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Molecular Structure of the Human Argininosuccinate Synthetase Gene: Occurrence of Alternative mRNA Splicing

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The human genome contains one expressed argininosuccinate synthetase gene and ca. 14 pseudogenes that are dispersed to at least 11 human chromosomes. Eleven clones isolated from a human genomic DNA library were characterized extensively by restriction mapping, Southern blotting, and nucleotide sequencing. These 11 clones represent the entire expressed argininosuccinate synthetase gene that spans 63 kilobases and contains at least 13 exons. The expressed gene codes for two mRNAs that differ in their 5′ untranslated sequences and arise by alternative splicing involving the inclusion or deletion of an entire exon. In normal human liver and cultured fibroblasts, the predominant mature argininosuccinate synthetase mRNA lacks sequences encoded by exon 2 in the expressed gene. In contrast, the predominant argininosuccinate synthetase mRNA in baboon liver contains exon 2 sequences. A transformed canavanine-resistant human cell line in which argininosuccinate synthetase activity is 180-fold higher than that in wild-type cells contains abundant amounts of both forms of the argininosuccinate synthetase mRNA. The mRNA lacking exon 2 sequences is the more abundant mRNA species in the canavanine-resistant cells. These observations show that splicing of the argininosuccinate synthetase mRNA is species specific in primates and varies among different human cell types.

Argininosuccinate synthetase is an enzyme involved in the urea cycle in liver and participates in arginine biosynthesis in nonhepatic tissues. In some cultured cell lines, argininosuccinate synthetase activity is repressed by the concentration of arginine in the medium (15, 27, 30). This arginine-mediated repression is regulated at the pretranslational level (31) and can change argininosuccinate synthetase activity over a 10-fold range. Cultured human cells having high levels of argininosuccinate synthetase activity can be selected by growth in medium containing canavanine, an arginine analog (16, 30). Canavanine-resistant (Can^r) cells have up to 300-fold-higher levels of argininosuccinate synthetase activity than wild-type cells do and have a parallel increase in the level of argininosuccinate synthetase mRNA (31). Unlike the enzyme overproduction described for other drug-resistant cell lines (1, 32), the increase in argininosuccinate synthetase expression in Can^r cells is not due to gene amplification (31). In Can^r cells, the increased expression of argininosuccinate synthetase is not dependent on the presence of canavanine, and the enzyme is not subject to metabolite regulation by arginine.

We previously reported the isolation and characterization of seven human genomic clones that represent argininosuccinate synthetase pseudogenes (14). All of these pseudogenes were of the processed type and were cloned from distinct genomic sites. Southern blot analysis of DNA isolated from human-Chinese hamster somatic cell hybrids has indicated that the human genome contains 14 argininosuccinate synthetase pseudogenes that are dispersed among 11 human chromosomes (3, 12; Su et al., Am. J. Hum. Genet., in press). Argininosuccinate synthetase pseudogenes are present in the genomes of other primates (S. P. Daiger and N. S. Hoffman, Genetics **104s**:20, 1983), and therefore many of these pseudogenes arose before the divergence of the great apes and hominids (14).

There have been numerous reports of single-copy genes that code for multiple mRNAs. Different mRNAs are synthesized by the use of different promoters (4, 9, 26), by the use of multiple polyadenylate [poly(A)] addition signal sequences (2, 13, 19, 24), and by alternative splicing involving the inclusion or deletion of exons and introns (11, 17). In some instances, the different mRNA forms are tissue specific (8, 26), are produced at specific times during the development of the organism (4, 25), or are expressed under different metabolic conditions (9). It has been observed that alternative splicing in a few viral and cellular genes can involve translated sequences and therefore provides a novel mechanism for changing the protein domains in the final gene product (28, 34). Thus, initiation of transcription and RNA splicing play important roles in gene expression by altering mRNA transcripts to yield mature mRNAs with different regulatory and coding potentials.

We now report the isolation and characterization of 11 human genomic clones that represent the expressed argininosuccinate synthetase gene. The expressed gene is 63 kilobases (kb) in length and contains at least 13 exons. Analysis of argininosuccinate synthetase mRNA in tissues from primates has indicated that the expressed gene codes for two different mRNAs that differ by the inclusion or deletion of 5′ untranslated sequences encoded by exon 2. Splicing of the argininosuccinate synthetase mRNA is species specific between primates and varies among cell types in humans.

