行政院國家科學委員會專題研究計畫　成果報告

GTP Cyclohydrolase I 突變及 Splicing 之分子結構機轉 (3/3)

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-002-101-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學醫學院小兒科

計畫主持人：胡務亮

報告類型：完整報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 11 月 17 日

ACADEMIC PRESS

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Biochemical and Biophysical Research Communications 306 (2003) 937–942

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Regulation of GTP cyclohydrolase I by alternative splicing in mononuclear cells ☆

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Received 22 May 2003

Abstract

GTP cyclohydrolase I (GCH, EC 3.5.4.16) regulates the level of tetrahydrobiopterin and in turn the activities of nitric oxide synthase and aromatic amino acid hydroxylases. Type II GCH mRNA, an alternatively spliced species abundant in blood cells, encodes a truncated and nonfunctional protein. When we stimulate peripheral blood mononuclear cells by PHA, the transcription of full-length GCH mRNA increased, but that of type II mRNA decreased transiently. We further demonstrated that the type II cDNA exerted a dominant-negative effect on the wild-type cDNA, similar to the effect of some GCH mutants. Therefore, type II mRNA may regulate GCH and then contribute to the regulation of NO production by BH4-dependent iNOS in mononuclear cells. Selection of the splicing sites may be coupled with transcriptional activation of the GCH gene.

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Keywords: GTP cyclohydrolase I; Alternative splicing; Dominant-negative effect; Regulation

Tetrahydrobiopterin (BH_4) is the essential cofactor of phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and nitric oxide synthase (NOS). The rate-limiting step of BH_4 biosynthesis is the cleavage of GTP to 7,8-dihydroneopterin triphosphate by the enzyme GTP cyclohydrolase I (GCH, EC 3.5.4.16) [1]. GCH is therefore necessary for the synthesis of the signaling molecules dopamine, norepinephrine, epinephrine, serotonin and nitric oxide as well as the detoxification of the amino acid L-phenylalanine [2–4].

Mutations of the GCH gene induce human diseases including malignant hyperphenylalaninemia (HPA) and dopa-responsive dystonia (DRD) [5]. HPA is induced by recessive GCH mutations and is associated with hyperphenylalaninemia, dystonia, seizure, and neurological degeneration, probably due to the lack of both catecholamines (dopamine, noradrenaline, and adrenaline) and serotonin [6]. Dystonia is the only symptom in DRD patients and the response to low-dose L-dopa is excellent. DRD is often caused by dominant GCH mutations with residual GCH activities up to 15% of normal [7–9]. GCH protein is a multimeric protein composed of 10 identical subunits [10], and we have demonstrated that some dominant GCH mutations exerted a dominant-negative effect toward the wild-type GCH protein [11].

Besides the control of neurotransmitter synthesis, BH_4 is a determining factor for NOS activity. GCH expression is enriched in brain, liver, and endothelial cells, and is inducible in other cell types and tissues. BH_4 synthesis and NOS are co-induced by proinflammatory cytokines [12]. Experiments employing GCH inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP) demonstrated that BH_4 is a limiting factor of nitric oxide generation in experimental endotoxic shock in rat [13]. Stabilization of BH_4 by L-ascorbic acid potentiates endothelial nitric oxide synthesis [14]. It has been shown

☆ *Abbreviations:* GCH, GTP cyclohydrolase I; BH_4, tetrahydrobiopterin; NOS, nitric oxide synthase; HPA, hyperphenylalaninemia; DRD, dopa-responsive dystonia; DAHP, 2,4-diamino-6-hydroxypyrimidine.

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doi:10.1016/S0006-291X(03)01091-X

that *cis*-elements required for cell type-specific cAMP-dependent enhancement of gene transcription are located along the GCH core promoter [15].

There are two alternatively spliced GCH mRNAs (types II and III) reported initially [16]. These mRNAs are identical at their 5′-regions, but diverge just after the break point between exons 5 and 6. The full-length (type I) mRNA contains a long 3′-noncoding region encoded by exon 6 [17]. A splicing between the 5′ donor site of intron 5 and the middle of exon 6 generates type II mRNA. Intron 5 is not spliced in type III mRNA. Two more types, types IV and V, of alternative splicing were reported in human myelomonocytoma cells (THP-1) [18]. In this paper, we studied alternative splicing of GCH mRNAs and their role in human peripheral blood mononuclear cells. We proved the previously described forms and demonstrated a new form of alternative splicing. We characterized the transcription of these mRNAs when blood cells were stimulated and conferred a role upon the type II mRNA in CGH regulation.

