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Identification of a liver-specific cAMP response element in the human argininosuccinate synthetase gene

Tai-Ru Guei [a], Mei-Chun Liu [a], Chun-Ping Yang [b], Tsung-Sheng Su [a,b,c,*]

[a] Faculty of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei, Taiwan
[b] Institute of Microbiology & Immunology, National Yang-Ming University, Taipei, Taiwan
[c] Department of Medical Research and Education, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-pai Road, Taipei 11217, Taiwan

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ABSTRACT

Argininosuccinate synthetase (ASS), a key enzyme in the urea cycle, participates in many metabolic processes including arginine biosynthesis and the citrulline–nitric oxide (NO) cycle. Factors like diets, hormones and pro-inflammatory stimuli are known to regulate *ASS* gene expression primarily at the transcription level. However, little is known about the *cis*-elements for transcriptional regulation of the *ASS* gene. In this study, we employed DNase I hypersensitive sites mapping to identify potential regulatory sites of the gene and revealed a site located at 10 kb upstream of the transcription start site which is responsible for liver-specific cAMP induction. Furthermore, a cAMP response element (CRE) highly conserved among mammals was identified and was experimentally verified. Our results show that liver-specific enhancement of *ASS* gene expression is mediated in part by the cAMP signaling pathway through a distal CRE site.

Argininosuccinate synthetase (ASS; EC 6.3.4.5) is an enzyme that functions to catalyze the conversion of citrulline and aspartate to argininosuccinate which is subsequently converted to arginine by argininosuccinate lyase. In addition to its function in arginine biosynthesis, ASS was also found to catalyze a potential limiting step in nitric oxide (NO) biosynthesis. ASS is present in all tissues and cultured cells studied but the highest enzyme activities are found in the liver where the enzyme is involved in the urea cycle to eliminate ammonia (reviewed in [1]).

One of the approaches to study the mechanism of liver-specific gene expression is by somatic cell fusion [2]. Chin and Fournier [2] showed that hybrids of rat hepatoma cells and mouse fibroblasts failed to express the entire set of known liver-specific genes. Nevertheless, a subset of genes was re-expressed following the loss of a specific chromosome of the mouse fibroblast cells [3]. It was known then that the extinction of expression of a particular set of liver-specific genes involved a specific genetic locus and most likely more than one locus existed to control the whole spectrum of liver-specific gene expression [3]. The best studied locus, the tissue-specific extinguisher 1 (*TSE1*) that maps on mouse fibroblast chromosome 11 and its human homolog on chromosome 17, was shown to extinguish liver-specific expression of the tyrosine aminotransferase (*TAT*), phosphoenolpyruvate carboxykinase (*PEPCK*), *ASS*, and other genes [2,3]. The *TSE1* gene was subsequently found to encode a regulatory subunit, R1α, of cAMP-dependent protein kinase A (PKA) [4]. The TSE1/R1 α-mediated extinction of *TAT* and *PEPCK* expression was shown to involve repression of the basal PKA activity resulting in the reduction of phosphorylation of the cAMP response element (CRE)-binding protein (CREB) and consequently a reduction of CREB binding at the CRE target located in the enhancer of the gene [5,6].

Liver-specific enhancement of *ASS* gene expression is known to be under *TSE1* regulation [2,3]. Moreover, transcription of the *ASS* gene in rat liver was shown to be stimulated by cAMP in nuclear run-on assays [7]. Thus, the effect of TSE1 or cAMP on *ASS* gene expression is most likely mediated through a CRE sequence on the gene. To define possible *cis*-acting elements in *ASS* gene, three major DNase I hypersensitive sites at the 5′ region of the human *ASS* gene were first identified in this study. Functional analysis of the DNase I hypersensitive sites further indicated that the cAMP response element of the human *ASS* gene is located at 10 kb upstream of the transcription start site.

Materials and methods

Cell lines. HuH-7 is a well-differentiated human hepatoma cell line established from a primary hepatocellular carcinoma [8].

