4. Analysis of erythrocyte band 3 5′-flanking sequences. Nucleotide numbers relative to transcription initiation site within *region A* (■) that gave the strongest signal in both primer extension and S_1 nuclease protection analyses are shown on *right*. Multiple transcription initiation sites are marked by brackets and labeled *A–I*. Sequences complementary to oligonucleotide *primers 1* and *2* used for primer extension analysis and for preparation of probes used in S_1 nuclease protection analysis are underlined and labeled. GT-rich regions conserved between rat and mouse are boxed. Potential binding sites for AP-1, AP-2, CACCC factor, and thyroid hormone receptor (THR) are underlined and labeled. All matches with consensus sequences are 100% unless otherwise indicated. First nucleotide of erythrocyte band 3 cDNA shown in Fig. 1 is indicated by ●. Also shown is nucleotide sequence of the erythrocyte specific exons 1–3, with a portion of exon 4, and their deduced amino acid sequence. Junctions between exons are marked by arrows. Amino acids are numbered below the sequence. (GenBank Accession no. for 5′-flanking sequence is LO2942; GenBank Accession no. for sequences included in mRNA is LO2943).

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We reported previously that the 5′-untranslated sequence of the major rat kidney band 3 mRNA contains sequences from intron 3 (16). Primer extension analysis indicated that the 4.8-kb mRNA extends ~28 nucleotides upstream of the 5′ end of the cDNA, suggesting that the kidney promoter is located in the 3rd intron of the band 3 gene. To test this hypothesis, we performed S_1 nuclease protection analysis of the 5′ end of the kidney mRNA using two single-stranded S_1 probes that were constructed using the same oligonucleotide primers (complementary to the sequences underlined in Fig. 6) that were utilized previously for primer extension analysis (16). Experiments with both probes gave identical results (Fig. 5) and were consistent with the results of primer extension analysis in our previous study (16). A small cluster of protected fragments was observed 25–31 nucleotides upstream of the 5′ end of the kidney band 3 cDNA. Thus the promoter for the major kidney band 3 mRNA is located within the 3rd intron of the band 3 gene, and 377 nucleotides of this intron serve as a part of the 5′-untranslated region of the 4.8-kb kidney band 3 mRNA.