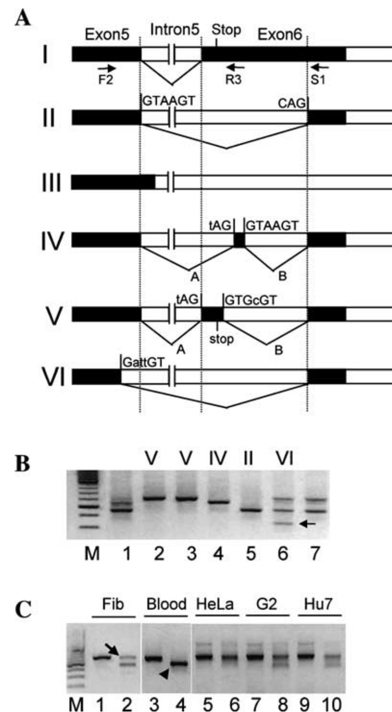

Fig. 1. (A) A schematic sketch of six types of GTP cyclohydrolase I gene alternative splicing. The dark boxes indicate sequences that are included in the mRNA. The position of the authentic stop codon is marked (Stop). The arrows indicate the positions of primers used in RT-PCR. (B) Amplification of GCH mRNAs from diluted template, which shows the presence of type V (lanes 2 and 3), type IV (lane 4), type II (lane 5), and type VI mRNA (lane 6, arrow). M denotes molecular weight markers. (C) Abundance of GCH mRNAs in cells. Lanes 1, 3, 5, 7, and 9 are amplifications for the full-length mRNA and lanes 2, 4, 6, 8, and 10 are spliced forms. Lanes 1 and 2 are from human skin fibroblast (Fib); lanes 3 and 4, human peripheral blood mononuclear cells (Blood); lanes 5 and 6, HeLa cells (HeLa); lanes 7 and 8, HepG2 cells (G2); and lanes 9 and 10, Huh7 cells (Huh7). The major spliced mRNAs are type IV (arrow) and type II (arrow head).

Materials and methods

Cells and cultures. BHK, HepG2, HeLa, Huh7, and T24 (bladder transitional-cell carcinoma) cells were obtained from the American Type Culture Collection directly or through the National Health Research Institute Cell Bank. HepG2, HeLa, and Huh7 cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% (v/v) fetal-calf serum (FCS) (Gibco, USA). T24 cells were grown in Eagle's minimal essential medium (MEM) with Earle's salts supplemented with 10% (v/v) heat-inactivated FCS (Gibco, USA). Confluent monolayer of T24 cells stimulated with 0–1000 IU/ml IFN-γ (PeproTech, England) in fresh medium for 24 h. Peripheral blood mononuclear cells were obtained by centrifuging diluted blood (1 part of blood + 1 part of phosphate-buffered saline) over Ficoll–Paque (Amersham–Pharmacia Biotech, USA). The interface region was separated and washed. Mononuclear cells purified from 20 ml blood were suspended in 30 ml RPMI 1640 (Gibco, USA) with 15% heat-inactivated FCS and then split into six T25 flasks. Peripheral blood mononuclear cells were stimulated by PHA (Murex, France) for up to 72 h. Nitrite concentrations in the culture medium were determined by the Griess reagent (Promega, USA).

Construction of plasmids. GCH cDNA was kindly provided by Dr. Nagatsu. This cDNA produced functional protein although it lacks a short 5′ piece [19]. Type II cDNA was produced by PCR using GCH cDNA as a template and cloned into a CMV promoter expression vector (pCMV-GCH-II). pCMV-A16-GCH-wt was constructed by inserting full-length GCH cDNA (*Nar*I/*Eco*RI fragment) into pCMV-AGP/EBP (replacing its *Bst*BI/*Eco*RI fragment) [20]. This generated an N-terminal AGP/EBP A16 epitope. The construction of pCMV-A16-GCH-249 and pCMV-A16-GCH-201 has been described [9,11].

