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Cloning and characterization of the cDNA encoding human adenylosuccinate synthetase

Steven M. Powell[a], Howard Zalkin[a] and Jack E. Dixon[b]

[a]*Department of Biochemistry, Purdue University, West Lafayette, IN 49707, USA* and [b]*Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109, USA.*

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Adenylosuccinate synthetase (AS) catalyzes the first committed step in the conversion of IMP to AMP. A cDNA was isolated from a human liver library which encodes a protein of 455 amino acids (M_r of 49,925). Alignments of human, mouse, *Dictyostelium discoideum* and *E. coli* AS sequences identify a number of invariant residues which are likely to be important for structure and/or catalysis. The human AS sequence was also 19% identical to the human urea cycle enzyme, arginosuccinate synthetase (ASS), which catalyzes a chemically similar reaction. Both human liver and HeLa AS mRNA showed signals of 2.3 and 2.8 kb. An unmodified N-terminus is required for function of the human AS enzyme in *E. coli* mutants lacking the bacterial enzyme. The human cDNA provides a means to assess the possible role of AS abnormalities in unclassified, idiopathic cases of gout.

Recombinant DNA; De novo purine nucleotide synthesis; Gout

1. INTRODUCTION

The genes for de novo purine nucleotide (nt) synthesis in bacteria have been analyzed in detail ([28] references therein). Only recently have molecular approaches been applied to characterize these genes in higher eukaryotes. Avian cDNAs encoding enzymes which catalyze eight of the ten steps leading to IMP have been isolated in our laboratory by functional complementation of *E. coli pur* mutants [1,2,6,18]. Similar attempts to complement an *E. coli purA* mutant were unsuccessful in isolating the eukaryotic cDNA for AS.

AS catalyzes the reaction shown in Fig. 1, which is the first committed step in the pathway leading from IMP to AMP, and is subject to feedback inhibition by AMP [21]. AS activity is present in most tissues but is highest in skeletal muscle, heart, and testes [5,15,17,21]. Elevated activity has been noted in tumors [10]. Two isozymes have been reported in tissues of rat, rabbit, and chicken [21]. A basic form, having a pI of 8.9 and low K_m for aspartate, has been assigned a role in an nt interconversion cycle and may function in purine salvage [20,21]. An acidic form, with a pI of 5.9 and a low K_m for IMP, is thought to have a biosynthetic role. However, only a single AS locus has been identified in CHO cells [22]. Somatic cell hybridization studies have localized the human AS gene to the long arm of chromosome 1, between the centromere and 1q12 [14]. Prior to the start of this work, the only AS sequence available was that from the cloned *E. coli* gene [27]. Our efforts to obtain the human cDNA were greatly aided by the availability of a mouse cDNA sequence [9]. During the course of our work, the sequence of AS from *Dictyostelium discoideum* was published [26].

This paper reports the isolation and analysis of the human cDNA encoding AS. This human cDNA will allow examination of the relationship of AS to human disease states. The human sequence is of interest clinically. In addition, the human enzyme has a predicted isoelectric point of ~7, as opposed to the suggested pI of 5 and 9 for the two rat isoforms. No information is available regarding the human isoforms and few mutations in AS are known in humans suggesting that the enzyme is important for growth and development. A mutated murine lymphoma cell line has been identified with a partial defiency in AS [23,24]. These cells synthesized large amounts of IMP which is secreted as inosine, thus suggesting a link between the AS deficiency and hyperuricemic states. A defective AS may contribute to

Abbreviations: Ade-H, adenylosuccinate lyase gene; AMP, adenosine monophosphate; AS, adenylosuccinate synthetase; ASS, arginosuccinate synthetase; ATP, adenosine triphosphate; bp, base pair; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CHO, Chinese Hamster Ovary; GDP, guanosine diphosphate; GTP, guanosine monophosphate; IMP, inosine monophosphate; kb, kilobase(s); kDa, kilodalton(s); nt, nucleotide; PCR, polymerase chain reaction; P_i, inorganic phosphate; PP_i, pyrophosphate; Pipes, piperazine-*N*,*N*'-bis[2-ethane-sulfonic acid]); *purA*, *E. coli* gene encoding adenylosuccinate synthetase; SDS, sodium dodecyl sulfate; SSC, sodium chloride, sodium citrate; SSPE, sodium chloride, sodium phosphate containing EDTA.