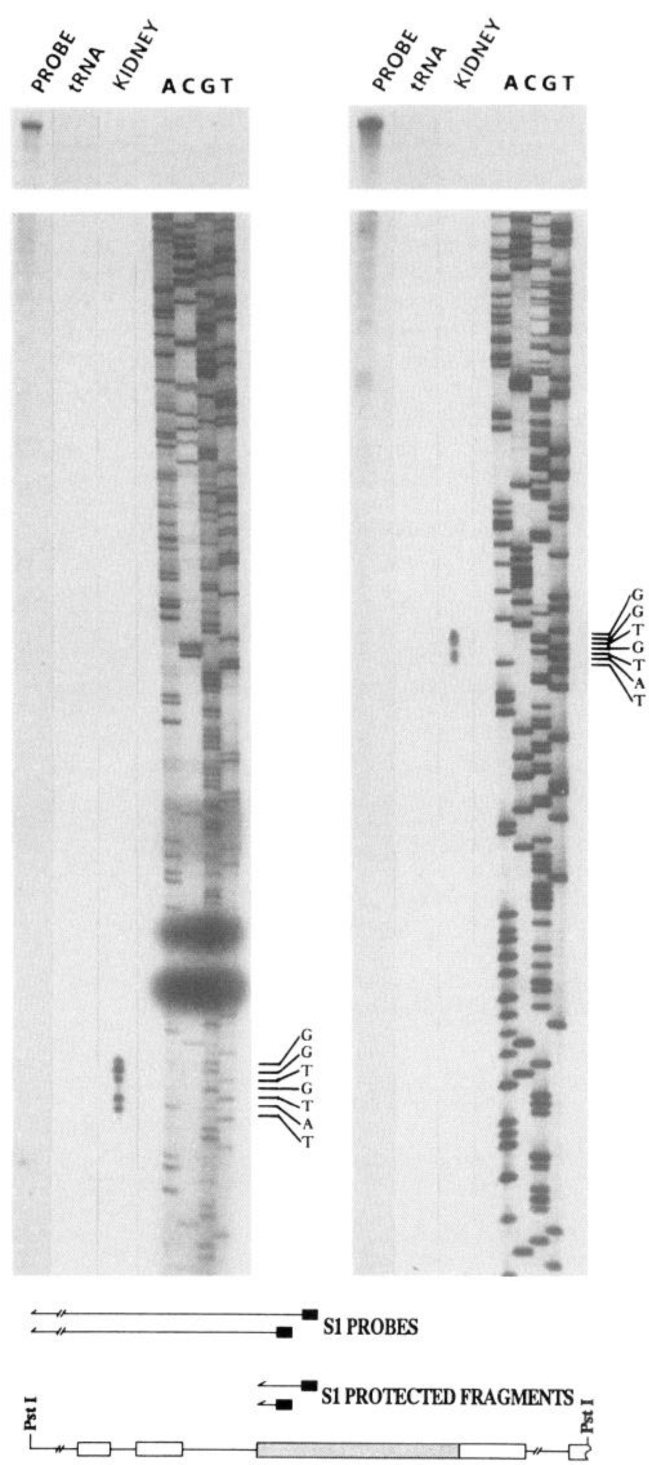

Fig. 5. S_1 nuclease protection analysis of kidney band 3 mRNA. *Left* and *right*, results of experiments performed using 2 different probes. Bands in lines labeled as kidney represent protected fragments of S_1 probes after annealing with kidney poly(A)$^+$ RNA followed by treatment with S_1 nuclease. Undigested probes and tRNA negative controls are also shown. Sequencing ladders (A, C, G, T) are labeled at *top* according to nucleotides on sense strand. Nucleotides that correspond to positions of protected fragments are marked on *right*. Strategy for S_1 nuclease protection analyses is drawn schematically at *bottom*. Results of experiments with longer probe are shown on *right* and with shorter probe on *left*.

The sequence of intron 3, which extended almost 600 nucleotides upstream of the transcription initiation site, was determined and searched for potential regulatory elements (Fig. 6). A potential TATA sequence (4) is located 25 nucleotides upstream of the transcription initiation site. A sequence that is identical at seven of eight positions to an AP-2 binding site (10) and one perfect AP-2 binding site are also present in this region. Also, several sites that match the core element of the thyroid hormone receptor binding site (21) were also found in this region.

Alternative splicing of 5′-untranslated sequence yields a 4.4-kb kidney band 3 mRNA. In addition to the major 4.8-kb mRNA, rat kidney contains a minor 4.4-kb mRNA. In a recent brief report, Sahr and Hanspal (24) noted that the major mouse kidney band 3 mRNA, which is the same size as the minor rat kidney band 3 mRNA, contains a 37-nucleotide exon that is derived from sequences in the 3rd intron. Interestingly, a potential splice donor site (vertical arrow in Fig. 6) is located at nucleotide 36 of the rat kidney mRNA. Splicing of this potential donor site to the splice acceptor site of exon 4 would yield an mRNA corresponding in size to that of the minor kidney mRNA. It seemed possible that low-frequency splicing of this potential donor site (nucleotide 36) to the splice acceptor site of exon 4 (nucleotide 378) might be responsible for production of the minor kidney band 3 mRNA. To test this hypothesis, PCR analysis of rat kidney cDNA was performed using an upstream primer that preceded the potential splice donor site and a downstream primer complementary to sequences from exon 7 (data not shown). PCR products of 789 and 448 bp in length were obtained and subcloned. DNA sequence analysis revealed that the larger product corresponded to the major kidney band 3 mRNA and that the smaller product corresponded to an mRNA in which nucleotides 37–377 were excised.

DISCUSSION

The results of this study demonstrate that the rat erythroid and kidney band 3 mRNAs are derived from a

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Intron3
TGAGACCCACCCCAGGCCTCCTGCCTCCGCCAGAAGAGCTGCTGGGTCCCCATAGGGACAGAAGGGACCCAGGCCTTCCCCTCTCCACAGCTCCGGTCCCAGCCCTGGGGCCAGTTCTCC  -459
          AP-2                              THR                 THR

CCACAATCTTGGCTGATTGAAGGATAAACAGTGTTTTGTTTGAAATGATCACAGGAGGTGATTGGGGGGCGGGAGAGGGGAAGGAGGCTGGGTCCCTTTCACATGTGACAGGGCAGTCAG  -339
                                                                                                THR

GTATGTCTGGGACAAGGGCACAGACCCAGCTGTTAGCCTGTCTGTCCCATATCAGTATTGAGGCCAAGCAGTAGGCCTGTTGGCTGAACGTCATTCAGTCTAGACGGCTGAAGATGCAGA  -219

GAAAGGAGAGAAAGCTGTGGGGACCCCTGAAGCAGAGACCCCAGGAGTCCATACCACCTTCCTTGGCCTGCCCAAGGTCTTAGATGGGGAGCCTGAGGTGGGGACCTGTTTACTGTCAAC   -99
                     THR                AP-2 87%

AGGGCTTAAGTGTTAGTGATTAGATTTGAAGGTAAGAGGTCCCCTTGGGGAAGGAGGGGTTGGGGTTGCGGGATATAAGGGACTCACCCCATCTGACTGGTGTATGGTAAGAGGCTGGCG    22

