Properties of Human Liver Cytosolic Aspartate Aminotransferase mRNAs Generated by Alternative Polyadenylation Site Selection†

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ABSTRACT: Human cytosolic aspartate aminotransferase (cAspAT) cDNA clones have been isolated from an adult human liver cDNA library. Among the clones, two cDNAs of 1550 and 1950 base pairs, respectively, have been characterized. These two cDNAs differ only in the lengths of their 3′ noncoding regions and by the presence of one or two putative polyadenylation signals AATAAA. Northern blot analysis revealed two different mRNAs of 2.1 and 1.8 kbp in several human tissues, whereas Southern blot analysis suggested the existence of a single gene for the human cAspAT. The two mRNA species result from the alternative use of two polyadenylation signals. In the liver, the relative ratio of these mRNAs varies among different species and, in humans at least, during development. The properties of the two mRNAs were compared. The half-lives of the 2.1 and 1.8 kbp mRNAs, in the HepG2 cell line, are 8 and 12 h, respectively. The two mRNAs have similar and rather short poly(A) tracts of 20–50 nucleotides. Both mRNAs are capable of directing the in vitro synthesis of the cAspAT protein. We conclude that both the 2.1 and 1.8 kbp cAspAT mRNAs are functional and exhibit similar properties.

Aspartate aminotransferase (AspAT,[1] EC 2.6.1.1) is a ubiquitous pyridoxal phosphate dependent enzyme. Two isoenzymes are present in animal cells: the cytosolic aspartate aminotransferase (cAspAT) and the mitochondrial aspartate aminotransferase (mAspAT) (Braunstein & Snell, 1985). Recently, we reported the nucleotide sequences of the rat cAspAT and pre-mAspAT (Pavé-Preux et al., 1988), and of the human pre-mAspAT (Pol et al., 1988). Northern blot analyses have revealed the presence of two different cAspAT mRNAs in the rat (Pavé-Preux et al., 1988) and in the chicken (Mattes et al., 1989), whereas a single cAspAT mRNA species has been identified in the mouse (Obaru et al., 1986) and in the pig (Nagashima et al., 1989). Several mechanisms can lead to the production of different mRNAs from the same gene [reviewed by Leff et al. (1986)]. Differential splicing events or the selective choice of several polyadenylation sites or both mechanisms can lead to multiple mRNAs generated from the same primary transcript (Barbas et al., 1988; Hashinaka et al., 1988; Kornfeld et al., 1989). Alternative RNA processing events may exhibit either tissue (Leff et al., 1987; Nawa et al., 1984; Streuli & Saito, 1989) or developmental specificities (Rozek & Davidson, 1983). Although the alternative use of distinct polyadenylation sites has been postulated to explain the presence of several mRNAs from a single gene (Zehner & Paterson, 1983; Schweizer et al., 1989; Yanagisawa et al., 1988), the regulatory function of this alternative processing event in vivo is still unknown. Indeed, the mRNAs generated by distinct polyadenylation signals usually differ in their 3′ noncoding region and are thought to encode for the same protein. Other mRNA parameters such as stability, poly(A) tract length, or translational capacity could be modified by different 3′ noncoding regions.

In this study, we have addressed these important questions. We report the cloning of different human cAspAT cDNAs from liver. The sequence of these cDNAs allowed us to show that they correspond to two different mRNAs of 2.1 and 1.8 kbp which are transcribed from the same gene, by using different polyadenylation signals. The mRNAs have slightly different half-lives and poly(A) tail lengths, and their relative ratio varies among different species and, in humans, during development. In an in vitro system, they are both equally efficient in directing the synthesis of the 44000-Dalton cAspAT monomer.

Materials and Methods

Screening of a cDNA Library from Human Liver. An adult human liver cDNA library (5.5×10^5 independent clones), constructed in λgt11, was purchased from Clontech Laboratories (Palo Alto, CA). One million clones were plated on NZY medium, supplemented with 50 μg/mL ampicillin, at a density of 142 plaque-forming units/cm^2 (Davis et al., 1986). Nitrocellulose replica filters were screened for human cAspAT sequences using a nick-translated rat cAspAT cDNA (4B21), recently isolated in our laboratory (Pavé-Preux et al., 1988). The nitrocellulose filters were prehybridized at 68 °C for 6–12 h in a medium containing 6 × SSC (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate), 1× Denhardt's solution [0.02% Ficoll, 0.02% poly(vinylpyrrolidone), and 0.02% bovine serum albumin], and 100 μg/mL denatured salmon sperm DNA. The filters were hybridized to 50×10^6 cpm of the labeled rat cAspAT probe (10^8 cpm/μg of DNA) in 2 × SSC, 1× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 25 mM NaH_2PO_4, pH 7.2, and 2 mM EDTA, for 16 h at 68 °C, and washed 3 times in 2 × SSC/0.1% SDS at 65 °C for 60 min. The screening of a second human cDNA library constructed in λgt11 (a kind gift of Dr. J. Edman) was performed under the same conditions using, as probe, a cAspAT cDNA obtained

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[1] Abbreviations: AspAT, aspartate aminotransferase; cAspAT, cytosolic AspAT; mAspAT, mitochondrial AspAT; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; Denhardt's solution [Ficoll (0.02%), poly(vinylpyrrolidone) (0.02%), and bovine serum albumin (0.02%)]; kbp (kb in figures), kilobase pair(s); bp, base pair(s); EDTA, ethylenediaminetetraacetate.

