行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：■個別型計畫 □整合型計畫

計畫編號：NSC 89－2314－B－002－102

執行期間： 88 年 8 月 1 日至 89 年 12 月 31 日

計畫主持人：蔡 偉

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中 華 民 國 90 年 12 月 14 日

政院國家科學委員會專題研究計畫成果報告

S蛋白缺乏症的基因研究（三）

Genetic Study of Protein S Deficiency (III)

計畫編號：NSC 89-2314-B-002-102
執行期限：88年8月1日至89年12月31日
主持人：蔡偉　國立台灣大學醫學院內科

中文摘要

S蛋白（PS）缺乏症是中國人遺傳性血栓好發症的最常見的原因。本研究之目的是發掘中國PS缺乏家族的基因缺陷及其機制。過去我們在26個PS家族裡共發現十一種不同的點突變(point mutations)。其中有一突變C(-56)T發生位置是在啟動子（promoter）上，重複發生在五個不同的家族，都表現為第三型S蛋白缺乏症。在此我們利用PCR的方法，複製啟動子的部分，再用運限制酵素產生幾種長短不同的片段；並使用類似方法產生C(-56)T突變的啟動子片段。再分別將這些片段構築到質體pGL_3上。我們利用三種培養細胞HeLa、HepG2、Hu-7，暫時表現其轉錄功能。結果證實啟動子遠端nt-511到nt-141，具很強的轉錄活性；但點突變C(-56)T卻能導致基因完全無法轉錄。由此我們推測，此突變必發生於啟動子極重要的部位，藉此我們得以瞭解啟動子的部分結構與功能。據我們知道，本研究是全世界首先發表PS基因的轉錄功能的研究報告。

關鍵詞：S蛋白缺乏症、基因缺陷、啟動子、基因轉錄。

Abstract

Protein S (PS) deficiency is the most common disorder of hereditary thrombophilia in Chinese. The aim of our studies is to detect the genetic defects and their molecular mechanism of our Chinese patients with PS deficiency. In previous studies, we totally found eleven different point mutations in 26 PS deficient families. Interestingly, a recurrent mutation C(-56)T at the promoter resulted in type III phenotype in five families. We amplified the wide or mutant C(-56)T sequence of PS promoter through polymerase chain reaction (PCR). We further cloned them into expression pGL_3. plasmid. Then, we construct a panel of serial-deletion expression plasmids. We detected transient trasnscription activities of each construct in HeLa, $HepG_2$, and Hu-7 cell lines, separately. Our results demonstrated that the distal portion of promoter from nt -511 to -141 had strong transcription activity, while mutant C(-56)T construct abolished all transcription activity. This point mutation must have a critical influence to PS promoter. We hypothesized a strong inhibitor bond to this mutant region. In addition, some promoter structure/function was disclosed. However, the detailed transcription mechanism needs further investigation. As we know, this is the first report about the transcription of human PS gene.

Keywords: protein S deficiency, genetic mutations, promoter, gene transcription.

Introduction

PS is a vitamin K-dependent plasma anticoagulant. Deficiency of PS is an established risk for venous thromboembolism[1]. Clinically, total and free PS antigen levels, and PS activity levels can be measured. According these plasma levels, PS deficiency can be classified into three phenotypes[2]. Patients who had reduced

antigen levels (total and free) and activities were defined as a type I PS deficiency. Patients who had normal antigen levels (total and free) but reduced activities were defined as a type II PS deficiency. Patients who had normal total antigen levels but reduced free antigen levels and activities of PS were defined as a type III PS deficiency.

We had previously reported that PS deficiency was the most common cause of hereditary thrombophilia in Taiwan[3]. In the prevous studies[4], we had succeeded to detect eleven point mutations in 26 families. Among them, one interesting recurrent mutation C(-56)T occurred at the promoter resulted type I or type III phenotype in five families.