Detection of GCH alternative splicing by reverse transcription-coupled PCR. Cytoplasmic RNA was extracted from cells with a Total RNA Extraction Miniprep System (Viogene, Taiwan). For the measurement of GCH mRNA transcribed from transfected plasmids, mRNA was extracted by a QuickPrep Micro mRNA Purification kit (Amersham–Pharmacia Biotech, USA). The upper strand PCR primer used was F2 (5′-GCCCCGCAGCGAGGAGGATAAC) and lower strand primers were R3 (5′-GACAGACAATGCTACTGGCAGT) for the full-length transcript and S1 (5′-GAAGCTATGGTTCTGCA GAC) for the spliced forms (Fig. 1).

LightCycler PCR. Quantitative PCRs were performed in a LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) [21]. In glass capillaries with a total volume of 20 μl, the reaction mixture consisted of 2 μl of FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 μM each of the primers, 3 mM $MgCl_2$, and 2 μl cDNA after a 1:10 dilution. In each run, one negative control, with water added instead of DNA, was included. Primers used for type I mRNA were GCH-SPL-1 (5′-GGTTCCATTTGTTGGAAAGG) and GCH-SPL-2 (5′-CACA TGTGTGTTGCTTCAACC); for type II were GCH-SPL-1 and GCH-SPL-3 (5′-GGTTCTGCAGACGTTGCTTC); and for type III were GCH-SPL-1 and GCH-SPL-4 (5′-TGCAGACTTACGTTGC TTCAA). All lower strand primers were designed to cross the splicing junctions. Human GAPD gene was used to normalize the cDNA levels. A standard thermal cycler program suggested by the manufacturer was used except that the annealing temperature was 60 °C. Concentrations were calculated by Roche LightCycler software ver3.5, with standard curves made by serial dilution of PCR fragments from 2.33×10^{-1} to 2.33×10^{-9} ng/μl (type I) and from 2.65×10^{-1} to 2.65×10^{-9} ng/μl (type II) per reaction.

Transfection and Western blot analysis. Calcium phosphate precipitation method was used for transfection [22]. BHK cells in 6 cm dishes were transfected with a combination of pCMV-A16-GCH-wt, pCMV-GCH-II, and pCMV. Forty hours after transfection, cells were

subjected to either mRNA extraction or Western blot analysis. Western blot analysis was performed with a GCH antiserum [9] by standard methods. Blots were developed using the ECL detection system (Amersham–Pharmacia Biotech, USA).

Amplification of splicing factors. Primers for RT-PCR of splicing factor SC-35 [23] were 5′-GAACATCTACAACGCCTGC and 5′-ACGTTCGGAACGGAAAGG; SF2 [24] 5′-GTTTACCGAGATGGCACTG and 5′-ATCAAAGACACGAAGGGAATG; hnRNP-A1 [24] 5′-GAATGCAAGGCCACACAAG and 5′-ACCATATCCACCACCACCAC; PTB [25] 5′-GAACATCTACAACGCCTGC and 5′-ACGTTCGGAACGGAAAGG; SRP30C [26] 5′-TTCAGGACTTCCTCCGTCAG and 5′-CACAAAGCAGCTCAGTTAACC; and Htra-2β1 [27] 5′-AGACCACATACGCCAACAC and 5′-GCCCACAAACAATATCCCAG.

Results

GCH gene alternative splicing

The six types of GCH alternative splicing are depicted in Fig. 1A. Types I to III mRNAs have been reported by Togari et al. [16] in liver, types IV and V mRNAs have been reported by Golderer et al. [18] in human myelomonocytoma cells, and type VI has not been described (GenBank AY137465). Type IV mRNA encodes a truncated protein. Type V mRNA contains the authentic stop codon thus encodes a full-length GCH protein. Type VI is less abundant, which uses an alternative 5′ donor site within exon 6, and the same 3′ acceptor site as in type II splicing. These mRNAs were also obtained separately by the amplification of diluted template (Fig. 1B) and were proved by direct sequencing.