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from the screening of the first library. In this experiment, the filters were washed under highly stringent conditions (0.1 × SSC/0.1% SDS, 68 °C for 60 min). The filters were autoradiographed using hyperfilm Amersham MP and two intensifying screens at −80 °C.

Characterization of the Positive Clones. Positive clones were further purified by four successive screenings at lower plaque density, and amplified in liquid NZY medium supplemented with 50 μg/mL ampicillin. The phage were isolated on a discontinuous cesium chloride gradient according to Maniatis et al. (1982) with the following modifications: after the poly(ethylene glycol)/NaCl precipitation of the phage from a 250-mL culture, the pellet was resuspended in 12 mL of 10 mM Tris-HCl, pH 7.5, and 10 mM $MgSO_4$. This solution was layered on a discontinuous cesium chloride gradient composed of 9 mL of 5 M CsCl and 15 mL of 3 M CsCl dissolved in 10 mM Tris-HCl, pH 7.5, and 10 mM $MgSO_4$. The tubes were centrifuged in a Beckman SW 27 rotor at 23 000 rpm for 2 h at 20 °C. The phage particles were collected, and the phage DNA was extracted according to Maniatis et al. (1982). The cDNAs were excised from the λgt11 vector by the restriction enzyme *Eco*RI, and their sizes were determined on a 0.8% agarose gel. The cDNAs were subcloned in the *Eco*RI site of M13mp18 or of the plasmid PGEM-4Z (Promega Biotechnology) for sequencing and for the synthesis of complementary DNA probes.

Sequencing. The cDNA inserts were sequenced according to the dideoxynucleotide chain termination method of Sanger et al. (1977) using [α-^{35}S]dATP. Linearized single-strand DNA of recombinant M13 was submitted to directed digestion by the T4 polymerase (Dale et al., 1985), so as to obtain overlapping clones. For the cDNAs isolated from the first library, the sequence was completed by using oligonucleotides, prepared on an Applied Biosystems synthesizer, as sequencing primers. The cDNAs isolated from the second human liver library were subcloned only in pGEM-4Z, and denatured plasmids were sequenced by using synthetic oligonucleotides as primers.

RNA Preparation. Total RNA was isolated from various tissues and liver-derived cell lines, using the guanidine thiocyanate extraction method described by Chirgwin et al. (1979).

Northern Blot Hybridization. Total RNA (20 μg) was electrophoresed on a horizontal 1.2% agarose/2.2 M formaldehyde gel (Lehrach et al., 1979), transferred to a Hybond-N$^+$ membrane (Amersham), and fixed with 0.05 M NaOH. The membrane was prehybridized for 16 h at 42 °C in 50% formamide, 5 × SSC, 1× Denhardt's solution, 50 mM sodium phosphate buffer, pH 6.8, 0.2% SDS, and 200 μg/mL denatured salmon sperm DNA. The membrane was then hybridized overnight at 42 °C in the same medium containing 10% dextran sulfate, 200 μg/mL denatured salmon sperm DNA, and 20 × 10^6 cpm/mL denatured cDNA probe. The membrane was washed in 1 × SSC/0.1% SDS at 68 °C and autoradiographed by using two intensifying screens and an Amersham MP film for 24–72 h at −80 °C. The Northern blots were dehybridized in a solution of boiling 0.1% SDS and then hybridized with a ribosomal RNA probe as control.

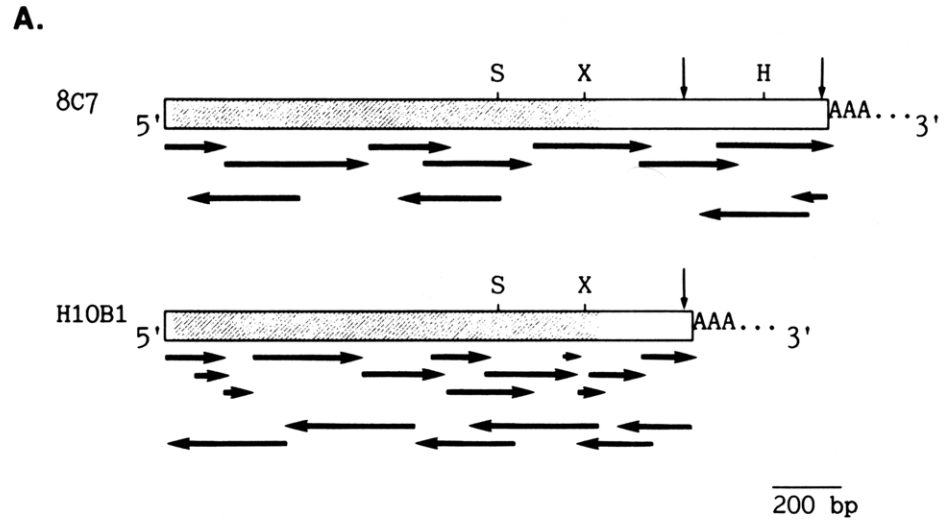
Genomic DNA Preparation and Southern Blot Hybridization. DNA from lymphocytes of caucasian blood donors was isolated, purified (Blin & Stafford, 1976), and digested with five restriction endonucleases (*Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pvu*II); the DNA fragments were then separated on a 0.8% agarose gel and transferred to a nylon membrane (Nytran, Schleicher & Schuell) according to Southern's procedure (Southern, 1975). The membrane was hybridized to a 532 base pair (bp) *Xba*I–*Hin*dIII restriction fragment corresponding to the 3′ end of the cAspAT 8C7 cDNA (see Figure 1A).