GCTTGGCTAAGCGGGTGGGGAGAAGAGAGGAGATACAGCAGGGAAAGGAGGGATGCTAGAATGGTTTCGAACACTGTGACTTGGGGCTCTCTCTCCTCATCTCAGAGGAGGGGTCTGGCT   142
                          primer 1                                                          primer 2

GGGGGCTCAGATCACCACAATAAATTCCAGGTTCACCAGTAAAATTCACGTGGAGAGTGGCAGCAGACTCATGTTAGGCCCAGAATAGGCACGGCTGTGGAACTGGGAAGAGGTCCCAGC   262

                                                                                                               Intron3 Exon4
CCAGGGGATGTGGAAGGAGTGGGTCTGGCTTCCTGAGGCTCCCTGAAAGTAGTACGGTAGGGCCTGAGCAGAGGGCCACATCCCAGGTGATACTAATTGGCTCTTGGGTTTTCAGCTCTT   382

                                                                      Exon4 Exon5
CCCACAGAGCAAACAGCCACAGACTACATCCCCACCAGTACCTCCACATCGCACCCAAGCTCCAGTCAGGTCTATGTAGAGCTGCAGGAACTGATGATGGACCAGAGGAACCAGGAACTA   502
                                                                                                         MetAspGlnArgAsnGlnGluLeu
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Fig. 6. Analysis of kidney band 3 5′-flanking sequences. Nucleotide numbers relative to transcription start site are shown on *right*. Transcription initiation site cluster is marked by a bracket. Sequences complementary to primers used to prepare probes for S_1 nuclease protection analysis are underlined. A potential TATA sequence is boxed. Potential binding sites for AP-2 and thyroid hormone receptor (THR) are underlined and labeled. All matches with consensus sequences are 100% unless otherwise indicated. First nucleotide of kidney band 3 cDNA (16) is indicated by •. Location of a splice donor site that seems to be involved in the generation of a minor kidney band 3 mRNA (see text) is marked by an arrow. Junction between intron 3 and exon 4 and between exons 4 and 5 are marked by arrowheads (GenBank Accession no. LO2944).

single gene by the use of alternative promoters as illustrated in Fig. 7. The erythroid promoter is located at the beginning of exon 1, and the transcripts generated from this promoter include all exons of the band 3 gene, starting with the erythroid-specific exons 1, 2, and 3. The promoter that controls transcription of the kidney band 3 mRNA is located in the 3rd intron of the band 3 gene (relative to the erythroid transcription unit). The 1st exon occurring in the major kidney mRNA consists of the last 377 nucleotides of intron 3 joined directly to exon 4. From the beginning of exon 4 to the polyadenylation site in exon 20 both mRNAs are identical, but because of the different 5′ ends the translation start site of the kidney mRNA corresponds to codon 80 of the erythroid mRNA. Thus the use of two highly tissue-specific alternative promoters leads to the production of erythrocyte and kidney band 3 variants that differ by the inclusion or exclusion of a 79-amino acid NH_2-terminal sequence.

Previous studies demonstrated that, in addition to the 4.8-kb mRNA, rat kidney also contains a minor 4.4-kb band 3 mRNA (5, 16), which is ~5–10% as abundant as the major mRNA. The minor rat kidney band 3 mRNA corresponds in size to the major band 3 mRNA in mouse kidney, which contains exons 4–20 (5) but has a shorter 5′ end that is reported to consist of only 37 nucleotides from intron 3 (24). Because a potential donor splice site is located 36 nucleotides downstream of the transcription initiation site of the major rat kidney band 3 mRNA, we performed PCR analysis to determine whether this site is involved in alternative splicing. A PCR product was identified in which nucleotide 36 was spliced to the acceptor site of exon 4. Thus the 4.4-kb rat kidney band 3 mRNA is apparently the result of a low-frequency splicing event, which excises much of the sequence derived from intron 3 but does not alter the coding sequence.

The rat erythroid 5′-flanking region exhibits a high degree of sequence similarity to that of the mouse (14), with 97% identity occurring in the 200 nucleotide sequence extending from the beginning of the G-rich element at position −200 to the major start site in *cluster A*. The multiple transcription initiation sites in both species closely parallel each other, and a number of potential regulatory elements are conserved. Two G-rich sequence elements that were noted in the mouse promoter are conserved in the rat with a single nucleotide replacement. Other sequences that are conserved between the two species and that may be involved in transcription regulation include a CACCC motif (position −131), an AP-1 site (position −51), an imperfect AP-2 binding site (position −28), and a half site for a potential thyroid hormone