Correspondence address: J.E. Dixon, Department of Biological Chemistry, The University of Michigan Medical School, Room 5416 Medical Science I Building, Box 0606, Ann Arbor, MI 48109-0606, USA. Fax: (1) (313) 7634-4581.

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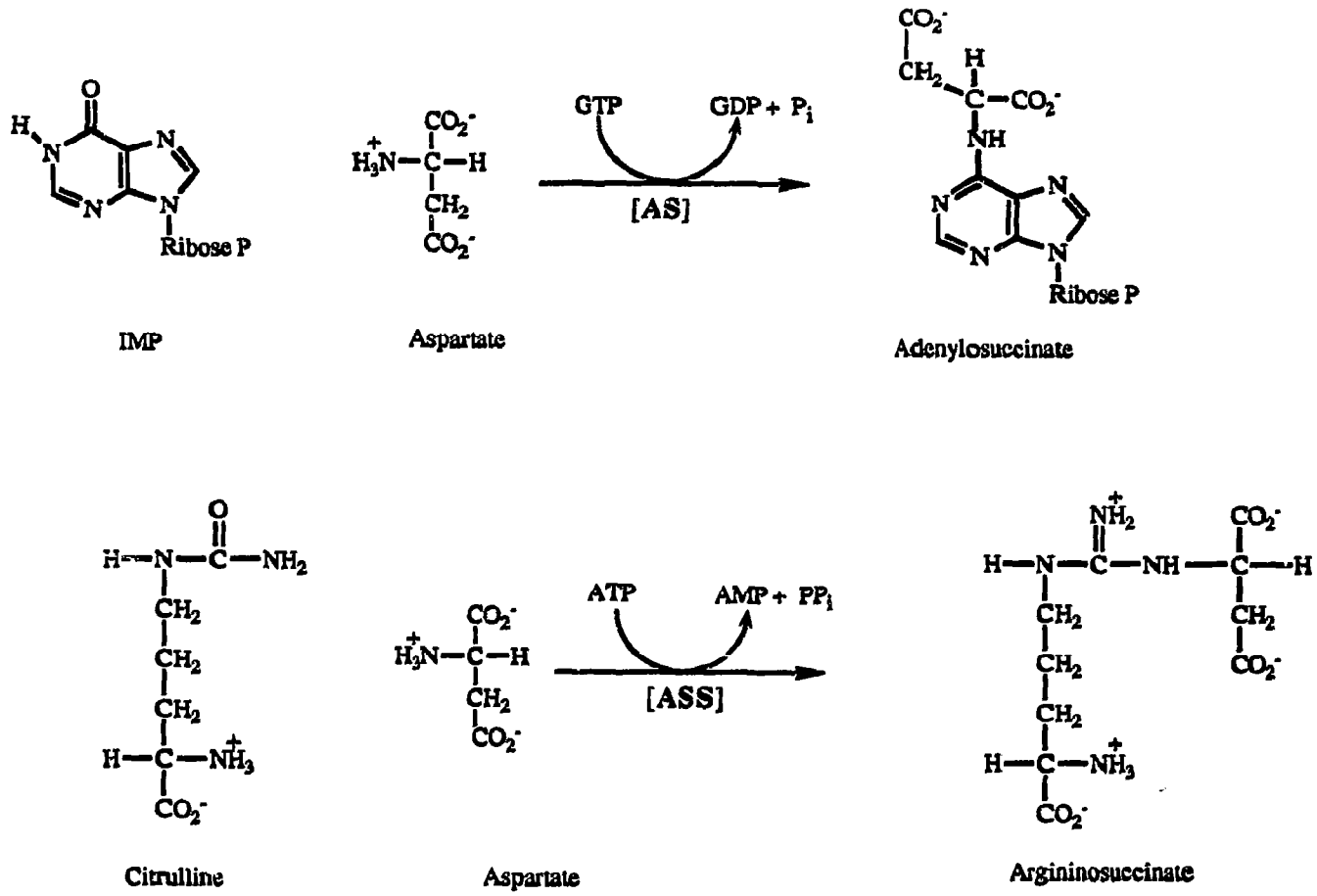