Complementary DNA Probes. The cAspAT cDNA clones, H10B1 isolated from the first library and 8C7 isolated from the second library (see Figure 1A), were subcloned into the *Eco*RI site of the plasmid pGEM-4Z. The inserts were purified on low melting point agarose gels (Bethesda Research Laboratories) following *Eco*RI digestion of the plasmids. The 1550 bp H10B1 cDNA covers the entire mRNA coding sequence, plus a 283 bp long 3′ noncoding region. The 532 bp *Xba*I–*Hin*dIII restriction fragment purified from the 8C7 insert was used as a specific 3′ sequence. It covers 310 bp of the 3′ noncoding sequence present in both cDNAs (H10B1 and 8C7), and an additional 220 bp sequence only present in the 8C7 clone.

Determination of the Half-Lives of cAspAT mRNAs. The human hepatoma cells, HepG2, were grown in a modified Ham's F12 medium (Gibco) (Coon & Weiss, 1969) supplemented with 10% fetal calf serum (Flow), 100 units/mL penicillin, 100 μg/mL streptomycin (Lab. Diamant), and 0.05 μg/mL fungizone (Squibb). The cells were treated either with 1.5 μg/mL actinomycin D or with buffer only. After 6, 22, or 30 h of treatment, the cells were harvested in 4 M guanidinium thiocyanate, 2.5 mM sodium citrate, and 0.1 M β-mercaptoethanol, pH 7. Total RNA was isolated and analyzed on a Northern blot using the nick-translated H10B1 insert as a probe. The intensity of the bands was determined by scanning the autoradiogram using a densitometer equipped with an integrator.

RNase H Experiment. The reactions were carried out according to Berkower et al. (1973) and Donis-Keller (1979) with some modifications. Briefly, total cytoplasmic RNA (40 μg) was heated at 85 °C for 5 min and hybridized to 2 μg of the oligonucleotide $_{5'}$TGCCCTGAGTTCAGATCT$_{3'}$, which is complementary to a cAspAT mRNA sequence including nucleotide +1027 to nucleotide +1044, with or without 10 μg of $(dT)_{18}$. Hybridization was performed in a 40-μL solution containing 40 mM Tris-HCl, pH 8, 4 mM $MgCl_2$, 1 mM dithiothreitol, and 30 μg/mL bovine serum albumin. Both oligonucleotides were made by using an Applied Biosystems synthesizer. After hybridization for 10 min at 42 °C, 2 units of RNase H (Amersham) was added, and the solution was incubated at 37 °C for 30 min. The reaction was extracted with phenol/chloroform and precipitated with ethanol. The products of the digestion were separated on a 2% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond-N$^+$ membrane (Amersham). The membrane was hybridized as described above.

In Vitro Transcription and Translation of cAspAT cDNA. Transcription of the plasmid 8C7 was performed by using the Riboprobe Gemini System (Promega). Six micrograms was linearized by *Bst*XI after the poly(A) tail or by *Sca*I immediately downstream from the first polyadenylation signal, and the RNAs were transcribed from the T7 polymerase promoter. The transcription products were analyzed by Northern blot as described above. The size of the mRNAs synthesized was approximately 2.1 kbp for the plasmid linearized by *Bst*XI and 1.6 kbp for the plasmid linearized by *Sca*I, and corresponded to the large and to the small cDNAs, respectively (data not shown). Increasing amounts of both mRNAs (6, 60, 180, and 600 ng) were used to direct in vitro translation in a rabbit reticulocyte lysate system (Stratagene). A negative control was performed by translating the antisense mRNA; this control RNA was transcribed from the SP6 promoter of the plasmid