Abundance of GTP cyclohydrolase I mRNAs in different types of cells

Since GCH is enriched in brain, liver, and endothelial cells, and is inducible in other cell types, we verify the spliced forms in different cells. Cytoplasmic RNA was extracted from human skin fibroblasts and cell lines, and the full-length and alternatively spliced GCH mRNAs were amplified separately (Fig. 1C). Besides the full-length GCH gene transcript, human skin fibroblasts expressed two major forms of the alternatively spliced mRNAs, type IV (arrow) and II; human peripheral blood mononuclear cells expressed predominantly the shorter type II mRNA (arrowhead); HeLa cells expressed predominantly the type IV mRNA; and HepG2 and Huh7 cells the type IV and a variable amount of type II mRNA (Fig. 2). Type V mRNA is less abundant and type VI mRNA is the rarest.

Differential induction of GCH transcripts in blood mononuclear cells by PHA

GCH activity has been routinely assayed in peripheral blood mononuclear cells after PHA stimulation, because the basal GCH activity in blood cells was low [28]. Under the stimulation of PHA, cell proliferation occurred as shown by the measurement of actin and ferritin mRNAs, however the full-length GCH mRNA increased to a much greater extent (Fig. 2A). Before PHA stimulation, type II mRNA (Fig. 2A, arrow) was the major spliced form in mononuclear cells, but its amount decreased paradoxically after PHA stimulation. On the contrary, the type IV and type V mRNA (Fig. 2A, open arrow) increased along with the full-length transcript. Nitrite accumulated in the culture medium after the cells were stimulated by PHA (Fig. 2B). In T24 cells, the type IV mRNA can also be stimulated by INF-γ along with the induction of the full-length transcript (Fig. 2C). Nevertheless, there was no visible type II mRNA both before and after INF-γ treatment. Therefore, the expression and induction of GCH mRNAs is cell type specific.

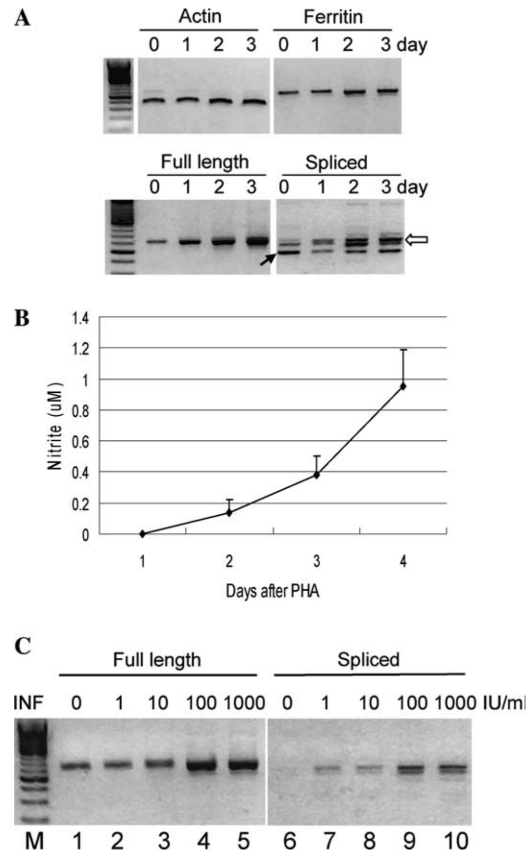

Fig. 2. Induction of GCH mRNAs. (A) Peripheral blood mononuclear cells were treated with PHA and harvested on days 0, 1, 2, and 3. RT-PCR was done for actin, ferritin, full-length GCH mRNA, and spliced GCH mRNAs. (B) Increase of nitrite concentration in culture mediums during the stimulation of blood cells by PHA. The bars on the data points indicate one standard deviation. (C) T24 cells treated with 0–1000 IU IFN-γ/ml for 24 h and GCH mRNAs amplified.

Quantification of the spliced forms of GCH transcripts

The quantities of types I, II, and III GCH mRNAs were determined by the LightCycle PCR. Before

treatment, the concentration of type II mRNA in mononuclear cells was higher than that of type I mRNA. One day after PHA stimulation, the concentration of type I mRNA increased, but that of type II mRNA decreased (Fig. 3A). While the concentrations of GCH mRNAs differed significantly between each independent experiment, when we calculated the changes (fold increase) of mRNA quantities, the tendency was pretty constant (Fig. 3B). The concentration of type III mRNA was much lower than that of either type I or type II mRNA (data not shown).