There were several published large-population genetic studies about PS deficiency[5]. However, the promoter function of gene PS-α was not reported yet. Here, we studied the transcriptional activity of mutant promoter to demonstrate the molecular mechanism of this gene defect.

Materials and Methods

PCR amplification of 5'-flanking region and contruction of serious-deletion plasmids

Genomic DNA was extracted from peripheral white blood cells by a commercial extraction kit. Store it at 1X TE buffer at 4℃.

We first designed two primers P5-2 5'-GAAGTCTTTATCGGAGCAAG and P3-3 5'-CGGAGCTGCGAGCCTGTG. We used normal genomic DNA as template to amplify the PS 5'-flanking region.. The detailed PCR condition had been described previously[4]. The PCR condition was the annealing cycle 61℃ 30sec, the extension cycle 72℃ 30 sec, the denature cycle 95℃ 1min, and total 35 cycles. A 554-bp DNA fragment, from nt(-511) to nt(+43), was got We cloned the amplified fragment into pCRII plasmid by TA cloning kit (Invitrogen). We further subcloned them into MP13mp19 phage to confirm the accuracy of DNA sequences. We then cloned this amplified fragment nt(-511 to +43) into a reporter vector pGL_3-basic (Promega). We further digested our MP13 phagemid or pGL3 plasmid by restriction enzymes BclI, StyI, StuI, HindIII to produce a serial of deletion report constructs pGL_3-A1, A2, A3, A4, and A5 as shown at Fig.1. We constructed another two variant reporter plasmids, pGL_3-B1 containing nt(-511 to -114), and pGL_3-B2 containing reverse sequence of nt (+43 to -511). We further used patient genomic DNA as template to get a mutant pGL_3-B3 reporter plasmid, which containing nt(-511 to +43) but having C(-56)T mutation.

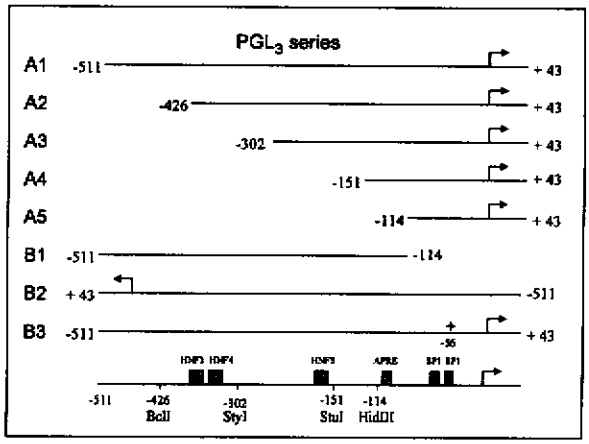

Fig.1 Serious-deletion and variant fragments of PS promoter

Cell culture, DNA transfection, and luciferase expression assays

HeLa, HepG2, and Hu-7 (a human hepatocelluar cell line) cells were cultured as a monlayer in DMEM medium with 10% fetal calf serum. DNA transfections were performed through Superfect transfection reagent (Qiagen, Tokyo), and the detailed procedures followed the recommendation by supplier. Plasmid pCMV-luciferase, or pGL_3-basic was used as a positive and negative control. In each 60-mm dish, 5 μg DNA was introduced. Cells were harvested 48 hr after transfection, and 30 μg of total protein was used to perform a luciferase assay (Promega) by luminometer.

Results

The transcription activities of those

reporter vectors were measured by the expression of luciferase activity. The following average results were derived from the average means of more than five transfection experiments.