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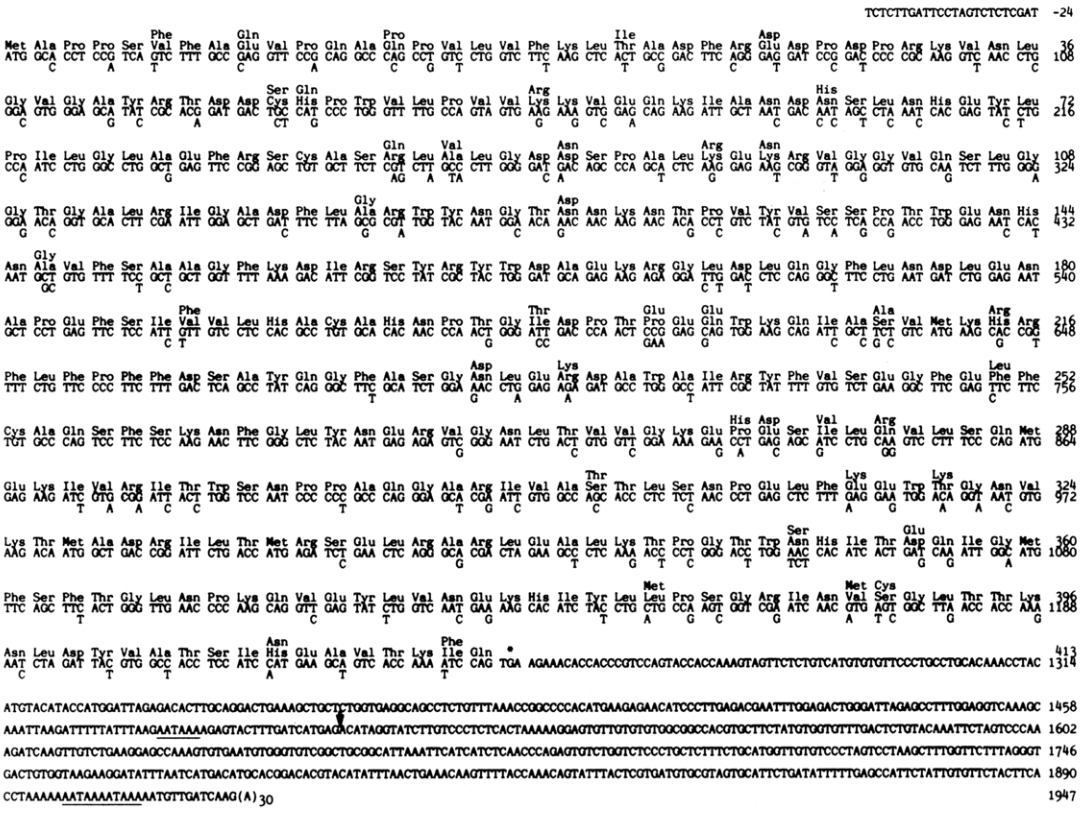

FIGURE 1: (A) Restriction map and sequence strategy for the two cytosolic AspAT cDNA clones. The hatched area corresponds to the coding region. Relevant restriction sites are indicated above the cDNAs (S = *Sac*I; X = *Xba*I; H = *Hin*dIII). Vertical arrows indicate the position of the AATAAA putative polyadenylation signals. The sequencing strategies are summarized beneath the maps; the direction and extent of each determination are shown by horizontal arrows. (B) Nucleotide and deduced protein sequences of the human cytosolic aspartate aminotransferase cDNA. The nucleotide sequence is the composite of the clones H10B1 and 8C7. The vertical arrow indicates the site of the poly(A) addition to the H10B1 cDNA. The predicted amino acid sequence is shown above the corresponding codons. Positive numbering of the protein and the nucleotide sequences starts at the putative initiation codon. Nucleotides and amino acids that differ in the rat cAspAT are indicated under and above the human sequences, respectively. Putative polyadenylation signals in the 3′ noncoding region are underlined. The stop codon is indicated by an asterisk.

8C7 linearized by *Kpn*I (data not shown). The in vitro translation products were analyzed on a 10% polyacrylamide–SDS gel according to Laemmli (1970).

RESULTS

Screening of Human Liver cDNA Libraries. Out of 10^6 clones screened from the first human liver cDNA library, 19 cAspAT clones were detected and isolated. These purified phages contained cDNA inserts of sizes ranging from 1.2 to 1.6 kbp. From the second human liver cDNA library, 19 clones were purified out of 700 000 phages. These positive phages contained cDNAs of sizes ranging from 1.5 to 2.1 kbp.

Sequence Analysis. Among the 38 cAspAT clones purified from the 2 libraries, 13 overlapping clones were sequenced at both ends and mapped to the RNA sequence. Among these clones, three covered only the coding region, and eight had a short 5′ untranslated region, the entire coding sequence, and a 3′ untranslated region containing a polyadenylation signal

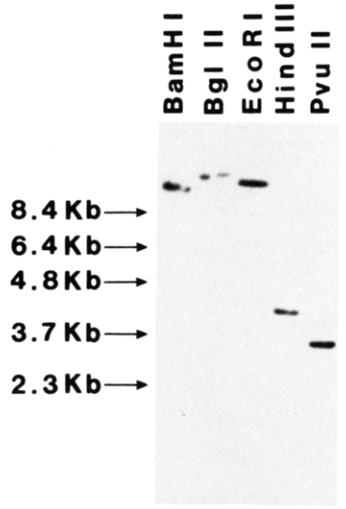

FIGURE 2: Southern blot analysis of human genomic DNA. The genomic restriction fragments obtained as described under Materials and Methods were hybridized to the *Xba*I–*Hin*dIII restriction fragment from the clone 8C7 as a 3′ probe. The sizes of molecular weight markers run on the same gel, which were stained with ethidium bromide, are indicated on the left (λDNA–*Bst*EII digest, Biolabs).