MATERIALS AND METHODS

Isolation and characterization of human genomic clones. A *Hae*III-*Alu*I Charon 4A-human genomic DNA library (18) was screened for argininosuccinate synthetase sequences as previously described (14). The bacteriophage were plaque purified, and DNA was prepared by a plate lysate or liquid lysate method (20). Phage DNA was digested with a battery of restriction enzymes, and the resulting DNA fragments were subjected to electrophoresis in 1% agarose gels containing 0.5 μg of ethidium bromide per ml. The DNA fragments were visualized and photographed under illumina-

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tion with UV light and then transferred to nitrocellulose (29). DNA fragments containing argininosuccinate synthetase sequences were identified by hybridization to the nick-translated cDNA probe, pAS4/1, as previously described (14).

Subcloning of genomic fragments and nucleotide sequencing of exons. The entire argininosuccinate synthetase gene was subcloned into plasmids pBR322 and pUC-8 by using standard procedures (20). Exons were subcloned and prepared for DNA sequencing as follows. A plasmid containing a subcloned genomic fragment was digested with a restriction enzyme that would generate a hybridizing fragment 200 to 400 base pairs (bp) in length. The resulting DNA fragments were shotgun cloned into a compatible cloning site in pUC-8. The recombinant plasmids were used to transform competent *Escherichia coli* JM83 cells (22), and bacteria containing recombinant plasmids were selected by growth on agar plates containing ampicillin (100 μg/ml) and X-gal (1 mg per plate). White colonies were picked and replica plated, and colonies carrying a plasmid containing the argininosuccinate synthetase gene sequences were identified by colony screening with ^{32}P-labeled pAS4/1 as a hybridization probe. Plasmid DNA was prepared from 5 ml of an unamplified overnight culture (5) and linearized by digestion with *Eco*RI or *Hin*dIII. The DNA was labeled at the 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase by standard procedures (21). All DNA sequencing was performed by the chemical degradation procedures of Maxam and Gilbert (21). For all sequences shown, both DNA strands were completely sequenced or the same DNA strand was sequenced twice.

S1 nuclease experiments and Northern blotting. Total cellular RNA was prepared by the procedures of Chirgwin et al. (10). S1 nuclease experiments were performed as described by Weaver and Weissmann (33) with the following modifications. After hybridization, the reaction mixture was incubated at 30°C with 1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals) for 30 min. The nucleic acid was precipitated with ethanol, and the RNA was hydrolyzed by incubation in 0.1 N NaOH–10 mM EDTA at 68°C for 1 h. The protected DNA fragments were precipitated with ethanol, dried under vacuum, and suspended in a solution containing 80% formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and 1 mg each of xylene cyanol and bromphenol blue per ml. The samples were applied to an 8% acrylamide DNA sequencing gel (21) and subjected to electrophoresis, and the radioactive DNA fragments were visualized by autoradiography.

For Northern blotting, 10 μg of poly(A)$^+$ mRNA was treated with 1 M glyoxal–50% dimethyl sulfoxide–10 mM $NaHPO_4$ (pH 6.8) at 50°C for 1 h. The denatured RNA was then subjected to electrophoresis in 1.5% agarose gels prepared in 10 mM $NaHPO_4$ (pH 6.8). The RNA was transferred to nitrocellulose, and argininosuccinate synthetase mRNA was detected by hybridization as previously described (31).

RESULTS

Characterization of genomic clones from the expressed gene. To understand how the human argininosuccinate synthetase gene is regulated by arginine and overexpressed in Can^r cells, we had to obtain structural information on the expressed gene. Twenty-five unique clones were obtained when a Charon 4A-human genomic DNA library was screened with the cDNA for argininosuccinate synthetase. Fourteen of these clones represented nine unique argininosuccinate synthetase-processed pseudogenes (14). Restriction mapping and Southern blotting showed that the remaining 11 clones overlapped and spanned a 63-kb region of DNA (Fig. 1). The clones were oriented by probing Southern blots with various nick-translated restriction fragments of the argininosuccinate synthetase cDNA (Fig. 2). In this example, three genomic clones (λAS2, λAS3, and λAS13) were used. Genomic clone λAS2 contained sequences representing the 5′ portion of the cDNA, whereas λAS3 and λAS13 contained sequences that extended further in the 3′ direction. After the initial screening of the genomic library, it was shown that λAS13 extended the furthest in the 3′ direction. However, this genomic clone did not contain sequences representing the 3′-most 500 bp of the cDNA (data not shown). To obtain a clone containing the 3′ end of the gene, the Charon 4A-human genomic DNA library was screened a second time, using a repeat-free intron probe (4.3-kb *Bam*HI-*Eco*RI fragment) isolated from λAS13 (see Fig. 1). Southern blot analysis of genomic DNA showed that this probe hybridized to a 31-kb *Eco*RI fragment and a 16-kb *Hin*dIII fragment that had been previously mapped to chromosome 9q34→qter, the location of the expressed argininosuccinate synthetase gene (data not shown). It also was known that these two hybridizing restriction fragments were in the 3′ portion of the expressed argininosuccinate synthetase gene. One clone was obtained when the genomic library was screened with the intron probe; it was designated λAS25. Probing this clone with a restriction fragment of the cDNA containing the 3′-most 220 bp suggested that λAS25 probably contained the 3′ end of the gene (data not shown). The entire 63-kb region of DNA shown in Fig. 1 was subcloned into plasmids pBR322 and pUC-8 on seven *Eco*RI, two *Hin*dIII, and two *Hin*dIII-*Eco*RI restriction fragments ranging in size from 2.2 to 13.0 kb. Detailed restriction mapping and Southern blotting of both phage DNA and the subcloned genomic fragments indicated that there were at least 13 exons in the expressed argininosuccinate synthetase gene.