Expression of the type II cDNA decreases the level of the wild-type GCH protein

Some GCH mutants exert dominant-negative effects on the wild-type protein [11], and the type II mRNA encodes a truncated protein with no enzyme activity [29]. When wild-type and type II cDNAs were introduced simultaneously into BHK cells by calcium phosphate precipitation method, the presence of type II cDNA decreased the level of wild-type GCH protein (Fig. 4A, lanes 1–4, and lanes 9 and 10). In the control experiments, GCH mutant G201E (lanes 11 and 12), but not R249S (lanes 7 and 8), also disclosed a dominant-negative effect. Type II GCH cDNA itself did not result in any visible GCH protein (lane 13). The levels of mRNA expressed from the transfected vectors were similar (Fig. 4B).

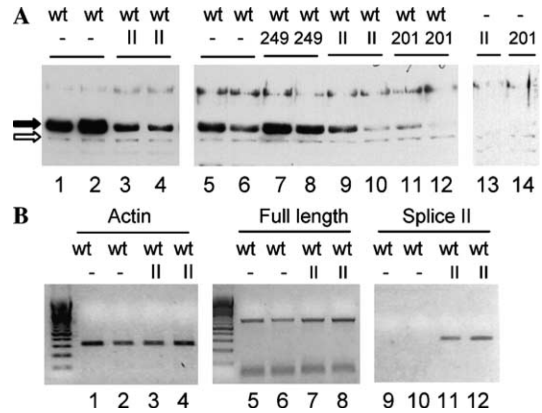

Fig. 4. Expression of GCH cDNAs in BHK cells. (A) Western blot analysis with GCH antibody. Wild-type GCH plasmid pCMV-A16-GCH-wt (wt) (0.8 μg in lanes 1–4 and 0.6 μg in other lanes) was transfected into BHK cells together with the spliced-form plasmid pCMV-GCH-II (II) (1.8 μg in lanes 3 and 4, and 0.6 μg in other lanes), R249S mutant plasmid (249, 0.6 μg), or G201E mutant plasmid (201, 0.6 μg). The arrow indicates the A16-tagged GCH protein and the open arrow indicates a cross-reacting band. (B) RT-PCR for actin (lanes 1–4), full-length GCH mRNA (lanes 5–8), and type II GCH mRNA (lanes 9–12) in BHK cells to show transcription of the transfected vectors.

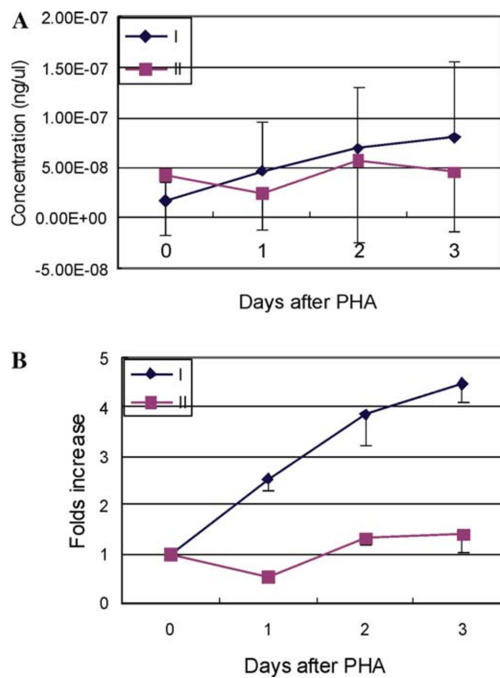

Fig. 3. Quantitative RT-PCR for GCH types I and II mRNA. Peripheral blood mononuclear cells were treated with PHA and harvested on days 0, 1, 2, and 3. (A) The concentrations of mRNAs. (B) Fold increases of GCH mRNAs after the treatment of cells by PHA. The bars on the data points indicate one standard deviation calculated from three independent experiments.

