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The Mouse Carbonic Anhydrase I Gene Contains Two Tissue-Specific Promoters

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We report the isolation and characterization of the mouse carbonic anhydrase I (CAI) gene. Direct RNA sequence analysis of the 5′ nontranslated regions of CAI mRNA from mouse colon and mouse erythroleukemia cells demonstrated tissue specificity in the lengths and sequences of CAI transcripts. Analysis of several mouse CAI genomic clones showed that the transcripts arose from a single CAI gene with two tissue-specific promoters and eight exons. CAI transcripts in the colon were found to initiate just upstream of the erythroid exon 2 of the CAI gene region sequence. Erythroid transcripts originated from a novel promoter upstream of exon 1, which was located more than 10 but less than 250 kilobases upstream of exon 2. Erythroid exon 1 contained only a nontranslated sequence, which was spliced to exon 2 via a cryptic splice acceptor site located in the region that encoded the colon mRNA 5′ nontranslated sequence. The remaining exon-intron junctions were conserved in comparison with those of the CAII and CAIII genes.

Carbonic anhydrase (CA; EC 4.2.1.1) is a zinc metalloenzyme involved in the reversible hydration of CO_2. In mammals, at least five distinct isozymic forms have been identified (CAI, -II, -III, -IV, and -V), which vary in tissue specificity and have markedly different CO_2 hydratase activities. All are monomeric enzymes with a molecular mass of approximately 29,000 daltons. CAI has been reported in such tissues as the intestinal epithelium, vascular endothelium, corneal epithelium and lens of the eye, and erythrocytes. The most widely occurring form, CAII, often referred to as the high-activity form, is expressed in nearly all tissues. CAIII has been found mainly in red skeletal muscle and the livers of some mammals (6, 17, 28). CAIV is a membrane-bound form, localized in the mammalian lung and kidneys (21, 32), and CAV has been found so far in kidney and liver mitochondria (11, 12, 31).

CA is the second-most-abundant protein in erythrocytes, where both CAI and CAII are present in different ratios, depending on the species. During in vitro differentiation of mouse erythroleukemia (MEL) cells induced by dimethyl sulfoxide, CAI protein levels appear to be highest in the uninduced state and then decrease somewhat as CAII protein levels increase during induction (26). CAI gene transcription is specifically down-regulated during MEL induction, with a concomitant and dramatic decrease in CAI steady-state mRNA (14). At the same time, CAII gene transcription increases five- to sevenfold, in parallel with increases in transcriptional activity for δ-aminolevulinic acid synthase, band 3, and globin genes (14). These results suggest that CAI may be a marker for an earlier step in erythroid differentiation. This statement agrees with studies of human CAI that used isozyme-specific antibodies which detected CAI in erythroid precursor cells lacking hemoglobin (30).

At present, cloned cDNAs for mouse CAI (13) and CAII (9), rabbit CAI (18), and human CAI (2), CAII (22), and CAIII (20) have been reported. In addition, genomic clones for mouse (29) and chicken (33) CAII and human CAIII (19) have been fully characterized. The overall gene structure is conserved among these genes and partially characterized CA genes. All contain seven exons and six introns and have the translational start site AUG codon in the first exon. The only major structural difference aside from intron length is exon 4 of the mouse CAII gene, which is shifted 14 bases 5′, interrupting codon 143 instead of codons 149 and 150. Here we report the unusual structure of the mouse CAI gene, which contains eight exons, the first of which is located more than 10 kilobase pairs (kbp) upstream of the remainder of the gene. A similar arrangement of exons has been noted in the human CAI gene (5). RNA sequence analysis of mouse CAI mRNA and structural analysis of the CAI gene demonstrate that the mouse CAI gene contains at least two tissue-specific promoters.

MATERIALS AND METHODS

Primer extension sequencing of poly(A)$^+$ RNA. Total RNA was extracted from MEL cells as described by Curtis and Weissmann (10). This procedure was modified as follows for isolation of RNA from whole mouse colons. The large intestine was removed from mice, washed briefly with ice-cold phosphate-buffered saline, taking care to remove solid material from the interior, and immediately frozen in liquid nitrogen. The frozen tissues were immersed in 20% sodium dodecyl sulfate (SDS) at 1 g/10 ml and immediately homogenized with a Polytron homogenizer. The solution was then diluted 10-fold with 20 mM Tris (pH 7.5)–1 mM EDTA–0.2 mg of Pronase per ml and incubated for 30 min at room temperature. The remainder of the preparation was performed as described above. Poly(A)$^+$ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography as previously described (8).