Defining the 5′ and 3′ boundaries of the expressed argininosuccinate synthetase gene. Primer extension experiments with mRNA isolated from Can^r cells indicated that the cDNA, pAS4/1, was missing ca. 20 to 25 bases of the 5′ end of the argininosuccinate synthetase mRNA (data not shown). The nucleotide sequence of the primer extension products also showed that a processed pseudogene, ψAS-3, contained the 5′ sequences that were present in the argininosuccinate synthetase mRNA but not in the cDNA. The 5′-most exon in the expressed gene was localized by probing the existing genomic clones with a *Pst*I-*Hin*fI restriction fragment of ψAS-3 that contained the 5′ gene sequences. These data showed that λAS17 and λAS15 contained a hybridizing region that was not previously identified with the cDNA, pAS4/1. This hybridizing sequence was localized on a 4.8-kb *Eco*RI fragment of λAS17 and was subsequently subcloned into plasmid pUC-8. The hybridizing material was subcloned further into pUC-8 on an 88-bp *Hae*III fragment and a 750-bp *Pst*I fragment. The nucleotide sequences of these subcloned fragments indicated that the 5′ end of the expressed gene was present in the 4.8-kb *Eco*RI fragment of λAS17 (Fig. 3A). The sequence of exon 1 in the expressed gene agreed with that of the 5′ region of ψAS-3 in 26 of 31 nucleotides and had perfect homology with the 5′-most eight bases of the sequence of pAS4/1. The cap site as determined by primer extension experiments (base +1, Fig. 3A) agreed well with the predicted cap site based on the homology between the expressed gene sequences and that of ψAS-3. Located 32 bases 5′ to the proposed cap site was the canonical TATAA sequence found in the promoter regions (−35 to −25 bp) of most RNA polymerase II-transcribed eucaryotic genes (7).

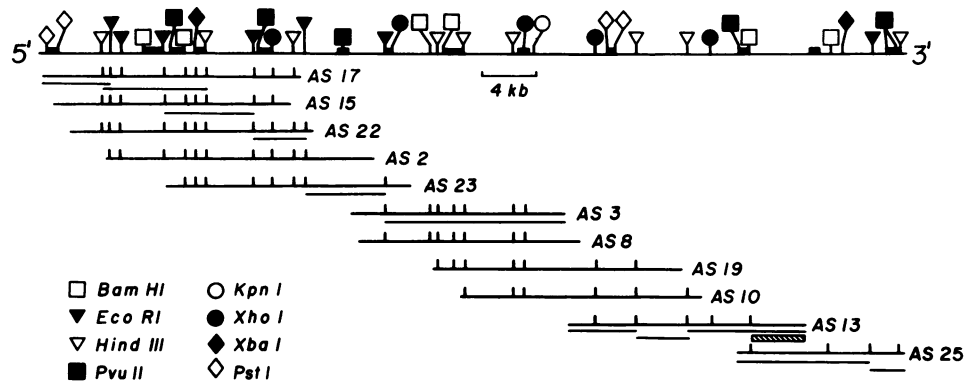

FIG. 1. Restriction map of the expressed argininosuccinate synthetase gene. Both phage DNA and subcloned genomic fragments were digested with eight restriction endonucleases. The hybridizing fragments were localized by Southern blotting with the cDNA, pAS4/1, as a hybridization probe. The underlined restriction fragments indicate those which were subcloned into plasmid pBR322 or pUC-8. The hatched bar indicates the repeat-free intron probe that was used to isolate the genomic clone containing the 3' end of the gene. The solid bars in the restriction map indicate exons. Not all the restriction sites mapped are shown.

The region 5' to the TATA box sequence (−152 to −32 bp) was 80% GC-rich and did not contain the canonical CAAT sequence that is found 70 to 90 nucleotides upstream from the cap site in some eucaryotic genes (7).