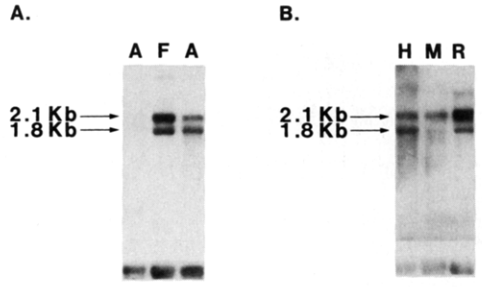

FIGURE 3: Northern blot analysis. Northern blots were prepared as described under Materials and Methods. Blots were hybridized to an *Eco*RI–*Sac*I fragment from the clone 8C7 as a cytosolic AspAT coding probe. Blots were dehybridized and then hybridized to an oligonucleotide specific for the rat ribosomal 18S RNA. The ribosomal RNA band is shown below each blot. In (A), 20 μg of total RNA from adult [right lane (A)] and fetal (F) human livers was loaded per lane, 10 μg of total RNA from adult human liver was loaded in the left lane (A), and 30 μg of total RNA from human (H), mouse (M), and rat (R) livers was used in (B). The size of the cAspAT mRNAs, reported on the left, was determined from molecular weight markers (RNA ladder, Bethesda Research Laboratories) run on the blot and stained with a solution of 0.02% methylene blue and 0.3 M sodium acetate, pH 5.5, after transfer to a nylon membrane.

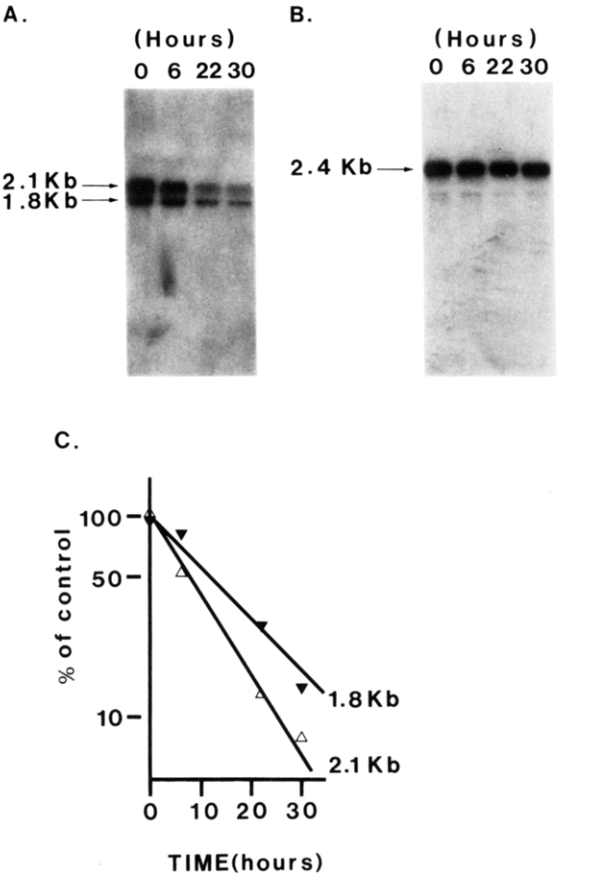

FIGURE 4: Half-lives of mRNA species in HepG2 cells. Total RNA (30 μg) from HepG2 cells treated with actinomycin D for 0, 6, 22, and 30 h was analyzed by Northern blot hybridization as described under Materials and Methods. The blot was hybridized to the cytosolic AspAT H10B1 cDNA (A), and after dehybridization, the same blot was rehybridized to a human mitochondrial AspAT cDNA (B). The blot was also hybridized to an oligonucleotide specific for the rat ribosomal 18S RNA (not shown). The amounts of the cytosolic AspAT mRNAs were determined by scanning densitometry of (A), and the relative values were plotted against the time of incubation with actinomycin D (C); the half-lives of the 1.8 kbp (▼) and the 2.1 kbp (△) cAspAT mRNAs correspond to the time necessary for a decrease in intensity of 50% for the cAspAT mRNA band following actinomycin D treatment (untreated cells, 100%).

followed by a poly(A) tail. The last two cAspAT cDNA clones perfectly matched the complete sequence of the eight previous clones, but, in addition, they had in the 3′ sequence a second polyadenylation signal followed by a poly(A) tail.