Fig. 1. Reactions catalyzed by AS and ASS.

unclassified idiopathic gout. Isolation of the human cDNA is the first necessary step in further exploring metabolic defects in AS.

2. EXPERIMENTAL

The template for first strand cDNA synthesis was human lymphoblast RNA. First strand synthesis was carried out using AMV reverse transcriptase as outlined by the manufacturer's specifications (Invitrogen). The resultant cDNA was used as template for PCR as suggested by the manufacturer's specifications (Perkin Elmer Cetus: Gene Amp kit). Degenerate oligonucleotide primers were designed based upon conserved amino acid sequences common to the *E. coli* [27] and mouse AS [9]. Two, 26 nt primers were synthesized with *Eco*RI and *Hin*dIII linkers: 5'-end primer, CATGAATTCCA(A/G)TGGGNNGA(T/C)GA(A/G)GG; 3'-end primer, ATGAAGCTTACNACNCC(G/A)TTNCC(A/G/T)AT. The first two thermo-cycles were as follows: 94°C, 1 min; 37°C, 2 min; 72°C over 2 min; 72°C, 2 min. The subsequent 40 thermo-cycles were 94°C, 1 min; 50°C, 2 min; 72°C, 2 min followed by an extension at 72°C for 20 min. The amplified, 200 nt fragment was then subcloned into M13mp18 for sequence analysis.

A λgt11 human liver cDNA library [13] was probed with the labeled PCR product described above. Approximately 200,000 plaques were plated and duplicate lifts were made onto nitrocellulose filters. Hybridization, washing of the filters and autoradiography was carried out as previously described [2]. Following plaque purification, the insert from the DNA was subcloned for sequencing.

RNA was extracted from HeLa cells and human liver as described [7]. Human liver poly(A)$^+$ RNA was selected by oligo d(T)-cellulose chromatography [4]. RNA was fractionated by electrophoresis in a 1.0% agarose gel containing formaldehyde and transferred to a nitrocellulose filter as described [16]. Hybridization with the 2.0 kb cDNA insert was conducted at 42°C for 18 h as previously described [2]. Filters were washed at room temperature in 0.1% SDS/0.25×SSPE for 30 min followed by autoradiography. Subsequent washes and autoradiographs were conducted at 37°C, 42°C, and 65°C. Functional complementation was carried out as follows. An *Nco*I (nt 73) to *Bam*HI restriction fragment containing an AS coding sequence (Fig. 2) was subcloned into the expression vector pTRC-97c [3]. The resulting plasmid was transformed into two *E. coli purA* strains: PCO950 (purA54, argI61, argF58, serB28, thr25, tonA49), and ES4 (purA45, lacY1 or Z4, tonA2, tsx-1 or tsx-70, supE44, gal-6, mtl2). The cells were grown in minimal medium [6] supplemented with 0.1% acid hydrolyzed casein, 0.2 μg/ml thiamine and ampicillin (50 μg/ml).