* Corresponding author. Address: Department of Medical Research and Education, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-pai Road, Taipei 11217, Taiwan. Fax: +886 2 2875 7435.
E-mail address: tssu@vghtpe.gov.tw (T.-S. Su).

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RPMI 2650, a human epithelium cell line [9], was obtained from the American Type Culture Collection. HeLa is a human cervical carcinoma cell line. Cells were cultured in DMEM medium with 10% fetal calf serum.

Analysis of DNase I hypersensitive sites of the human ASS gene. Preparation of nuclei, DNase I digestion and indirect end-labeling were performed essentially as described [10]. The DNA probe was a 524-bp HindIII–EcoRI fragment located in intron 1 where the EcoRI site is at 2 kb downstream of the transcription start site of the human *ASS* gene. The DNA fragment was labeled by [α-^{32}P]dCTP using a random prime labeling system [11].

Plasmid construction. A human genomic library in a lambda vector (kindly provided by Dr. S.F. Tsai) was screened to obtain clones covering the 5′ region of the *ASS* gene. A AS6B-1 clone containing sequences 12 kb upstream and 2 kb downstream of the transcription start site of the human *ASS* sequence was used as the starting material for subsequent constructions. DNA fragments covering DNase I HS sites of the gene were inserted into pBLCAT5 [12], a reporter plasmid with the bacterial chloramphenicol acetyltransferase (CAT) gene driven by a herpes simplex virus (HSV) thymidine kinase (*tk*) promoter. The construction strategy involved insertions in the forward and reverse orientations of a 3.9-kb BamHI fragment covering the HS+1 region to generate the CAT constructs pBB3.9CAT and pBB3.9(R)CAT, respectively. Similarly, a 2.2-kb BamHI fragment covering the HS-10 region was employed to generate pBB2.2CAT and pBB2.2(R)CAT. To map the sequence required for cAMP responsiveness, constructs with serial deletions of a 2.2-kb BamHI sequence were produced.

Transcriptional analysis of the ASS regulatory sequences. Plasmid DNA was transfected to cultured cell lines by the calcium phosphate co-precipitation method [11]. Twenty hours after transfection, cells were washed once with Hanks' balanced salt solution and cultured in DMEM without serum and induced with 10 μM forskolin or mock induced with 0.2% ethanol. Cells harvested 28 h later were used for CAT assays [11] and for total DNA isolation for quantification [13]. CAT activities were analyzed and expressed as percent conversion of [^{14}C] chloramphenicol into acetylated chloramphenicol after product separation by thin-layer chromatography and quantified with an ImageQuant software (Molecular Dynamics). The amounts of transfected plasmid DNAs were quantified by DNA slot blot analysis using the CAT DNA as the probe [13]. The level of CAT activity in each transfection was normalized to the amount of plasmid DNA transfected into cells.

Mutagenesis. Site-specific mutagenesis was performed with the Altered Sites II *in vitro* Mutagenesis System (Promega) according to the manufacturer's instructions. Sequences of the single-stranded oligonucleotides used in the mutagenesis of each putative transcription factor binding sites in the HS-10 region are as follows with the mutated bases indicated in bold letters: bHLH^{m}: 5′-TGCCACAGCAGG**GA**GCGTGGACCAAG-3′; C/EBP^{m}: 5′-ACGATGACGCTCCG**AGG**GCC**T**CAGCAGGTGGCGTGGA-3′; Sp1^{m}: 5′-CGCTGACGCACGG**TT**CGGGAGACGATGAC-3′; CRE-1^{m}: 5′-AAGG TCTCGCTGAC**CA**CACGGGGCGGGAGACGA-3′; HNF4^{m}: 5′-CAGGTC CTCGCTGT**ATT**G**TC**TCAAGGCTGTGCGCAGG-3′. The nucleotide alterations in the bHLH, C/EBP and HNF4 sites were as described [14–16], whereas CRE and Sp1 mutations were based on sequences of the mutated forms of oligonucleotides designed for electrophoretic mobility shift assays (Santa Cruz Biotechnology).