Two overlapping cAspAT cDNAs (H10B1 and 8C7) were completely sequenced and are shown positioned with respect to each other (Figure 1A). The complete nucleotide sequence of these clones is shown in Figure 1B. In this 1947 bp nucleotide sequence, the first ATG (+1) was identified as the initiation codon by comparison with the cAspAT nucleotide sequence from other species (Obaru et al., 1986; Pavé-Preux et al., 1988). This codon defines a short 5′ untranslated region of 24 nucleotides, a single open reading frame of 1239 nucleotides, and a 3′ noncoding region composed of 678 bp plus a poly(A) tail. The amino acid residues of cAspAT which are required for catalysis have been highly conserved during evolution (Obaru et al., 1986; Pavé-Preux et al., 1988; Nagashima et al., 1989); they are Tyr-71, Asp-223, Lys-259, and Arg-387 (Figure 1B). Furthermore, these functionally important residues are also invariant in the human mitochondrial isoenzyme (Pol et al., 1988). The 3′ untranslated region contains two putative polyadenylation signals (AAUAAA), one at position 1480 and the other at position 1899. Interestingly, at this second position, there are two successive putative polyadenylation signals (AAUAAAAUAAA) (Figure 1B).

Southern Blot Analysis of Human Genomic DNA. Southern blot analysis of human genomic DNA, digested by the *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pvu*II restriction endonucleases, showed that a single band hybridized to the 532 bp *Xba*I–*Hin*dIII fragment from 8C7 (Figure 2).

Northern Blot Analysis. Total RNA isolated from human, mouse, and rat livers was analyzed by Northern blotting using the cAspAT H10B1 cDNA as a probe (Figure 3A,B). Two distinct cAspAT mRNAs were identified in the RNA samples, with respective sizes of 1.8 and 2.1 kbp. The 1.8 kbp mRNA species is more abundant in the human adult liver (Figure 3B), whereas in the human fetal liver the 2.1 kbp species is predominant (Figure 3A).

Half-Lives of mRNA Species in HepG2 Cells Treated with Actinomycin D. RNAs from HepG2 cells treated with actinomycin D for 0, 6, 22, and 30 h were analyzed by Northern

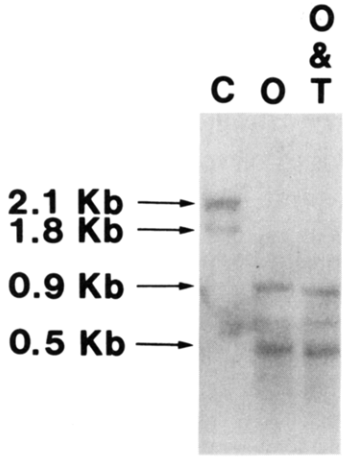

FIGURE 5: Poly(A) tail length estimation. Thirty micrograms of total fetal liver RNA was loaded as a control (lane C). In lanes O and O&T, 40 μg of the fetal liver RNA was hybridized to an oligonucleotide complementary to a cAspAT mRNA sequence from nucleotide +1027 to nucleotide +1044. In lane O&T, the 40 μg of fetal liver RNA was also hybridized to $(dT)_{18}$. Then the RNA–DNA hybrids were submitted to RNase H digestion (see Materials and Methods). The resulting fragments were separated on a Northern blot, and the resulting 3′ fragments were hybridized to the *Xba*I–*Hin*dIII fragment from 8C7. The sizes of the fragments are indicated to the left of the blot.

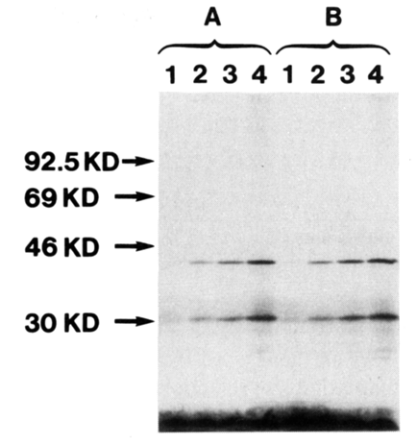

FIGURE 6: In vitro translation of the two cAspAT mRNAs. Increasing amounts of RNAs (lane 1, 6 ng; lane 2, 60 ng; lane 3, 180 ng; lane 4, 600 ng) corresponding to the short (A) and to the long (B) mRNAs were translated in a rabbit reticulocyte lysate. The translation products were analyzed by SDS–polyacrylamide gel electrophoresis, followed by autoradiography. Arrows and numbers to the left refer to the molecular weight markers.

blotting for cAspAT and mAspAT (Figure 4A,B). The amounts of the cAspAT mRNAs were determined by densitometric analysis, and the relative values were plotted against the time of incubation with actinomycin D (Figure 4C). The half-life of cAspAT mRNA corresponds to the time necessary for the disappearance of 50% of the amount of cAspAT mRNA in the cells treated with actinomycin D as compared to the control (100%). The half-lives of the 2.1 and 1.8 kbp species are slightly different (8 and 12 h, respectively). In contrast, the amount of the mAspAT mRNA was only slightly decreased after a 30-h treatment with actinomycin D (Figure 4B), thus indicating that this mRNA is much more stable than the cAspAT mRNAs.