Dideoxy sequencing of poly(A)$^+$ RNA was performed exactly as described by Geliebter et al. (16), using a 17-base oligonucleotide (oligo CAI-2; see Fig. 2) complementary to the mouse CAI cDNA sequence from nucleotides 48 to 64 (13). Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer.

Isolation of mouse CAI genomic clones. Three mouse genomic DNA lambda libraries (mouse embryo DNA in λ Ch4a, mouse brain DNA in λ EMBL3, and mouse lympho-

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cyte DNA in λ EMBL3) were screened with mouse CAI cDNA (13) and with a 30-base oligonucleotide complementary to nucleotides 33 to 63 of the 5′ noncoding region of erythroid CAI mRNA. λ CAI-4 was obtained from the λ Ch4a library, and λ CAI-12 and -21 were obtained from λ EMBL3 libraries.

DNA sequencing. Restriction fragments containing intron-exon junctions were subcloned into the appropriate M13 vectors and sequenced by the dideoxy-chain termination method of Sanger et al. (23).

Transverse-field alternation gel electrophoresis. High-molecular-weight DNA from MEL cells was prepared by incorporation into low-melting agarose and poured into Tygon tubing. After extrusion of the solidified agarose, the cells were lysed and deproteinized, and the DNA was digested by restriction enzymes in situ (15). Plugs containing digested DNA were transferred to slots in a 0.9% agarose gel and subjected to pulse-field gel electrophoresis (15), using 330 V and 180 mA, with field alternation at 4-s intervals for 30 min, and then 250 V and 150 mA, with alternation every 1 min for 26 h. The DNA was transferred to nitrocellulose.

DNA and RNA blots. Total cellular RNA (15 μg) was electrophoresed in a formaldehyde-agarose gel at 5 V/cm for 3 h and transferred to nitrocellulose. For both RNA and DNA, the filters were prehybridized for 4 h at 65°C in the hybridization solution (3.5× SSC [1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate], 0.5% SDS, 1× Denhardt solution, 50 μg of salmon sperm DNA per ml, 10% [wt/vol] dextran sulfate), followed by hybridization at 65°C for 18 h with probes labeled by random priming (27). Filters were washed at 65°C for 15 min in 2× SSC–1% SDS twice, followed by 5 min in 0.5× SSC–0.5% SDS.

RESULTS

RNA sequence analysis of the 5′ noncoding region of the mouse erythroid CAI mRNA. The mouse CAI cDNA, pMCAI, is 1,224 bp long and contains 45 bp of 5′ nontranslated sequence (13). To determine the full sequence of the 5′ end of the erythroid CAI mRNA and identify the cap site, the CAI mRNA was sequenced directly. A 17-base complementary oligonucleotide was synthesized from the coding region of the CAI cDNA in the vicinity of the translation initiation codon and used in primer extension dideoxy-chain sequencing reactions with poly(A)$^+$ RNA from uninduced MEL cells. The oligonucleotide was end labeled with polynucleotide kinase and annealed to MEL cell poly(A)$^+$ RNA at 5 degrees below the melting temperature as previously described (16). Four separate primer extension sequencing reactions were carried out, each with a different dideoxynucleotide. A fifth primer extension reaction containing only deoxynucleotides was performed to identify the strong-stop characteristic of the mRNA 5′ cap site structure and indicate any premature termination sites. All reactions were performed at 50°C to minimize the effects of RNA secondary structure on reverse transcriptase activity. The reactions terminated in a very strong stop, indicating the 5′ end of the mRNA (Fig. 1A). The complete sequence of the 5′ nontranslated region of the erythroid CAI mRNA is shown in Fig. 2. The mRNA sequence confirmed the 45 nucleotides of the 5′ noncoding region found in the 5′ end of the cDNA and revealed an additional 42 nucleotides up to the cap structure. Therefore, the entire 5′ nontranslated region of the mouse erythroid CAI mRNA is 87 nucleotides long.