Results

Identification of DNase I hypersensitive sites at the 5′ region of the human ASS gene

DNase I hypersensitive site mapping was applied to detect potential regulatory sites in the human *ASS* gene. Nuclei prepared from a human hepatoma cell line, HuH-7, and a human epithelial carcinoma cell line, RPMI 2650, were treated with increasing concentrations of DNase I, the genomic DNA subsequently purified from the cell lines was digested with EcoRI for Southern blot analysis. Using as a hybridization probe of a HindIII–EcoRI DNA fragment where the EcoRI site was located at 2 kb downstream of the transcription start site (Fig. 1A), the region which could be scored for DNase I hypersensitive sites was found to be extended from this probe-generating EcoRI site to the next EcoRI site located 34 kb upstream of the transcription start site. When the genomic DNA was digested with EcoRI without prior incubation of the nuclei with DNase I, a presumptive 36-kb fragment could be detected (Fig. 1B, open arrow). Treatment with DNase I led to the appearance of shorter fragments that increased in intensity with increasing DNase I concentrations. These fragments were estimated to be of sizes 12 kb, 2.2 kb, and 1 kb, respectively. Since the fragments were indirectly end-labeled at the EcoRI site that was located at 2 kb downstream of the transcription start site, the DNase I hypersensitive sites represented by these fragments were deduced to be located at about 10 kb and 0.2 kb upstream and 1 kb downstream of the transcription start site and are designated as HS-10, HS-0.2 and HS+1, respectively. HS-0.2 covers the proximal promoter elements that bear an open chromatin structure [17]; a smeared band after DNase I treatment was indeed observed (Fig. 1B). Hypersensitive site HS+1, resided in the first intron, was more prominent in the hepatoma cell line HuH-7 and was just discernible in the RPMI 2650 cells.

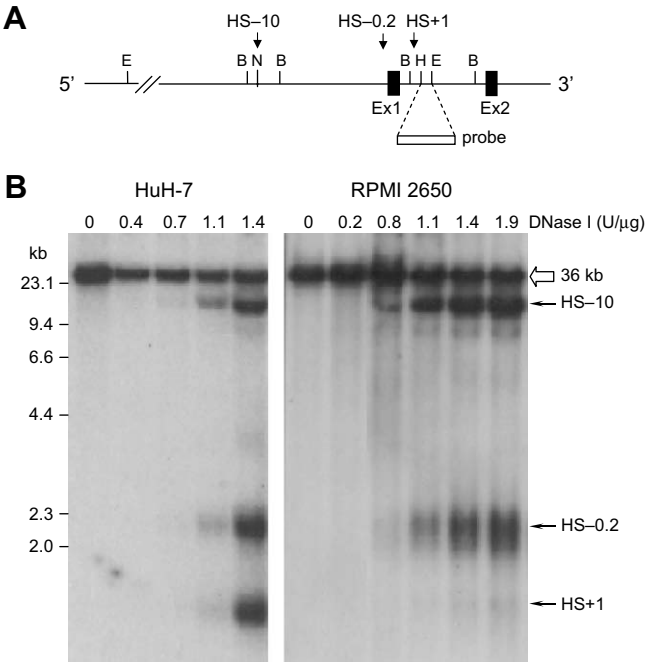

Fig. 1. Identification of DNase I hypersensitive sites at the 5′ region of the human *ASS* gene. (A) Schematic representation of the identified DNase I hypersensitive sites. The DNase I hypersensitive sites (HS) are indicated by arrows each with a designation number representing its distance (in kb) from the *ASS* transcription start site. Black boxes represent exons; the open rectangle represents a HindIII–EcoRI DNA fragment used as the indirect end-labeled probe. E, EcoRI; B, BamHI; N, NotI; H, HindIII. (B) Identification of DNase I hypersensitive sites by indirect end-labeling. Nuclei isolated from HuH-7 or RPMI 2650 cells were incubated with increasing amounts of DNase I after which genomic DNA was extracted and digested with EcoRI for Southern blot analysis with a [^{32}P] -labeled DNA probe as specified in (A) above. The DNA fragments produced as a result of DNase I digestion are indicated by arrows designated with the approximate distances (in kb) from the transcription start site. The open arrow indicates the presumptive 36-kb EcoRI fragment generated without prior DNase I incubation of the nuclei.