	HeLa	HepG2	Hu-7
A1	103.1%	50.7%	114.7%
A2	81.4%	85.8%	107.6%
A3	100.0%	100.0%	100.0%
A4	63.0%	73.3%	65.1%
A5	35.7%	35.3%	26.2%
A6	78.9%	110.9%	291.4%
A7	6.9%	5.1%	9.0%
A8	0.01%	0.02%	0.01%

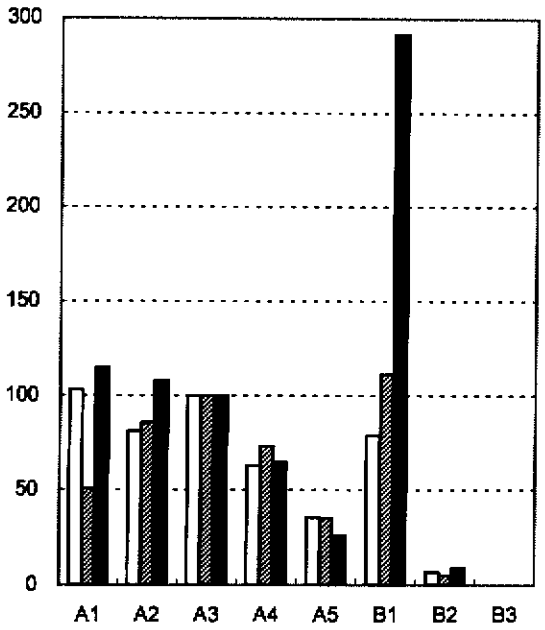

Fig. 2 Relative transcription activities of each reporter plasmid in three cell lines. The empty box is labeled for HeLa cells, the hatched box for HepG2 cells, the filled box for Hu-7 cells. The vertical bar is relative luciferase activities. The luciferace activity of wild-type construct A3 is arbitrarily assumed as 100%.

Discussion

Up date, no mutation reported at PS promoter. In previous studies, we found that the mutation C(-56)T was recurrent in five unrelated Chinese families. All of them did not have other mutations in the coding region of PS-α gene. All probands exhibited the similar phenotype of PS deficiency. Family study also demonstrated the cosegregation.

The PS gene had been cloned since 1990[6], but only one preliminary study analyzed the PS promoter[7]. In present study, we tried to analyze the transcription activity of PS promoter and to find the biological significance of our novel mutation C(-56)T. Serial deletion of 5'-portion of promoter from nucleotide position -511 to -302 had less influence to transcription activities. However, further deletion from nt -302 to -151, the activity lost about 30% more. We compared the sequence of this region, a HNF-3 like motif may be located at this region. Further deletion from nt -151 to -114, the activity lost to original 30% level. It suggested that the nucleotides between -151 to -114 contained some positive cis-elements for transcription. By our analysis, an androgen-response element-like motif may be located here. Hall has found the PS promoter activity can be increased by co-transfection of plasmid encoding androgen or steroid hormone receptor[7].

As we knew, PS could be synthesized and secreted by human endothelial cells[8], hepatocytes[9] and megakaryocytes[10]. PS promoter has similar behavior as other none TATA-box promoter. Multiple transcription initiation sites had been hypothesized by Hall et al[7]. The different cells may have different transactivation mechanisms. In this study, we used HeLa cells, HepG2 and Hu-7 hepatoma cell lines. We confirmed that PS promoter was functioning in these cell lines. The expression activities of the panel of fragments in these three cell lines were quite similar.

From the activity of B1 reporter, it suggested that nt -511 to -141 contained a strong transcription activity, especially in heptaoma cell line. In other word, the proximal portion of promoter (nt -114 to +43) have down-regulation activity in Hu-7 cell

lines. From the behavior of B2 reporter, it demonstrated that PS promoter activity is determined by its orientation, that is, the reverse direction nearly lost total activity.

Most surprising, the point mutation C(-56)T caused complete depletion of transcription activity. At the beginning, we analyzed the sequence between nt -64 to -24, three putative SP-1 sites was noted. However, we failed to demonstrated SP-1 binding to oligonucleotide of nt -64 to -50 by electrophoretic mobility shift assays. Our preliminary data suggested that this effect was not caused by destruction of putative SP-1 site. We hypothesized that it may associate with some strong inhibitor(s), which bind to the mutant region. But, further investigated is necessary to confirm this possibility.

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