A 2.2-kb *Eco*RI fragment of λAS25 was subcloned into plasmid pBR322 and probed with a 3'-most restriction fragment of the argininosuccinate synthetase cDNA. The Southern blot (not shown) indicated that this subcloned fragment probably contained the sequences coding for the 3' untranslated region of the argininosuccinate synthetase mRNA. Since the argininosuccinate synthetase cDNA contains a *Pvu*II site 40 bases from the poly(A) region (6), the subcloned genomic fragment was sequenced from a *Pvu*II site which proved to be the same site as in the cDNA. This sequence in the expressed gene had perfect homology with that of the cDNA up to the site of poly(A) addition, and it had homology with a processed pseudogene sequence, ψAS-1, 8 bases further in the 3' direction than it had with the cDNA (Fig. 3b). Therefore, it is possible that there are two poly(A) addition sites in the argininosuccinate synthetase mRNA sequence. This hypothesis is consistent with the observation of two putative poly(A) additional signal sequences, ATAAAA and AATTAAAA, at positions 1,549 and 1,560 in the expressed gene. These sequences are also present in the cDNA and pseudogene sequences (Fig. 3B).

Exons 1, 2, 3, 4, and 9 were completely sequenced, and

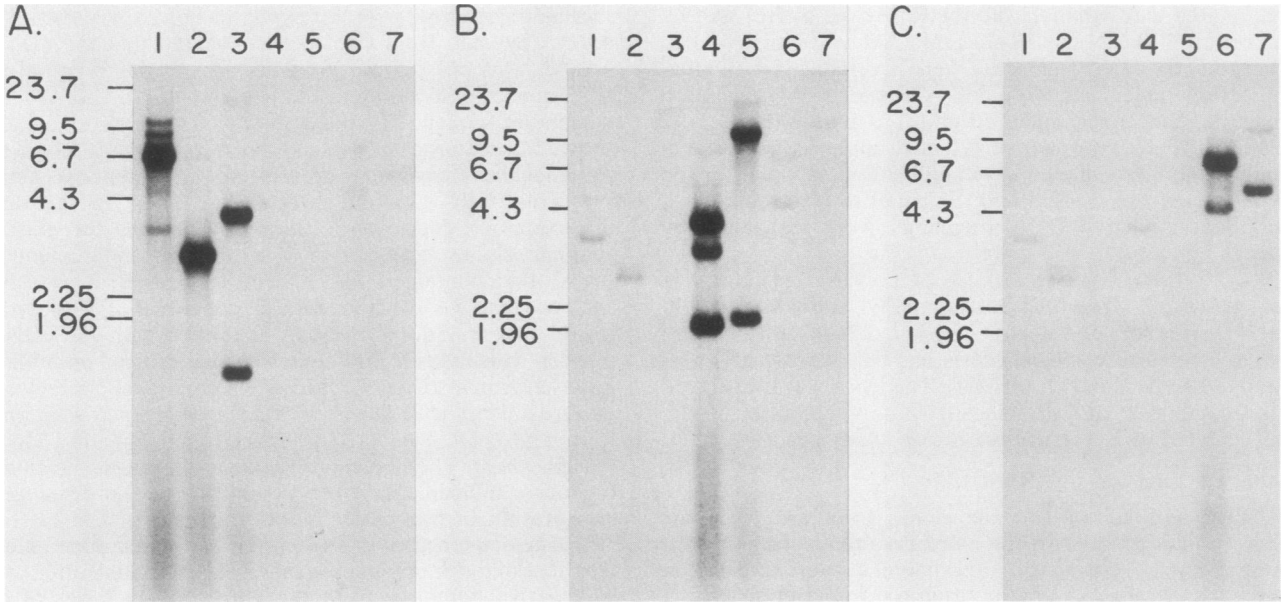

FIG. 2. Southern blotting of genomic clones from the expressed gene. Phage DNA (1 to 2 μg) was digested with various restriction endonucleases and subjected to electrophoresis in 1% agarose gels. The DNA fragments were transferred to nitrocellulose and probed with various nick-translated restriction fragments of pAS1. Lanes 1 to 3, λAS2 DNA digested with *Eco*RI, *Eco*RI-*Hin*dIII, and *Bam*HI, respectively; lanes 4 and 5, λAS3 DNA digested with *Eco*RI-*Hin*dIII and *Bam*HI, respectively; lanes 6 and 7, λAS13 DNA digested with *Eco*RI-*Hin*dIII, and *Bam*HI, respectively. (A) The probe used was a *Pst*I-*Hin*dIII restriction fragment of pAS1 (base 95 to 257 in the mRNA). (B) The probe used was an *Eco*RI-*Xho*I restriction fragment of pAS1 (base 545 to 767). (C) The probe used was an *Ava*I-*Acc*I restriction fragment of pAS1 (base 933 to 1,063). Numbers to the left indicate DNA markers in kb.