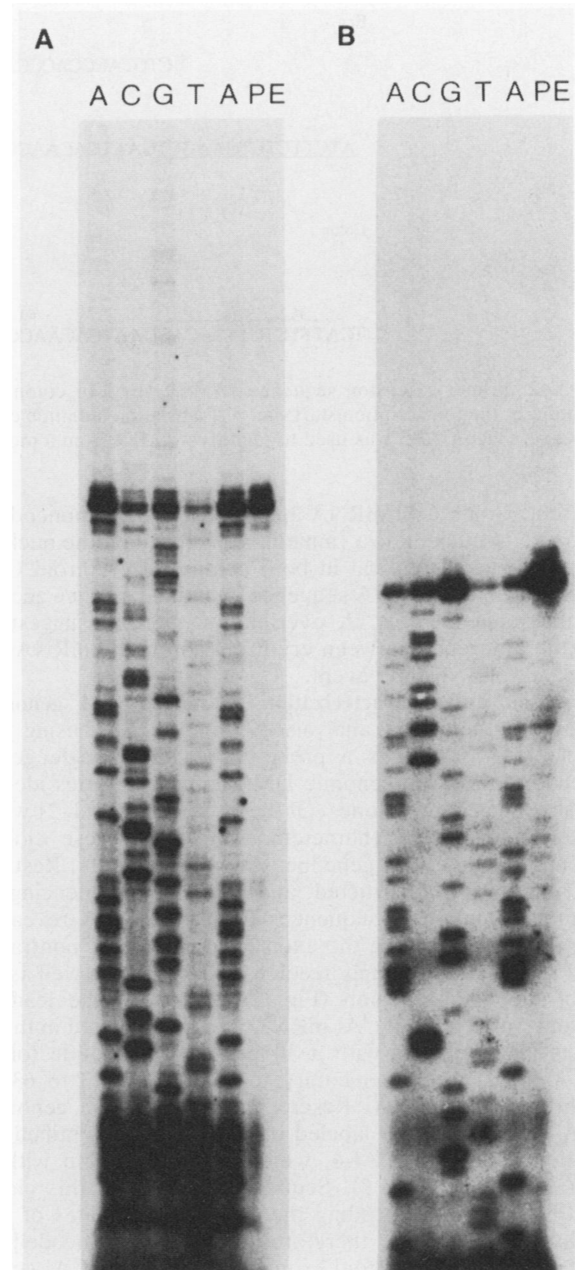

FIG. 1. Primer extension sequencing of CAI mRNA. (A) Poly(A)$^+$ RNA (10 μg) from uninduced MEL cells was annealed with oligo CAI-2 (complementary to nucleotides 48 to 64 of the mouse CAI cDNA sequence given in reference 13) and extended with avian myelocymatosis virus reverse transcriptase in the presence of A, C, G, or T dideoxynucleotide triphosphates, as indicated. Lane PE, Primer extension without dideoxynucleotide triphosphates. (B) As above, using 10 μg of poly(A)$^+$ RNA from mouse colon tissue.

RNA sequence analysis of the 5′ noncoding regions of CAI mRNA from mouse and rat colons. Since CAI is expressed in a few tissues in addition to erythrocytes, RNA-sequencing reactions similar to those described above were carried out on poly(A)$^+$ RNA isolated from mouse colon (Fig. 1B). The result showed a sequence shorter than and different from that observed with RNA from uninduced MEL cells. The colon RNA sequence was identical to the cDNA and erythroid RNA sequences for 24 nucleotides of the 5′ noncoding region preceding the initiation ATG (Fig. 2). Beyond this point, however, the colon RNA sequence was completely

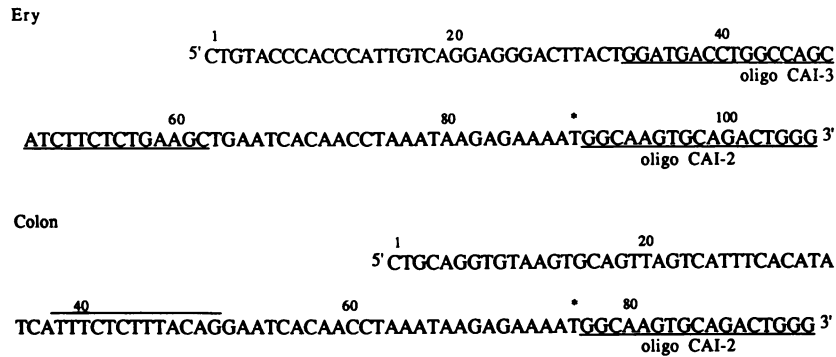

FIG. 2. Primer extension sequence of MEL (Ery) or colon poly(A)$^+$ RNA, using oligo CAI-2 primer. The nucleotides are numbered beginning at the transcription start site. *, Translation initiation codon. The overlined sequence is the cryptic acceptor site in the colon mRNA sequence. Oligo CAI-3 was used to identify λ CAI-4 from a mouse genomic library.

different. Colon CAI mRNA apparently has a 5′ noncoding region of 74 nucleotides. Immediately preceding the nucleotides that were identical in both colon and erythroid CAI mRNAs, the colon RNA sequence contained a splice acceptor-like sequence (Fig. 2, overlined sequence), suggesting that the difference between erythroid and colon mRNAs is the result of a splicing event.