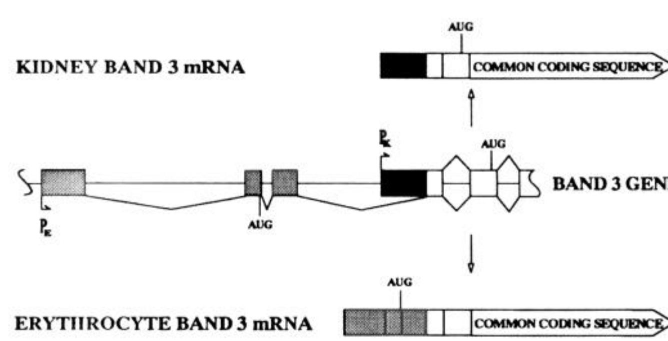

Fig. 7. Erythrocyte and kidney band 3 mRNAs are generated by the use of alternative promoters. Erythrocyte transcript is generated by splicing the first 3 erythrocyte-specific exons (shaded area) to 1st exon common to both transcripts (exon 4). Kidney band 3 transcript initiates within intron 3 and includes part of this intron (solid area) as a portion of its 5′-untranslated region. Codons common in both transcripts are represented by open areas. Locations of erythroid (P_E) and kidney (P_K) transcription initiation sites and initiation methionine codons (AUG) for each mRNA are labeled.

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response element (position −484).

The kidney band 3 transcription initiation site is located near the middle of the third intron. A potential TATA recognition site for factor TFIID (4) is located at an appropriate distance 25 nucleotides upstream of the initiation site, suggesting that it serves as a functional TATA box. A potential Sp1 binding site (7) and an inverted CCAAT sequence (8) are present at positions −387 and −394, respectively, but may be located too far from the transcription initiation site to have functional significance. Except for one sequence that is identical at seven of eight positions to an AP-2 binding site (10), one perfect AP-2 binding site, and several sites that match the core element of a thyroid hormone receptor binding site (21), no other elements specific for RNA polymerase II promoters were identified.

Transcription from alternative promoters, which results in the production of proteins that differ in their NH_2-terminal sequence, has been described for several genes expressed in eukaryotic cells (6, 11, 18, 19, 22). Most of the genes that give rise to different mRNAs are tissue specific, and the alternative use of multiple promoters is either developmentally regulated (22) or restricted to a few tissues (19). Also, there are reports where one promoter acts as a housekeeping promoter while the other is specifically regulated during development (6). The kidney and erythroid band 3 promoters are clearly used in a tissue-specific manner, although there is some evidence that the promoter which initiates transcription of the erythrocyte band 3 mRNA is also active in nonerythroid tissues (14).

Transcription of erythrocyte and kidney band 3 mRNAs is only one of several known examples of the use of alternative promoters within the band 3 family of genes. Two chicken erythrocyte band 3 mRNAs are generated by a combination of alternative promoters and alternative splicing (11). AE3, another member of the AE (anion exchanger) family of genes, uses alternative promoters in combination with alternative splicing to produce mRNAs encoding neuronal and cardiac-specific AE3 isoforms (19). In both of these cases the proteins produced as a result of alternative transcription and splicing differ from each other only at their NH_2 terminals. A similar pattern of transcription initiation may be involved in the production of additional AE1 (band 3) and AE2 mRNAs (2, 15). At least two additional AE1 mRNAs besides the 4.8- and 4.4-kb kidney mRNAs and the 4.6-kb erythroid mRNA are expressed in rat tissues. A 4.1-kb band 3 mRNA is expressed at low levels in rat heart, and a 3.6-kb mRNA is expressed at low levels in heart, kidney, liver, and small intestine (15). There is evidence for two additional AE2 mRNAs besides the 4.4-kb mRNA that has already been characterized (15). Whether these alternative mRNA species are transcribed from alternative promoters, or by other mechanisms such as alternative splicing, has not been determined.

In summary, the work presented here demonstrates that the 4.8-kb kidney and 4.6-kb erythroid band 3 mRNAs from rat are transcribed from alternative promoters and also provides genomic clones and sequence information that will be necessary for detailed studies of band 3 gene regulation. Of particular interest for future studies will be the molecular mechanisms controlling the tissue and cell type specificity of the kidney promoter, as well as the mechanisms by which kidney band 3 mRNA levels are modulated in response to respiratory acidosis (27). At the present time virtually nothing is known about kidney-specific gene expression or the mechanisms by which alterations in acid-base balance modulate gene expression. Analysis of band 3 gene expression in kidney should contribute to our understanding of these phenomena.

We thank Jeannette Greeb for performing the experiments that identified the splicing pattern for the minor kidney band 3 mRNA and Ginny Chasteen for typing the manuscript.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-39626.

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Received 1 June 1992; accepted in final form 28 September 1992.

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