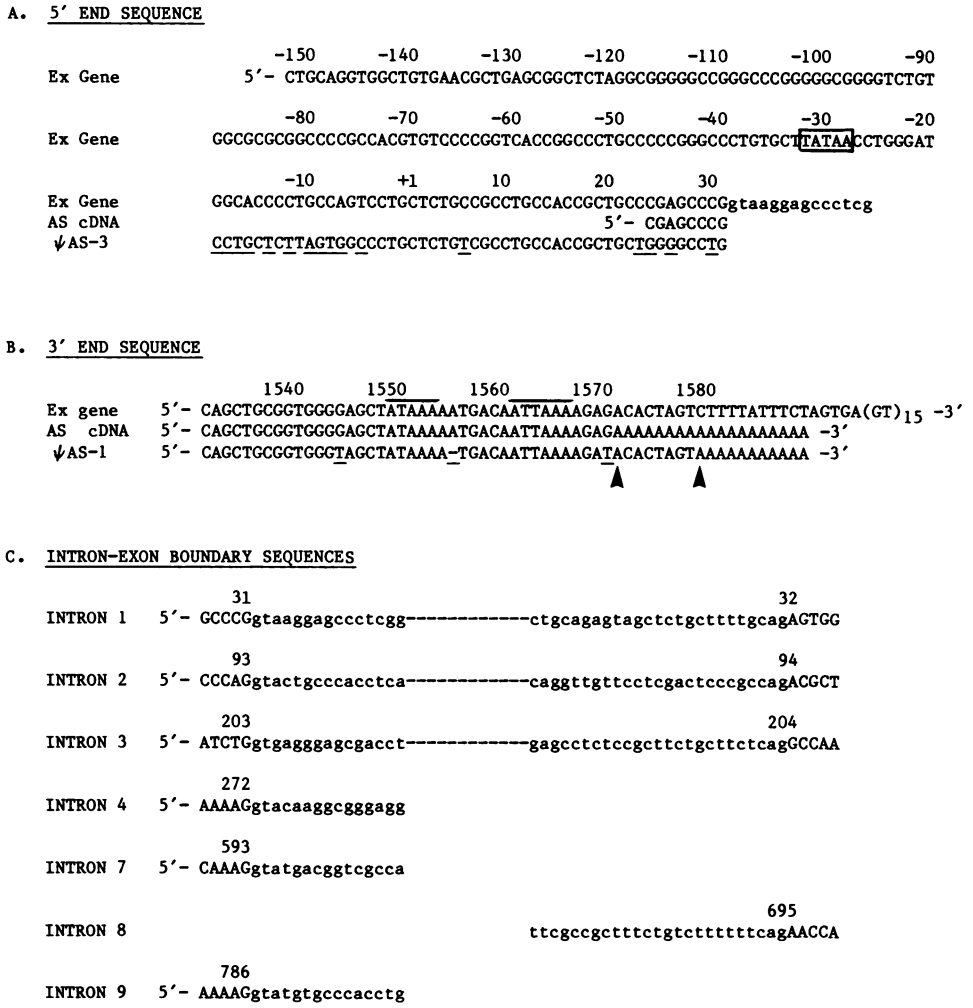

FIG. 3. The 5′ and 3′ boundaries and several exons in the expressed gene. Exon and flanking sequences are shown in capital letters; intron sequences are shown in smaller letters. (A) 5′ Promoter sequence. Base 1 indicates the cap site as determined by primer extension. The boxed region in the expressed gene sequence indicates the TATAA sequence. Underlined bases in ψAS-3 sequence differ from the expressed gene sequence. (B) 3′ End of the gene. Arrows indicate positions of poly(A) addition. Overlined bases in the expressed gene sequence identify putative poly(A) addition signal sequences. Underlined bases in the ψAS-1 sequence indicate bases that differ from the expressed gene sequence. (C) Exon-intron boundary sequences. The numbers indicate positions with respect to the mRNA sequence. Base 24 in exon 1 corresponds to base 1 in the consensus cDNA sequence described previously (6).

partial sequences for exons 7 and 13 have been obtained. The nucleotide sequences of these exons were in exact agreement with that of the cDNA, and their positions with respect to the nucleotide sequence of the argininosuccinate synthetase mRNA are indicated in Fig. 3C. Six intron donor sequences and four intron acceptor sequences showed that the exon-intron boundaries in the argininosuccinate synthetase gene complied with the well-documented GT-AG splice junction rule (7). As observed with most intron sequences, the regions 3′ to the donor sites were purine rich and the regions 5′ to the acceptor site were pyrimidine rich.