Isolation and characterization of mouse CAI genomic clones. Southern blot analysis of mouse DNA, using the mouse CAI cDNA as a probe, revealed a single gene. Screening of mouse genomic DNA lambda libraries identified several genomic clones. Of these, λ CAI-12 and -21 were chosen for detailed characterization, since these clones encompassed the CAI gene locus most completely. Restriction maps were constructed, and nucleotide sequencing of fragments containing sequences from the cDNA revealed seven exons, including the exon encoding the 5′ nontranslated region (74 bp) found in colon CAI mRNA as well as 37 bp of the coding region (Fig. 3). However, the leading sequence of erythroid CAI mRNA was not detected in these clones when probed with a 30-mer oligonucleotide (oligo CAI-3; Fig. 2) complementary to nucleotides 33 to 63 of erythroid CAI mRNA. Rescreening of a mouse genomic DNA library with the labeled oligonucleotide identified an additional clone, λ CAI-4, which did not overlap with λ CAI-12 and -21 (Fig. 3). Sequence analysis of this clone identified an exon encoding the first 63 nucleotides of the erythroid CAI mRNA; therefore, this mRNA is encoded by eight exons. The erythroid exon 1 is novel to the CAI gene, consisting entirely of nontranslated sequences, and was spliced to exon 2 (61 bp) by using the cryptic acceptor site present in the first exon of colon CAI mRNA at the exact site of divergence between erythroid and colon CAI mRNA (Fig. 2). The remaining exon-intron boundaries interrupted the same codons as in the chicken CAII (33) and human CAIII (19) genes. The nucleotide sequence of the intron boundaries fit the consensus donor and acceptor sequences (Fig. 4).

DNA sequence analysis of the putative erythroid promoter region revealed TATAA and CCAAT box motifs at −30 and −70 nucleotides, respectively. The hexanucleotide CCGCCC occurred at −47 (Fig. 5). This sequence has been found in the promoter regions of the mouse CAII, human CAIII, and a number of other eucaryotic genes. Of further interest is the sequence CCACACCC located at −212. This sequence element is found in all mammalian adult β-globin genes and the chicken β-globin gene and is also present in the promoter of the mouse CAII gene, which is also expressed in erythroid tissues. The colon promoter contained a TATAA motif at −33 and had several potential CCAAT box motifs located upstream, none of which fit the consensus GCCAAT sequence exactly.

Linkage relationship of the mouse CAI genomic clones. λ CAI-12, which encodes exons 2 to 6 of the mouse CAI gene, also contains approximately 8 kbp of intron 1. λ 4, which encodes exon 1, contains approximately 2 kb of intron 1. Together, these clones indicate that the first intron of the mouse CAI gene is longer than 10 kbp. Attempts to establish a linkage relationship between these two clones by Southern blot analysis techniques were not successful (because no fragments containing both intron 1 5′ and 3′ sequences could

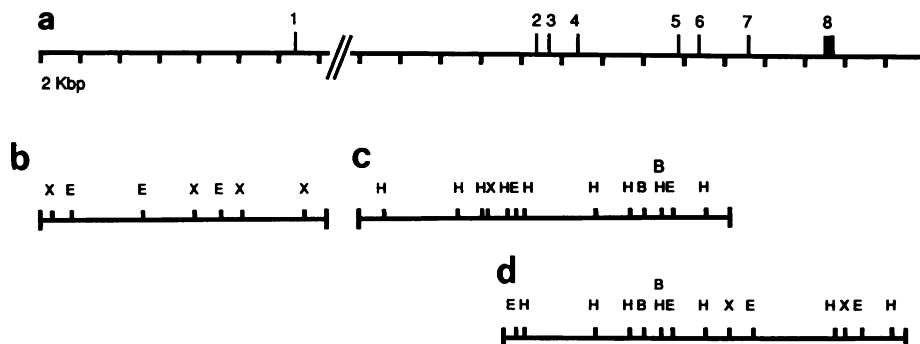

FIG. 3. Exon structure of the mouse CAI gene. (a) Locations of the mouse CAI gene exons 1 to 8. The discontinuity indicates the undetermined size of intron 1, which is more than 10 kbp but less than 250 kbp. (b to d) Restriction maps of λ CAI-4 (b), -12 (c), and -21 (d). Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