3. RESULTS AND DISCUSSION

3.1. *Isolation and sequence of the human cDNA encoding AS*

A human PCR product of approximately 200 bp was amplified using degenerate oligonucleotide primers based on conserved regions in *E. coli* and mouse AS amino acid sequences [9]. Sequence analysis demonstrated that the PCR product was similar but not identical to both *E. coli* and mouse AS. The PCR product

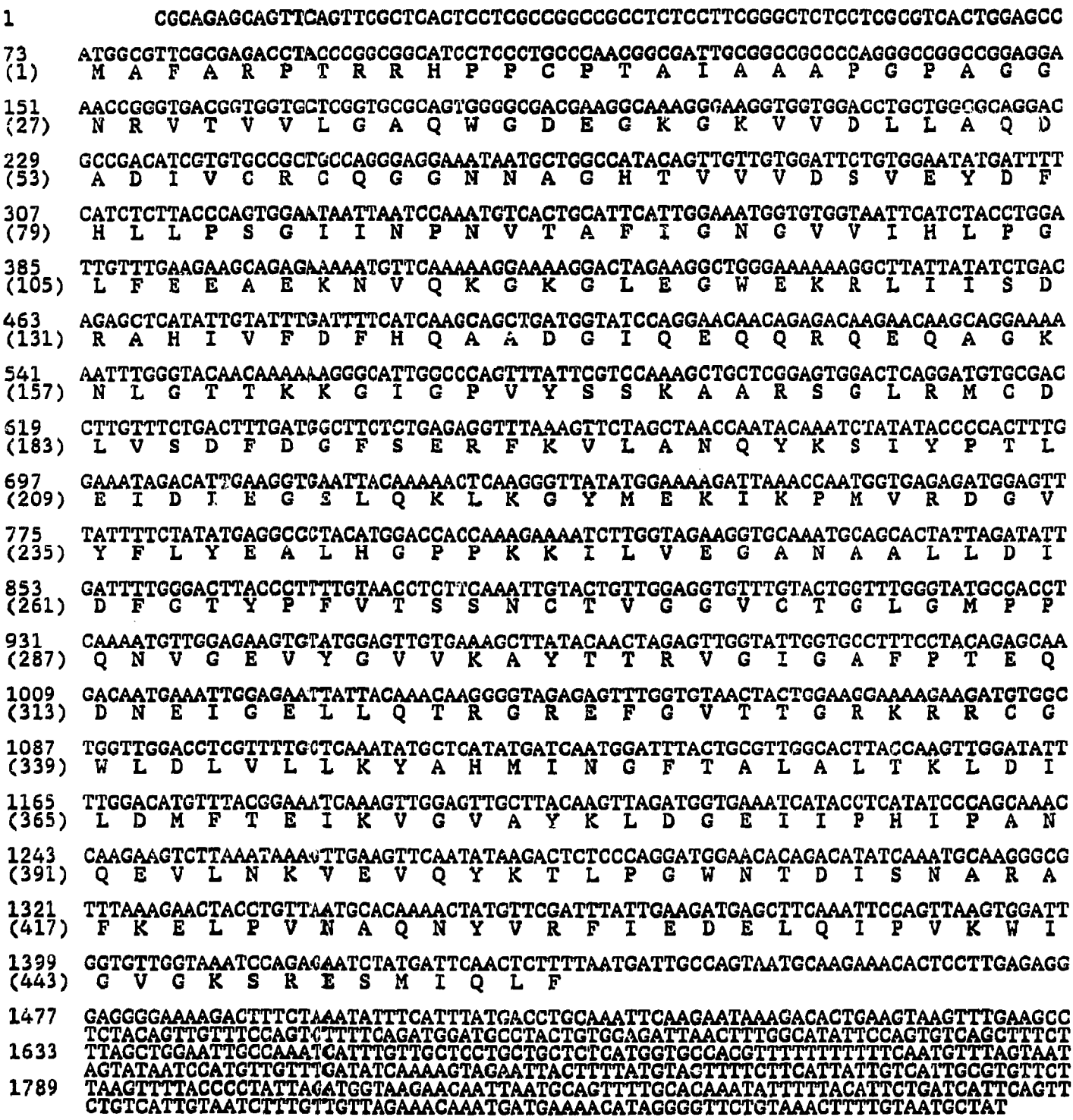

Fig. 2. The nt sequence of the human AS cDNA and the derived amino acid sequence. The numbers shown correspond to nt while the numbers in brackets refer to amino acid residues.

was used as a probe to screen a human liver cDNA library and a single positive clone was identified. Following plaque purification, the clone was shown to contain an insert of approximately 2.0 kb. This 2.0 kb fragment was subcloned into a phagemid for sequencing.