Alternative splicing of argininosuccinate synthetase mRNA. Primer extension experiments with Can^r mRNA showed that the nucleotide sequence 5′ to base 94 (the first base in exon 3) did not correspond to the sequence of exon 2 in the expressed gene. However, it was observed that the primer extension sequence 5′ to base 94 agreed perfectly with the first eight bases of a cDNA (pAS4) that extended the furthest in the 5′ direction (6). When the nucleotide sequence of exon 1 was obtained, we found that the first eight bases in pAS4 corresponded to the last eight bases of exon 1. These data indicated that argininosuccinate synthetase mRNA isolated from Can^r cells lacked exon 2 sequences. This observation was not consistent with the fact that exon 2 sequences were present in pAS4 which was isolated from a cDNA library prepared from Can^r cell mRNA. Furthermore, all of the human argininosuccinate synthetase pseudogenes in which DNA sequence data are available have exon 2 sequences. Therefore, it seemed likely that there were two populations of argininosuccinate synthetase mRNA in Can^r cells, one containing exon 2 sequences and serving as a template for pAS4 and another lacking exon 2 sequences and serving as a template in the primer extension experiments. S1 nuclease experiments with a 5′ restriction fragment of pAS4/1 as a hybridization probe showed that Can^r cell mRNA contained two populations of argininosuccinate synthetase mRNA (Fig. 4). In this experiment, a 5′ restriction fragment of pAS4/1 was hybridized to mRNA isolated from baboon liver

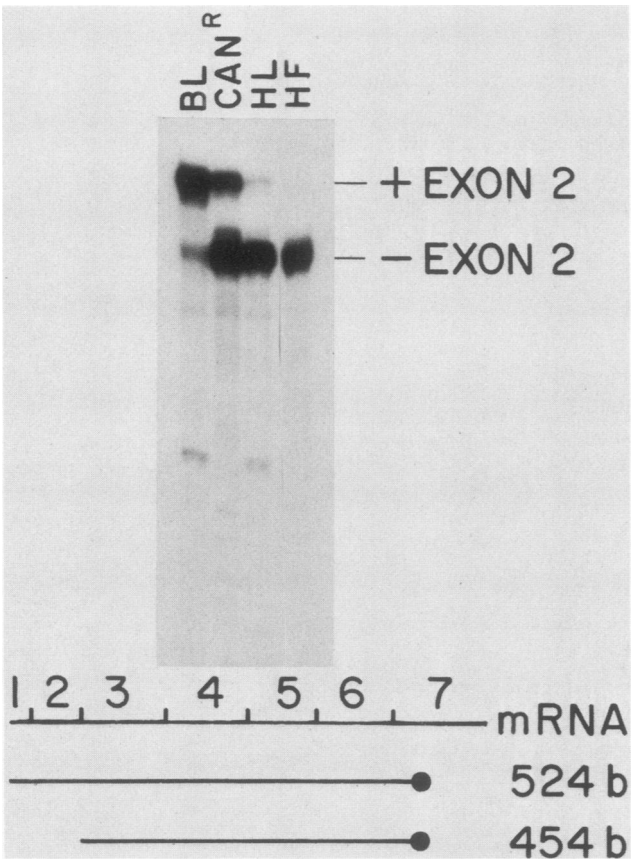

FIG. 4. S1 nuclease assays probing for exon 2 sequences. A 4.7-kb *Eco*RI restriction fragment of pAS4/1 was 5′ end-labeled at the *Eco*RI sites. This fragment contains 524 bases of argininosuccinate synthetase sequence, and the remaining portion is pBR322. One *Eco*RI site corresponds to sequences in exon 7 of the expressed gene. Poly(A) mRNA (10 μg) was used in the assays for baboon liver, Can^r cells, and human liver, and 50 μg of total RNA was used in the human fibroblast assay. BL, baboon liver mRNA; CAN^R, Can^r cell mRNA; HL, human liver mRNA; HF, human fibroblast mRNA. The products were separated on an 8% acrylamide sequencing gel and visualized by autoradiography for 16 h with an intensifying screen.