5′ codon no.	5′ exon / intron	Intron #	3′ intron / exon	3′ codon no.
	GCT gtgagta·····	1	·····tttacag GAA	
11	AAT G gtaagag·····	2	·····catgtag GT CCT	13
77	TCT G gtgagct·····	3	·····atttcag TT CTG	79
117	GAG gtaatgc·····	4	·····ctttcag CTT	118
149	AAG gtgagtt·····	5	·····tatctag GTT	150
170	AAG gtaaatt·····	6	·····gttttcag GGA	171
222	CAG gtatcaa·····	7	·····cctacag CTG	223

FIG. 4. Mouse CAI intron-exon structure. Codon numbering starts after the initiation codon ATG. Capital letters represent exon sequences; lowercase letters represent intron sequences.

be detected). We therefore chose to establish a maximal separating distance between exons 1 and 2 by use of pulse-field gel electrophoresis. Figure 6 shows the autoradiograms of two filters from the same pulse-field gel run with two sets of identical digestions. The exon 1 probe (Fig. 6A) hybridized to the same 800-kbp *Sal*I, 300-kbp *Cla*I, and 250-kbp *Sac*II fragments as did the exon 2 to 6 probe (Fig. 6B); the exon 2 to 6 probe also hybridized to a 50-kbp *Apa*I fragment that did not contain exon 1 sequences. These results indicate that the erythroid exon 1 of the mouse CAI gene is less than 250 kbp from exon 2. To determine whether the upstream location of exon 1 was specific to erythroid cells, DNA from MEL, NIH 3T3, and mouse HEp-2 cells was digested with *Eco*RI, *Hin*dIII, and *Kpn*I and analyzed by the Southern blot method. The patterns of hybridized bands were the same in

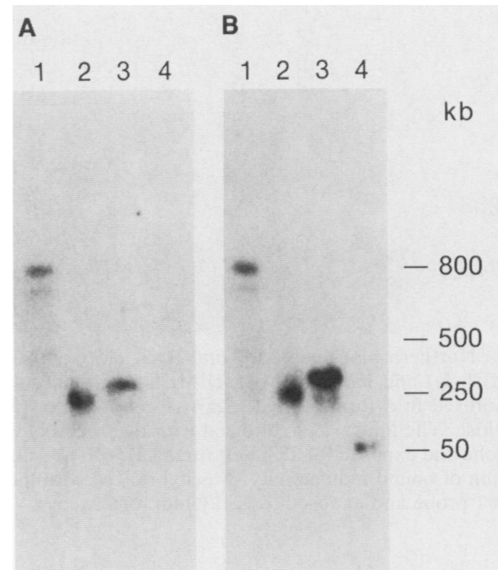

FIG. 6. Pulse-field gel electrophoresis. MEL cell DNA was digested with restriction enzymes, electrophoresed, transferred to a nitrocellulose filter, and hybridized with labeled probe: a 300-bp fragment containing exon 1 (A) or CAI cDNA (B). Lanes: 1, *Sal*I; 2, *Sac*II; 3, *Cla*I; 4, *Apa*I. Size markers were concatemerized lambda DNA.

these three types of cells and corresponded to restriction fragments found in the genomic clones.

RNA encoded by the erythroid exon 1. The location of an exon in erythroid CAI mRNA at a considerable distance upstream of the rest of the CAI gene suggests many interesting possibilities, one being that this exon is part of another gene expressed in erythroid cells. Total RNA from uninduced and induced MEL cells and mouse colon was analyzed by Northern (RNA) blot analysis. The filter was first hybridized with the 3′ fragment of the CAI cDNA and then, after washing, with a 300-bp fragment containing exon 1

FIG. 5. Nucleotide sequence of the mouse CAI gene surrounding exons 1 and 2. The first two exons of mouse erythroid CAI mRNA are indicated by capital letters. The transcription initiation sites for the CAI mRNAs are identified by arrows; the nucleotide sequence overlined is the presumed splice acceptor site of intron 1, which is located within the first exon of colon CAI mRNA.