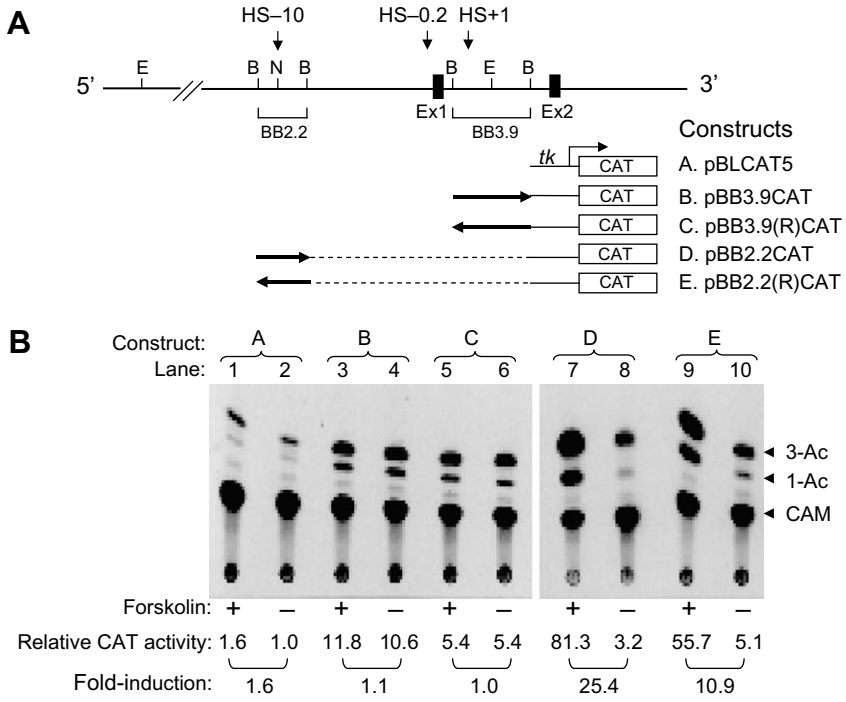

Fig. 2. Analysis of enhancer activities of the DNase I hypersensitive sites of the human *ASS* gene. (A) Schematic diagram of the 5′ region of the human *ASS* gene and the plasmid constructs used in the transfection experiments. The constructs were generated by placing the 3.9-kb (BB3.9) or 2.2-kb (BB2.2) BamHI fragments covering the HS+1 and HS-10 regions, respectively, in front of thymidine kinase (*tk*) promoter of pBLCAT5 in both the forward and reverse orientations as indicated by the arrows. (B) Analysis of CAT activities in transient-transfected HuH-7 cells using the plasmid constructs A–E as described in (A). Twenty hours after transfection, cells were induced with 10 μM forskolin (+) or mock induced with 0.2% ethanol (−). Transfected cells were harvested 28 h after induction and crude extracts were subjected to CAT assays and DNA analysis. The CAT activity of uninduced pBLCAT5 (construct A, lane 2) was arbitrarily set as 1. The fold-induction by forskolin treatment is indicated. All CAT activities were normalized with the amount of plasmid DNA in the transfected cells. The CAT data were the averages of two sets of transfection experiments each using independently prepared plasmid DNAs.

Regions of DNase I hypersensitivity show enhancer activities and the HS-10 region contains a liver-specific cAMP response motif

To test for possible enhancer activities in HS-10 and HS+1, a 2.2-kb and a 3.9-kb BamHI fragments (Fig. 2A, BB2.2 and BB3.9) covering HS-10 and HS+1, respectively, were placed in front of the minimal viral thymidine kinase (*tk*) promoter driving the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene in either the forward or reverse orientation (Fig. 2A). The constructs were transiently transfected into HuH-7 cells for CAT activity assays (Fig. 2B). The data indicated that in the presence of the HS+1 sequence in either orientation (Fig. 2, constructs B and C), the *tk* promoter activity was stimulated 5- to 10-fold (Fig. 2B, lanes 4 and 6) comparing to the parental control plasmid pBLCAT5 (Fig. 2B, lane 2), whereas constructs with the HS-10 sequence in either orientation (Fig. 2, constructs D and E) enhanced CAT activity 3- to 5-fold (Fig. 2B, lanes 8 and 10). The enhancements of *tk* promoter activities in an orientation-independent manner suggest that these DNase I hypersensitive site sequences are associated with enhancer activities.