cells, Can^r cells, human liver cells and human fibroblasts. Argininosuccinate synthetase mRNA containing exon 2 sequences would protect a fragment 524 bases in length, whereas argininosuccinate synthetase mRNA lacking exon 2 sequences would protect a fragment 454 bases in length. The predominant argininosuccinate synthetase mRNA in baboon liver contained exon 2 sequences, and a minor amount of mRNA lacking exon 2 sequences was also present (Fig. 4). The converse result was obtained with mRNA isolated from both human liver and human fibroblasts. In these tissues, the predominant argininosuccinate synthetase mRNA lacked exon 2 sequences. An interesting result was obtained with mRNA isolated from Can^r cells. In these cells, both forms of the argininosuccinate synthetase mRNA were present in significant amounts, and the relative amount of mRNA containing exon 2 sequences was increased with respect to human liver or human fibroblasts. This result was further substantiated by Northern blotting with a genomic fragment containing exon 2 sequences as a hybridization probe (Fig. 5). These data indicated that there was a population of argininosuccinate synthetase mRNA in baboon liver and Can^r cells that contained exon 2 sequences and that the argininosuccinate synthetase mRNA from human liver lacked exon 2 sequences. The Northern blot also confirmed that the relative level of argininosuccinate synthetase mRNA containing exon 2 sequences in baboon liver was higher than that observed in Can^r cells. This observation is consistent with the S1 nuclease experiments shown in Fig. 4.

DISCUSSION

The alternative splicing patterns of the argininosuccinate synthetase mRNA are depicted in Fig. 6. Exons 1 and 2 encode 5′ untranslated sequences since the initiation codon is located in the 5′ region of exon 3 at position 99. It is likely that both forms of the argininosuccinate synthetase mRNA are translatable in vivo since baboon liver and human liver have 99% of form A or B, respectively, and livers from both species contain high levels of argininosuccinate synthetase enzymatic activity. In baboons, and perhaps in other lower primates, splicing pattern A is preferred, yielding mRNA 1,578 bases in length (not including the poly(A) region) which contains 98 bases of 5′ untranslated sequence that includes exon 2 sequences. In contrast, the human tissues we examined used predominantly splicing pattern B, produc-

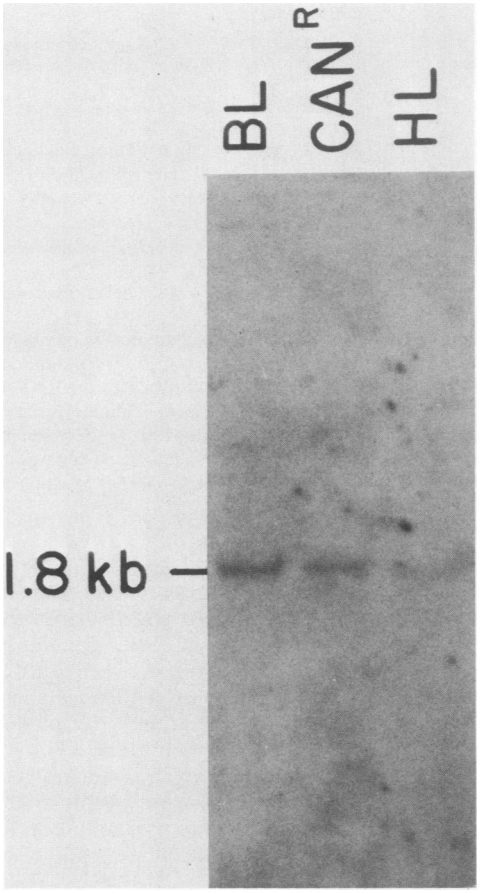

FIG. 5. Northern blot with exon 2 probe. Poly(A)$^+$ mRNA (10 μg) from baboon liver (BL), Can^r cells (CAN^R), and human liver (HL) were denatured and subjected to electrophoresis in 1.5% agarose. The RNA was transferred to nitrocellulose and probed with a nick-translated plasmid that contained exon 2 sequences cloned as an 190-bp *Pst*I fragment.

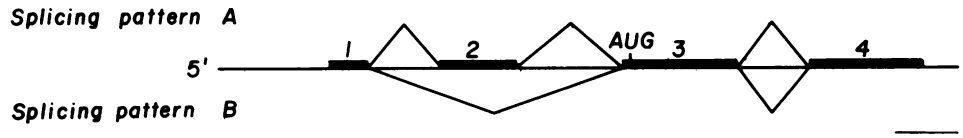

FIG. 6. Alternative splicing patterns of argininosuccinate synthetase mRNA. Exons are designated by the solid bars. Percent splicing patterns are as follows. Baboon liver, 99% pattern A, 1% pattern B; human liver, human fibroblast, 1% A, 99% B; human Can^r cells, 20% A, 80% B, human ancestors (10^7 years ago), 99% A, 1% B. These numbers represent estimates based on the data shown in Fig. 4.