Both strands of DNA were sequenced. The single open reading frame encodes a protein of 455 amino

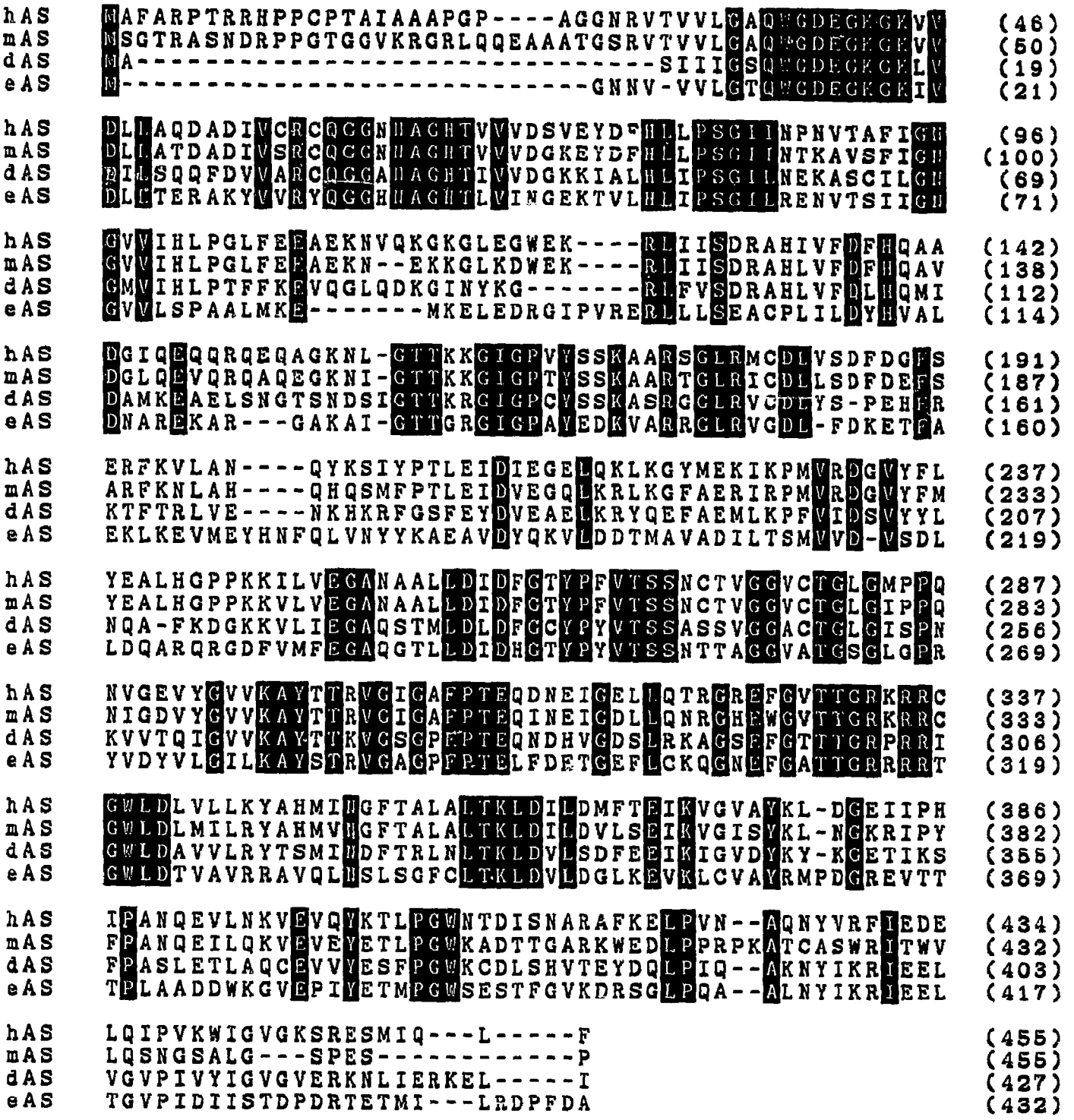

Fig. 3. Amino acid sequence alignment of human (hAS), mouse (mAS), *Dictyostelium discoideum* (dAS), *E. coli* (eAS). Black boxes indicate identity in all three sequences.

acids with a M_r of 49,925 Da (Fig. 2). The predicted M_r is in agreement with 50 kDa reported for AS from several species [21]. The cDNA contained a 5′ untranslated region of 72 nt and a 3′ untranslated region of 425 nt, and it did not have a poly(A) tail. The putative initiation codon at nt 73 is not preceded by a stop codon, but it is the first ATG in the sequence. The G/C-rich sequence surrounding the first ATG conforms to the consensus eukaryotic initiation sequence [12]. The calculated pI of the human enzyme is 7.0.

Alignment of the human AS sequence with those from *Dictyostelium discoideum* [26] and *E. coli* [27] dem-