To determine if the enhancer element(s) are responsible for cAMP induction, transfected HuH-7 cells were treated with the cAMP-generating agent forskolin (10 μM) to increase the intracellular cAMP. The results of CAT assay showed that when constructs containing the HS-10 sequence were transfected into HuH-7 cells, forskolin induction led to 10- to 25-fold enhancement in CAT activities compared to results obtained in the mock induction control (Fig. 2B, constructs D and E); no enhancement was observed when HS+1 constructs were similarly assayed (Fig. 2B, constructs B and C). The data indicate that the HS-10 region contains cAMP induction element(s).

To more accurately map the cAMP response element (CRE) in the HS-10 region, serial deletions from both ends of the BamHI 2.2-kb DNA fragment were generated and the resulting deletion fragments were cloned in front of the *tk* promoter of pBLCAT5 (Fig. 3A). In the CAT assays, cAMP responsiveness in HuH-7 cells was unchanged when HS-10 region was narrowed down to a 135-bp sequence of HindIII–EcoNI fragment, designated as HS-10E (Fig. 3B, panel HuH-7). To test whether such cAMP responsiveness was liver-specific, transfection experiments were further carried out using two non-liver human cell lines, RPMI 2650 and HeLa. No cAMP responsiveness was discerned in these two cell lines (Fig. 3B). Thus, the cAMP responsiveness located in HS-10E is liver-specific. It is noted that that basal enhancer activity of the HS-10E sequence, i.e., CAT activity in the absence of forskolin induction, was similar in HuH-7 and RPMI 2650 cells yet it was low in HeLa (Fig. 3B, lane 1 in each panel). Such low CAT activities in the HeLa cells may be a result of negligible *tk* promoter activity in these cells (Fig. 3, right panel, lane 3).

Localization of cAMP response element in the HS-10 region

Inspection of the 135-bp sequence for potential transcription factor binding sites using the web-based algorithms of TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and MatInspector (http://anthea.gsf.de/biodv/matinspector.html) [18] revealed the presence of two putative binding sites for CRE-binding protein (CREB) and were designated as CRE-1 and CRE-2 (Fig. 3C). To confirm participation of these CRE sequences in cAMP inductions, nucleotide substitution was introduced into the CRE-1 site of pHS-10E that carrying the 135-bp sequence to generate the mutant construct CRE-1^{m}. For the CRE-2 site, a CAT construct with deleted CRE-2, i.e., CRE-2del, was created by PpuMI restriction enzyme digestion (Fig. 3C). When these plasmids were transfected into the HuH-7 cells, the construct carrying the