Estimation of the Poly(A) Tail Length. A reduction in size of cAspAT mRNAs was performed by RNase H treatment of cAspAT mRNA sequences hybridized to an oligonucleotide complementary to nucleotides +1027 to +1044 of the cAspAT mRNA sequence. The resulting RNA fragments, hybridizing with the *Xba*I–*Hin*dIII fragment from 8C7 as a 3′ probe, are 896 and 480 nucleotides long (Figure 5, lane O). Further digestion of the poly(A) tract by addition of oligo(dT) and RNase H digestion decreased the size of both fragments (Figure 5, lane O&T). The difference between the size of the fragments with and without oligo(dT) addition gives an estimate of the length of the poly(A) tract. From this experiment, the length of the poly(A) tail can be estimated to be approximately 20–50 bases for both the 1.8 kbp mRNA and the 2.1 kbp mRNA. We did not detect mRNAs that were not polyadenylated (Figure 5).

In Vitro Translation of cAspAT mRNAs. Translation products of the two cAspAT mRNAs were separated on a 10% polyacrylamide–SDS gel (Figure 6). Increasing amounts of the 1.6 kbp mRNA (Figure 6A, lanes 1–4) and of the 2.1 kbp mRNA (Figure 6B, lanes 1–4) code, with a similar efficiency, for a 44 000-dalton polypeptide. The 30 000-dalton polypeptide, which was also synthesized, could be derived either from the initiation of translation at an internal methionine codon or from the transcription of the Amp^r gene of the vector. The latter possibility is very likely since this polypeptide was also present when the vector with an inverted insert was used as a template for the transcription–translation reaction (data not shown).

Discussion

We report here the nucleotide sequence of the human cAspAT cDNA. The overall homology between the human and the rat cAspAT nucleotide sequences is approximately 79%. The homology in the coding region is higher (87%) than in the 3′ noncoding region (69%). The predicted amino acid sequence is 90% identical with the rat cAspAT (Pavé-Preux et al., 1988); this confirms the high conservation of this protein in different species (Joh et al., 1985; Kondo et al., 1987; Horio et al., 1988; Mattes et al., 1989). The homology between human cytosolic and mitochondrial (Pol et al., 1988) AspAT is 50%; the same degree of homology was found in the rat between the cAspAT and the mAspAT (Pavé-Preux et al., 1988). The two cDNAs (H10B1, 8C7) that we sequenced were 100% identical in their coding region and therefore encode for the same protein; moreover, all the other cDNAs that were partially sequenced were also derived from one of the two cAspAT mRNA species. Therefore, the different isoenzymes for cAspAT that have been reported (Denisova & Polyanovsky, 1973) are probably due to posttranslational modifications of the protein.

Several data suggest that the cytosolic aspartate aminotransferase is encoded by a single-copy gene in the human genome: (i) with the use of in situ chromosomal hybridization, the cAspAT gene has been localized to a unique chromosomal site at the 10q24 locus (Pol et al., 1989); (ii) Southern blot analysis of human genomic DNA digested with several restriction enzymes always reveals a single band hybridizing with a cAspAT probe (Figure 2); (iii) all the overlapping sequences of the various cDNAs are identical. These results are consistent with those obtained by Obaru et al. (1988), who have isolated five genomic clones all of which were derived from the same gene in the mouse.

The Northern blot analysis presented here reveals two mRNA bands for the human cAspAT. Previously, we obtained similar results when analyzing the rat cAspAT mRNAs, and, using various probes, we showed that these mRNAs differed in the lengths of their 3′ noncoding regions (Pavé-Preux et al., 1988). In the present study, we have succeeded in cloning two human cAspAT cDNAs corresponding to both mRNAs. The sequence of these cDNAs demonstrates that

Table I: Nucleotide Sequence Located Downstream of the First Polyadenylation Signal of cAspAT cDNAs in Various Species[a]

species	sequence
human	AATAAAAGAGTACTTTGATCATGAGACATAGGTATCTTGTCCCTCTCACTAAAAAGGAGTGTTGTGTGTGGCGGC
pig	AATAAAAAGGTCCTTTGATCATGAGATGTAGATGTCTTGCCCCCTCACTAGAAGCAGGAGTATTGCCTGTGTCACT
rat	AATAAAAAAACCCTTTGATATGAGATGTAATCATCTTGCCTTCCTCTGTAGTATTCTGCAGGAGTGTTGCCCACG
mouse	AATAAAAAGAACTTTGGCTATGAGATGTAATCACCTTGCCTTCCTCTCTAGCATTCTGCAGGAGTGATGCCCACG

[a] The nucleotide sequence starts at the polyadenylation signal AATAAA (position 1480 in the human cDNA). The sequences of the cAspAT cDNAs are from the following references: human (our results), pig (Nagashima et al., 1989), rat (Pavé-Preux et al., 1988), and mouse (Obaru et al., 1986).

the two mRNAs are generated from a single gene by differential polyadenylation site selection. This posttranscriptional event is not present in all species; indeed, while the 2.1 kbp form is readily detected in human (our results), mouse (Obaru et al., 1986), rat (Pavé-Preux et al., 1988), and pig (Nagashima et al., 1989), the 1.8 kbp form is readily detected only in human; lesser amounts of the 1.8 kbp form are found in the rat, while it is barely detectable in the mouse (Figure 3B) and is not detected at all in the pig (Nagashima et al., 1989). In all these species, two consensus polyadenylation signals (AAUAAA) are found at similar positions in the 3′ noncoding region.