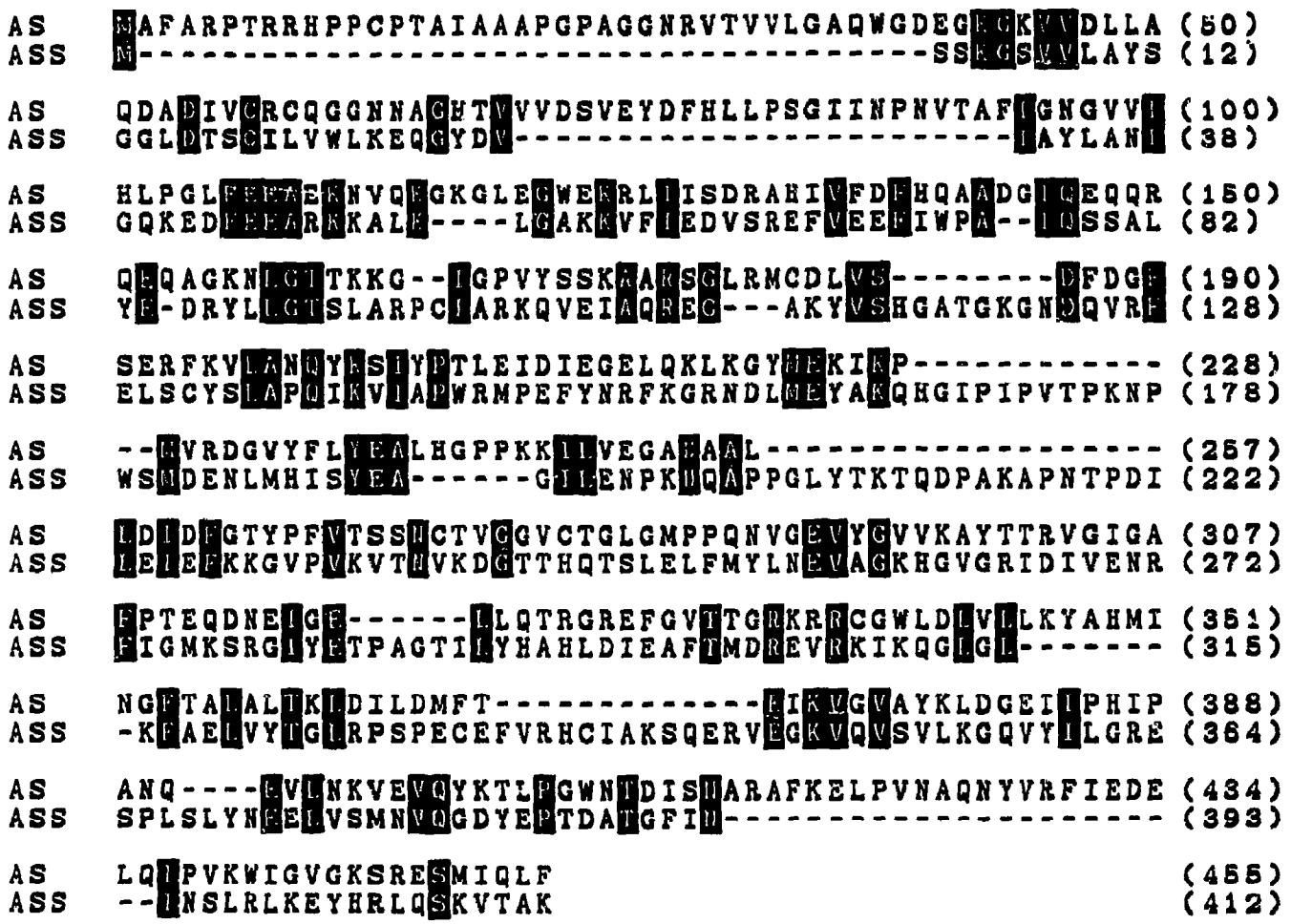

Fig. 4. Alignment of human AS and ASS sequences. Identical residues are shown in black. The numbers on the right represent the positions of the residue at the extreme right for each enzyme.

onstrates 47 and 41% amino acid identity, respectively (Fig. 3). The mouse and human sequences have an even higher degree of identity as would be expected (Fig. 3). The degree of positional identity supports the idea that structural properties and catalytic mechanism have been retained for this enzyme throughout the course of evolution. Three motifs at residues 38–44, 314–317 and 360–363 correspond to the expected consensus elements for GTP binding. GX_4GK at residues 38–44 and DNX_2G at residues 314–317 are thought to be involved in phosphoryl binding. A guanine recognition motif (TKKD) is present between residues 360 and 363 [26]. Also well conserved is the lysine residue at position 172 which was recently shown to be important for enzyme activity in *E. coli* by site directed mutagenesis [8].

Argininosuccinate synthetase (ASS), a urea cycle enzyme, uses aspartate as an amino donor and catalyzes reactions similar to AS (Fig. 1). Amino acid alignment of the human AS sequence demonstrated 19% identity with the human ASS [19]. The sequence identity between human AS and ASS occurs throughout the entire sequence (Fig. 4). Conserved residues are likely to have roles in maintaining structure as well as participating in substrate binding and catalysis. The fact that both enzymes utilize aspartate as a substrate suggests that some of the invariant residues within the two enzymes are likely to be required for binding.

Deficiency in ASS results in citrullinemia and a complete absence of the enzyme would be incompatible with life [11]. Several ASS mutations leading to citrullinemia have recently been characterized [11]. It was of interest to determine if any of these mutations corresponded to conserved positions found in both ASS and AS (Fig. 4). Several point mutations occur within the ASS sequence which are not conserved in AS [11]. However, an R307W substitution in patient RI corresponds to the invariant Arg^{340} in AS. No detailed kinetics of ASS from this patient are available [11]. We can predict, however, that the corresponding R340W mutation in AS would likely have an effect on enzyme activity similar to that noted for the R307W substitution in ASS. The AS/ASS sequence alignment also identifies additions residues