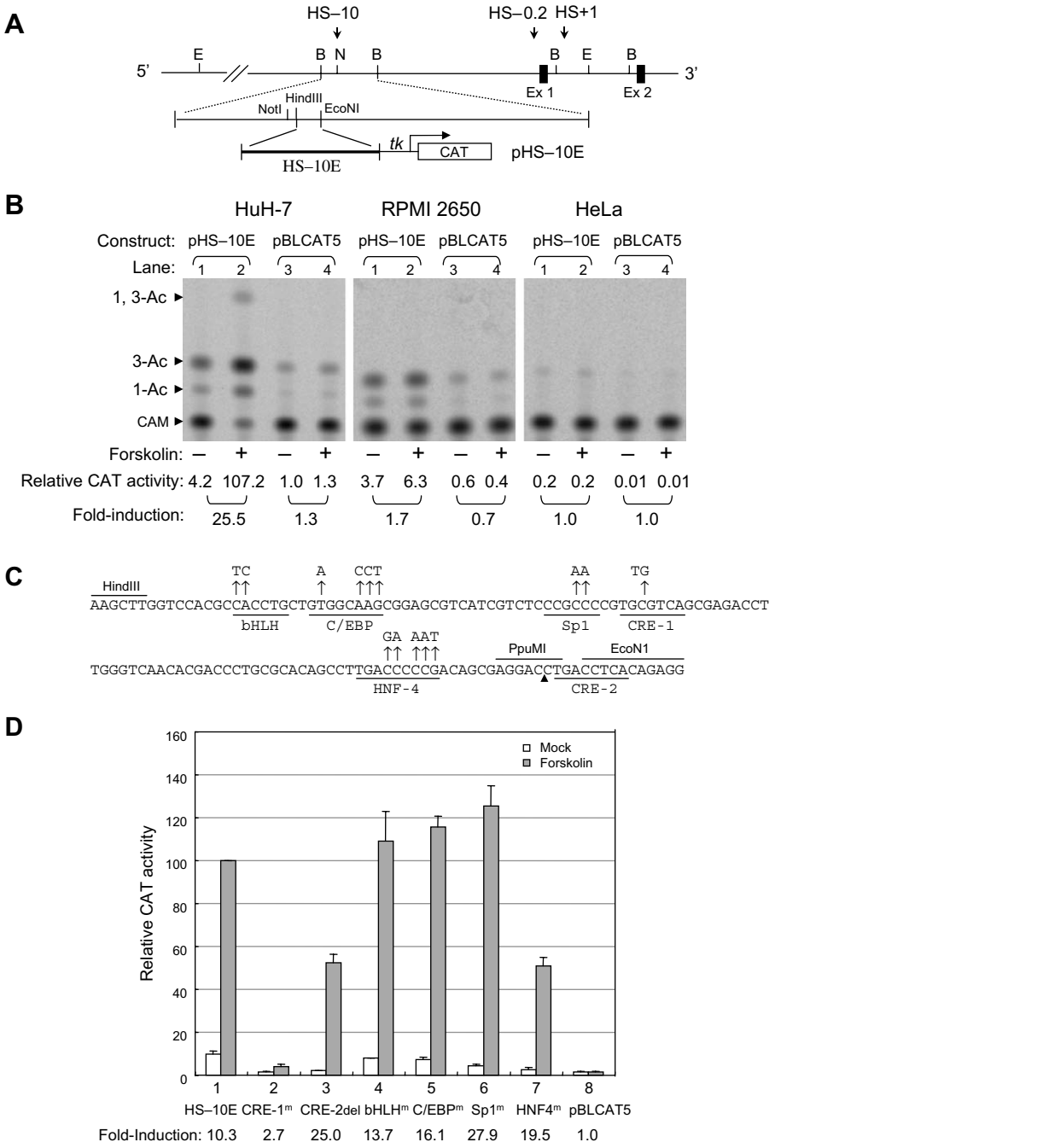

Fig. 3. Analysis of the cAMP response enhancer and the putative cAMP response element (CRE) of the human *ASS* gene. (A) Map of the 5′ region of the *ASS* gene including the DNase I HS sites. A 135-bp HindIII–EcoNI fragment designated as HS-10E derived from the HS-10 region was cloned into pBLCAT5 to generate the plasmid pHS-10E. (B) CAT assays of transient transfection of pHS-10E or pBLCAT5 plasmids into HuH-7, RPMI 2650 and HeLa cell lines, respectively. CAT assays were performed as described in the legend to Fig. 2 using the activity of the uninduced pBLCAT5-transfected HuH-7 cells set as 1. The data were the averages of two independent transfection experiments. (C) Nucleotide sequences of HS-10E contained within the 135-bp HindIII–EcoNI fragment. The putative transcription factor binding sites are underlined; the mutations introduced are indicated by upward pointing arrows. The CRE-2 deletion was performed with PpuMI restriction enzyme digestion with the arrowhead marking the end of the deleted sequence. (D) CAT activities of pHS-10E and mutant derivatives transfected into HuH-7 cells. The CAT activity of forskolin-induced pHS-10E-transfected cells was set as 100. The CAT data were the averages from three sets of transfected cells each using independently prepared plasmid DNAs.