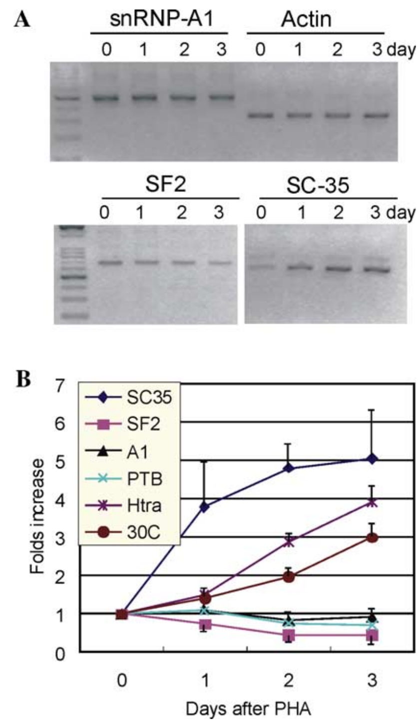

Fig. 5. Transcription of splicing factors by PHA treatment. Peripheral blood mononuclear cells were treated with PHA and harvested on days 0, 1, 2, and 3. (A) RT-PCR was performed for hnRNP-A1, actin, SF2, and SC-35. (B) Data normalized by actin mRNA for hnRNP-A1 (A1), SF2, SC-35, PTB, Htra-2β1 (Htra), and SRP30C (30C). The bars on the data points indicate one standard deviation.

Changes in splicing factor expression after PHA stimulation

From the results presented in the previous sections, it is clear that the selection of splicing points changes when peripheral blood mononuclear cells were stimulated by PHA. Therefore, we measured the transcription of several splicing factors by semiquantitative RT-PCR. The data revealed that SC-35, Htra-2β1, and SRP30C were induced, hnRNP-A1 and PTB stayed steadily, while the transcription of SF2 was repressed by PHA (Fig. 5A). The data were also quantified and normalized by the levels of actin mRNA (Fig. 5B).

Discussion

Alternative splicing is a major mechanism to enable a single gene to increase its coding capacity [30], to facilitate its tissue-specific function [31], or to contribute to its regulation (as revealed in this study). In this study, we demonstrated complex alternative splicing forms over the 3′-end of the GCH gene. Along with the increase in the full-length GCH transcript, the type II mRNA decreased by PHA treatment, showing a fine-tune regulation for GCH in mononuclear cells.

GCH catalyzes the rate-limiting step of BH_4 biosynthesis, and the induction of GCH leads to an elevation in tissue BH_4 concentration, which in turn contributes to the syntheses of neurotransmitters and nitric oxide to cope with acute stress to the body. Among the alternatively spliced GCH mRNA species, the type II mRNA is abundant in several tissues and cells ([18] and this study). It encodes a nonfunctional polypeptide lacking the C-terminal part of β-sheet domain. Since the β-sheet domain is responsible for both enzyme activity and complex formation, the type II mRNA does not give rise to active enzyme [29]. Our results demonstrated that type II cDNA could exert a dominant-negative effect on the full-length GCH protein, so type II splicing may erratically decrease GCH activity. One would ask why this mRNA exists. In this study, the response of type II mRNA to PHA stimulation may help to resolve the question. In mononuclear cells, the production of NO by inducible NO synthase (iNOS) is important for the function of these cells during infection or inflammation. We hypothesize that the presence of type II mRNA contributes to the regulation of GCH by reducing its basal activity and then allowing a bigger amplitude of induction. Differential splicing of the GCH RNA has also been implicated in the variation of clinical symptoms in DRD [32].

When we compared the sequence at the splicing junctions, we found that the alternative 3′ acceptor site used by type II splicing actually fits the consensus better than that used by the full-length splicing (Fig. 1A). This could explain the abundance of the type II mRNA. It is intriguing that how these two 3′ acceptor sites are selected in different conditions. The heterogeneous nuclear ribonucleoproteins (hnRNPs) have been implicated in splicing repression [33]. The SR proteins, which are required for constitutive splicing, also influence alternative splicing regulation [34]. The SR protein SF2/ASF and hnRNP A1 could be functional antagonists in several examples [24]. In our study, we could observe a specific pattern of changes in mRNA for a panel of splicing factors, including the large induction of SC-35, for which the "SC-35 domain" is a place where processing of many pre-mRNAs occurs. Besides, alternative splicing has also been shown to be coupled with the transcription machinery [35]. Since the existence and the role of alternative splicing are rapidly emerging, this study should contribute significantly to both the mechanism of GCH regulation and to the understanding of alternative splicing.

Acknowledgments

We thank Dr. Nagatsu for the GCH plasmid. This work was supported by National Science Council Grants NSC 90-2314-B-002-163 and NSC 91-2314-B-002-147.

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