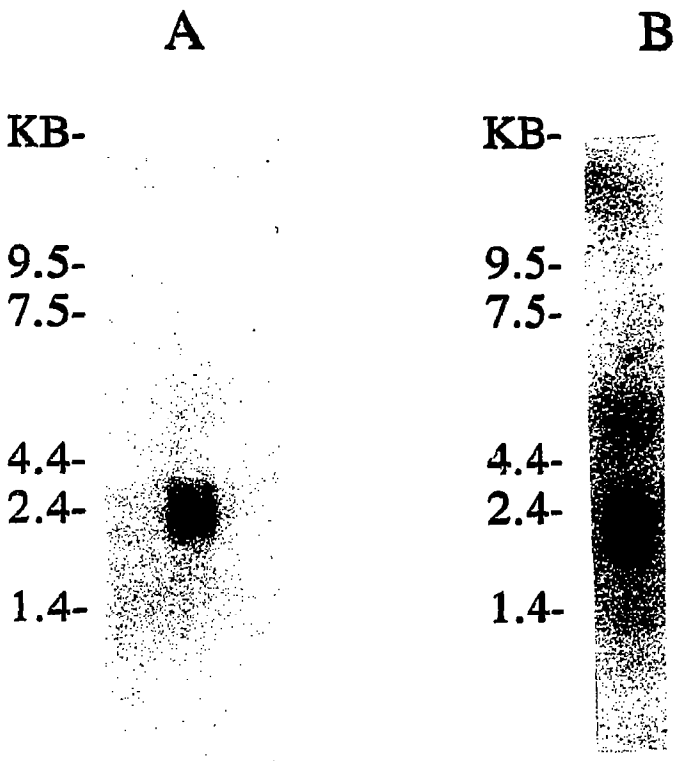

Fig. 5. RNA analysis. (A) Northern blot of human Poly(A)$^+$ RNA (5 μg). Exposure was for 72 h at −80°C. An RNA ladder (BLR) was used as a size standard (kb). (B) Northern blot of HeLa RNA (20 μg total RNA).

which might be important for structure and/or catalysis and therefore suggests possible site-directed mutations to address the function of these invariant residues.

The AS cDNA hybridized to two RNAs from human liver and HeLa cells with approximately equal intensities (Fig. 5A,B). At elevated 'washing' temperatures neither RNA is selectively eluted from the filter. This suggests that the two RNAs encode a single AS isoform as opposed to encoding two distinct isoforms of AS.

3.2. *Functional complementation and conclusions*

Previous efforts to isolate AS cDNA by complementation of an *E. coli purA* mutant with an avian expression library were not successful. Functional complementation of *E. coli pur* mutants has been used to isolate a number of enzymes in the de novo purine nt synthetic pathway from higher eukaryotes [1,2,6,18]. Functional complementation was attempted using the full-length human cDNA. The human cDNA clone was expressed as an in-frame, *lacZ* fusion. Complementation of *purA* was not obtained. However, when the complete coding sequence was inserted into the expression vector pTRC-97c, which produces the human enzyme with the correct amino terminus, complementation of two purA strains was obtained. Plasmids were isolated from *Pur*$^+$ transformants, then re-transformed into the same *purA* strains. This plasmid complemented each *pur* mutant with high efficiency. As expected, no *Pur*$^+$ transformants were noted using the pTRC-97c vector alone. This result demonstrates that the correct amino terminus of AS is necessary for activity.

The cDNA encoding human AS can be used as a tool to investigate the potential role of the enzyme in human disease. A select population of previously unclassified idiopathic, overproducing, hyperuricemic gout patients would be prime candidates for such a study. Evidence supporting a possible deficiency of AS underlying some of these cases comes from the genetic model developed with A-100 cells [23,24]. This cell line is a mutated mouse T cell lymphoma cell which is 80% deficient in AS activity. These cells contain four-fold elevated IMP levels and secrete massive amounts of inosine into the media [23–25]. Having the human cDNA encoding AS will now make it possible to determine if any of the idiopathic gout patients' pathophysiology is associated with alterations in the AS gene.

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