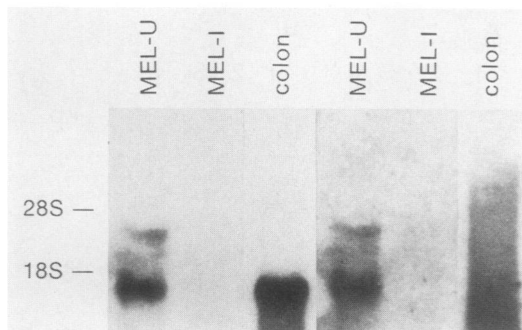

FIG. 7. Northern blot analysis. Total RNA (15 μg) from uninduced (MEL-U) and induced (MEL-I) MEL cells and colon was electrophoresed in a formaldehyde-agarose gel and transferred to nitrocellulose. The filter was hybridized with the 3′ end of the CAI cDNA probe and exposed for 18 h (left three lanes). The same filter, after elution of bound radioactivity, was hybridized with the erythroid exon 1 probe and exposed to X-ray film for 2 weeks.

(Fig. 7). The cDNA probe detected a predominant 1.5-kbp RNA, CAI mRNA, as well as a 3.3-kbp RNA, presumably a splicing intermediate, in uninduced MEL cells but no signal in induced cells. In colon RNA samples, only the 1.5-kbp CAI mRNA was detected. The erythroid exon 1 probe gave exactly the same result as did the cDNA probe with uninduced and induced MEL cell RNA. However there was no band corresponding to the 1.5-kbp CAI mRNA in colon RNA; only a smear of RNA of large to small molecular size was seen.

DISCUSSION

Nucleotide sequencing by primer extension analysis of CAI mRNA from two different tissues, erythroid cells and intestinal epithelium (colon), revealed a difference in the sequences of the 5′ noncoding regions of the mRNAs. Analysis of mouse genomic CAI clones showed that these two mRNAs were transcribed from two different promoters within a single gene. The colon mRNA is encoded by seven exons whose boundaries are identical to those found in chicken CAII (33) and human CAIII (19) genes. The erythroid mRNA is encoded by eight exons. The additional 5′ exon of 63 bp is spliced to a cryptic acceptor site located in exon 1 of the colon mRNA sequence, while the remaining exons are identical in the two mRNAs.

The mouse α-amylase gene was the first gene shown to have two tissue-specific promoters (34). Since then, several examples of genes with multiple promoters have been described (24). However, the alcohol dehydrogenase gene of *Drosophila melanogaster* is the only gene that has the same structural arrangement of promoters and 5′ noncoding exons as does the mouse CAI gene (3). In most genes with more than one promoter, one promoter acts constitutively, while the other is inducible or tissue specific. The colon transcriptional unit is similar to that of the CAII gene, which is expressed in a wide variety of tissues. However, CAII is considered a characteristic protein of erythrocytes, since it is expressed at a much higher level in these cells, but the CAII mRNA is expressed from a single promoter.

The question to be answered is why the mouse CAI gene has acquired an erythroid-specific promoter that is far upstream of the rest of the gene. The erythroid promoter could be more active than the colon promoter. Alternatively, the different 5′ noncoding sequences might confer differing levels of mRNA stability or translatability. However, the existing evidence suggests that there is no remarkable difference in the levels of CAI in erythrocytes and colon epithelial cells (6, 7, 25). Another explanation for this structure is that the erythroid promoter and exon have been acquired from an erythroid-specific gene by duplication and translocation; alternatively, the promoter and exon could belong to an erythroid-specific gene located within the large first intron. However, the only examples of genes within genes are those encoded by opposite strands of the DNA (1). In addition, the exon 1 probe detected only the two RNA species seen with the 3′ end of CAI cDNA: the CAI mRNA (1.5 kbp) and presumably a splicing intermediate of CAI mRNA (3.3 kbp). As expected, the exon 1 probe did not detect CAI mRNA in colon RNA; interestingly, however, the probe revealed a smear of RNA. Since the exon 1 probe does not contain any detectable repeat sequences, this result implies that the region containing the erythroid exon 1 is transcribed in colon epithelial cells.

The large first intron raises additional questions that have been considered before with respect to the human c-*abl* gene, which has an intron of at least 200 kbp (4). The questions related to the possibility that transcription of genes with very large introns might be discontinuous, jumping from DNA encoding exon 1 to DNA encoding exon 2. These regions would be brought into close proximity by looping of chromatin, resulting in a much smaller transcribed intron such that the donor and acceptor splice sites in the RNA transcript would be the only possible choices for splicing. If transcription is continuous, it is not obvious how the correct donor and acceptor sites are identified in such a very long transcript. Further analysis of the CAI gene should provide answers to these questions and identify the *cis*- and *trans*-acting factors that determine the timing of CAI erythroid-specific expression.

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