CRE-1 mutation was found to significantly affect cAMP inducibility while the CRE-2 deletion had no effects on cAMP responsiveness (Fig. 3D, fold-induction) indicating that CRE-1 is the *cis*-element responsible for liver-specific cAMP induction in the human *ASS* gene. Besides the CRE sites, we also explored possible contribution of putative binding sites for the basic helix-loop-helix (bHLH) class of DNA-binding proteins, C/EBP family transcription factors, Sp1 proteins and hepatocyte nuclear factor 4 (HNF4) [14–16,19] for cAMP responsiveness. The data showed that mutations in the putative binding site for bHLH or C/EBP affected neither cAMP responsiveness nor basal enhancer activity (Fig. 3D). On the other hand, mutations in the putative site for Sp1 or HNF4 showed normal cAMP responsiveness although the basal enhancer activity was affected as also observed in the case of the CRE-2 deletion. This phenomenon is particularly obvious in the construct harboring HNF4 mutation. It is possible that these sequences also play a role in *ASS* gene expression with an unknown mechanism.

Discussion

The liver plays a central role in metabolism. When the diet is rich in protein, glucagon, a hormone released by the pancreas, stimulates hepatic enzyme activities for amino acid catabolism when the amino group is converted to urea and the carbon skeletons are converted to glucose by gluconeogenesis (reviewed in [20]). The elevation of activities of the urea cycle enzymes in response to glucagon stimulation is known to go through the cAMP-dependent signaling pathway [21] the action of which was shown to be at the step of transcription initiation for the *ASS* gene [7]. Likewise, the tissue-specific extinguisher TSE1, a dominant negative regulator that represses a subset of liver-specific gene expression including *ASS* gene in hepatoma-fibroblast hybrid cells [2,3], has been shown to target CRE-binding protein (CREB) at the CRE sites in the *TAT* and *PEPCK* genes [5,6]. Thus, cAMP signaling plays an important role to enhance liver-specific *ASS* gene expression in response to diet or hormonal factors.

We show in this study that the CRE-1 sequence located at 10 kb upstream (HS-10E) of the transcription start site of the human *ASS* gene is most likely the target site of the CRE-binding protein (CREB) to mediate glucagon action and TSE1 extinction of the *ASS* gene. Moreover, measurement of evolution conservation by alignment of the sequences of 18 species of mammals or 28 vertebrate species using the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/) reveals two regions of high degrees of sequence conservation, designated as HS-10EI and HS-10EII, within the 135-nt of HS-10E sequence (Supplementary Fig. S1). HS-10EI covers the putative Sp1 and CRE-1 sites (Supplementary Fig. S1). Importantly, the CRE-1 sequence in HS-10EI is found to be identical among the *ASS* genes in mammals (Supplementary Fig. S1). It is noteworthy that only mammals and adult terrestrial amphibians, i.e., the ureotelic animals, excrete amino nitrogen as urea [22]. The particular evolutionary conservation of the CRE-1 sequence among placental mammals suggests that this site is critical for cAMP action to regulate urea synthesis. On the other hand, the HS-10EII region contains a putative binding site for HNF4 (Supplementary Fig. S1) which has a similar DNA-binding specificity as the retinoid X receptor (RXR) [23]. Both HNF4 and RXR are members of the steroid hormone receptor superfamily that are critical for developmental regulation [23]. The sequence conservation of this site suggests that HNF4/RXR may act on HS-10EII to regulate *ASS* gene expression.

In addition to the urea cycle, the ASS enzyme participates in metabolic processes including arginine biosynthesis and citrulline–NO cycle (reviewed in [1]). Factors such as hormones, nutrients and pro-inflammatory stimuli are known to regulate *ASS* expression [1]. Identification of *cis*-element(s) responsible for the action of these regulators is important to elucidate the molecular mechanism(s) involved. The identification of DNase 1 hypersensitive sites at the 5′ region of the *ASS* gene should facilitate the search of additional regulatory elements involved in controlling *ASS* gene expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.118.

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