ing mRNA that was 1,508 bases in length and contained 36 bases of 5′ untranslated sequence that lacked exon 2 sequences. The fact that pAS4, which contains exon 2 sequences, also contains the last eight bases in exon 1 showed that exon 1 sequences were present in the larger mRNA. Similarly, the nucleotide sequence of primer extension products in Can^r cell mRNA showed that the smaller argininosuccinate synthetase mRNA that lacked exon 2 sequences also contained exon 1 sequences. Therefore, alternative splicing of the argininosuccinate synthetase mRNA involves the exact inclusion or deletion of only exon 2 sequences. It is interesting to speculate why, in baboons, the argininosuccinate synthetase mRNA is spliced in the opposite way than in humans. One explanation could be that the acceptor sequence in intron 1 of the human argininosuccinate synthetase mRNA is a poor acceptor site. Examination of this acceptor site sequence showed the occurrence of three AG dinucleotides in close proximity to the acceptor AG dinucleotide (Fig. 3c). These sequences occurred 14 to 19 bases 5′ to the AG acceptor dinucleotide in intron 1. A survey of 97 intron sequences showed that the average distance between the acceptor AG dinucleotide and the nearest 5′ proximal AG was 41.5 bases, with the shortest distance being 14 bases in all introns surveyed and 17 bases when only human introns (n = 16) were surveyed (23). Therefore, it is possible that the occurrence of AG dinucleotides within a given distance of the acceptor AG dinucleotide causes infrequent splicing at that acceptor site. It is not likely that the different splicing seen between baboons and humans is due to a polymorphism in the acceptor sequence in the human gene, since splicing pattern B was the predominant pattern in 15 different human individuals representing 30 different genes (unpublished data).

In Can^r cells, both mRNAs are present, with the mRNA that lacks exon 2 sequences being the predominant form. It is known that the increased level of argininosuccinate synthetase in Can^r cells is controlled at the pretranslational level and probably involves an increase in gene transcription, stabilization of argininosuccinate synthetase mRNA, or both. S1 nuclease experiments with nuclear RNA isolated from Can^r and wild-type cells has shown a higher steady-state level of precursor RNA in the nucleus of Can^r cells (T. S. Su, unpublished data). These data imply that the increased level of mature argininosuccinate synthetase mRNA in Can^r cells is regulated at the level of nuclear RNA processing, nuclear RNA stability, or gene transcription. Although it is possible that nuclear precursors (or the mature message) containing exon 2 sequences are more stable than mRNAs lacking these sequences, it is not likely that the increased level of argininosuccinate synthetase activity in Can^r cells is due to a change in the abundance of the mRNA containing exon 2 sequences. S1 nuclease experiments with mRNA isolated from Can^r and wild-type cells have indicated that the increased level of mature argininosuccinate synthetase mRNA in Can^r cells is predominantly due to an increase in the mRNA lacking exon 2 sequences (unpublished data). Furthermore, since enzyme activity is directly proportional to the level of mature mRNA, it is also unlikely that a change in the rate of translation of the argininosuccinate synthetase mRNA has a role in the Can^r phenotype. Therefore, the biological significance, if any, of alternative splicing of the argininosuccinate synthetase mRNA in Can^r cells is uncertain at this time.

Analysis of clones isolated from a human genomic DNA library has led us to conclude that there is a single expressed argininosuccinate synthetase gene and at least nine argininosuccinate synthetase-processed pseudogenes in the human genome. Chromosomal mapping data suggest that there are in fact 14 argininosuccinate synthetase-like sequences that are dispersed to 11 human chromosomes (Su et al., in press). Analysis of human structural gene mutations causing enzyme deficiency (30a) and analysis of human pseudogenes (14) support the interpretation that there is a single expressed gene. Since all of the genomic clones either represent pseudogenes or can be aligned to form one linear DNA map, there is no evidence for more than one functional gene.

We previously reported the complete nucleotide sequence of two argininosuccinate synthetase processed pseudogenes ψAS1 and ψAS3 (14). Both of these pseudogenes contained exon 2 sequences and were 93% homologous to the cDNA sequence. On the basis of this homology, it was estimated that these processed pseudogenes arose 10 to 11 million years ago, probably before the divergence of the great apes and hominids. Since nearly all of the argininosuccinate synthetase mRNA in the human tissues we examined lacked exon 2 sequences, we would predict that any processed pseudogene transcribed from human mRNA might also lack exon 2 sequences. Probing human genomic DNA with an exon 2 probe should distinguish between pseudogenes containing exon 2 sequences and those lacking exon 2 sequences. Therefore, this analysis might allow one to distinguish between processed pseudogenes of a more recent origin (lacking exon 2 sequences) compared with those which arose earlier in evolution (containing exon 2 sequences). By comparing the sequence of a pseudogene lacking exon 2 sequences with that of pseudogenes containing exon 2 sequences, one might estimate the time in evolution when the argininosuccinate synthetase mRNA of primates began splicing in a fashion similar to that of humans.

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