It has recently been shown that an efficient polyadenylation is dependent on GT-rich elements located downstream of the polyadenylation sites (McDevitt et al., 1986). A comparison of the sequences located downstream of the first polyadenylation signal, in human, pig, rat, and mouse cAspAT cDNAs (Table I), shows a longer stretch of GT-rich sequences located 30–40 bases after the AATAAA site in the human cAspAT. This could explain the increased amounts of the 1.8 kbp mRNA form found in man as compared to the other species.

The polyadenylation site selection in cAspAT appears to be regulated in humans during development. Indeed, the 2.1 kbp form is more abundant than the 1.8 kbp form in the fetal liver while the opposite is found in the adult liver (Figure 3A). A similar regulation has been observed for the pyruvate kinase (Marie et al., 1986) and the vimentin (Capetanaki et al., 1983) cDNAs. This implies that polyadenylation is an additional step whereby the regulation of RNA processing may occur. In the present case, polyadenylation is not coupled to splicing as has been described for other mRNAs (Peterson & Perry, 1989; Barbas et al., 1988; Hashinaka et al., 1988; Kornfeld et al., 1989; Adami & Nevins, 1988).

We have also investigated the physiological relevance of these multiple polyadenylation sites. The stability of both cAspAT mRNA forms was assessed in a human hepatoma cell line. The 2.1 and 1.8 kbp mRNA species exhibit half-lives of 8 and 12 h, respectively. This small difference is not due to the poly(A) tail length as both mRNAs have similar and rather short poly(A) tracts (20–50 A). One possible explanation for the difference in half-life is the presence of AU-rich sequences in the 3′ noncoding region of the cAspAT mRNAs. The sequence motif AUUUA is believed to trigger mRNA instability [reviewed by Brawerman (1989)]. Four such elements are found in the 2.1 kbp form whereas only one is present in the 1.8 kbp form. However, these sequence elements are also found in the mitochondrial AspAT mRNA (Pol et al., 1988) which is more stable (Figure 4B). The ability of both types of mRNAs to direct cAspAT protein synthesis was also tested and found to be similar in an in vitro system.

In conclusion, we have demonstrated by sequence analysis of several human cAspAT clones that the presence of two cAspAT mRNAs was due to alternative polyadenylation site selection. These mRNAs differ in their 3′ noncoding region; they differ slightly in their stability but have the same translational capacity. These data suggest that the additional 400 bases in the longer mRNA do not significantly modify the properties of cAspAT mRNAs, at least under basal conditions.

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Chemical Cross-Linking Study of Complex Formation between Methylamine Dehydrogenase and Amicyanin from *Paracoccus denitrificans*†

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ABSTRACT: Two soluble periplasmic redox proteins from *Paracoccus denitrificans*, the quinoprotein methylamine dehydrogenase and the copper protein amicyanin, form a weakly associated complex that is critical to their physiological function in electron transport [Gray, K. A., Davidson, V. L., & Knaff, D. B. (1988) *J. Biol. Chem. 263*, 13987–13990]. The specific interactions between methylamine dehydrogenase and amicyanin have been studied by using the water-soluble cross-linking agent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). Treatment of methylamine dehydrogenase alone with EDC caused no intermolecular cross-linking but did cause intramolecular cross-linking of this $\alpha_2\beta_2$ oligomeric enzyme. The primary product that was formed contained one large and one small subunit. Methylamine dehydrogenase and amicyanin were covalently cross-linked in the presence of EDC to form at least two distinct species, which were identified by nondenaturing polyacrylamide gel electrophoresis (PAGE). The formation of these cross-linked species was dependent on ionic strength, and the ionic strength dependence was much greater at pH 6.5 than at pH 7.5. The effects of pH and ionic strength were different for the different cross-linked products. SDS–PAGE and Western blot analysis of these cross-linked species indicated that the primary site of interaction for amicyanin was the large subunit of methylamine dehydrogenase and that this association could be stabilized by hydrophobic interactions. In light of these results a scheme is proposed for the interaction of amicyanin with methylamine dehydrogenase that is consistent with previous data on the physical, kinetic, and redox properties of this complex.

Paracoccus denitrificans, when grown on methylamine as the sole source of carbon and energy, synthesizes an inducible methylamine dehydrogenase which functions in the periplasmic space of this Gram-negative bacterium (Husain & Davidson, 1987). It catalyzes the oxidation of methylamine to formaldehyde and ammonia and donates electrons to an inducible periplasmic type I copper protein, amicyanin (Husain & Davidson, 1985). Methylamine dehydrogenase is an oligomeric protein with an $\alpha_2\beta_2$ structure and subunit molecular weights of 46 700 and 15 500 (Husain & Davidson, 1987). Each small subunit contains covalently bound pyrroloquinoline quinone (PQQ).[1] Amicyanin is a monomeric protein with a molecular weight of 15 000 (Husain & Davidson, 1985).

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; PAGE, polyacrylamide gel electrophoresis; PQQ, pyrroloquinoline quinone.

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