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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※ GTP CYCLOHYDROLASE I 基因轉殖小鼠 ※
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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-002-387
執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：胡務亮
共同主持人：

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□國際合作研究計畫國外研究報告書一份

執行單位：國立台灣大學醫學院(附設醫院)優生保健部

中　華　民　國 90 年 10 月 31 日

行政院國家科學委員會專題研究計畫成果報告

GTP CYCLOHYDROLASE I 基因轉殖小鼠啟動子表現及表現型分析

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一、中文摘要

GTP cyclohydrolase I (GCH)負責tetrahydrobiopterine(BH4)合成的第一個步驟。BH4是一個很重要的分子，它是phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase以及nitric oxide synthase的輔因子。GCH基因突變會引起惡性苯酮尿症或Dopa-敏感性肌肉張力不全症，兩者和巴金斯症一樣，都需要接受L-dopa治療。大部分Dopa-敏感性肌肉張力不全症是顯性遺傳，其分子機轉為dominant-negative。基於BH4生理作用的廣泛性，其引發疾病之複雜性，以及與巴金斯症的相關性，我們希望能製造出缺乏GCH的動物模型。

我們在過去的時間中，我們自人類基因庫中篩選到GCH基因前方一斷13kb長的序列。並且證實2.8kb及5kb(包含部分exon 1)的片段可以表現出啟動子活性。我們將2.8kb片段後方接上LacZ基因後注入小鼠受精卵中，結果LacZ F1小鼠腦部無法見到LacZ基因的表現。因此我們將含有Dopa-敏感性肌肉張力不全症G201E突變的GCH基因cDNA，接在5kb片段後方。小鼠F0及F1均已篩選完畢。

在F0及F1的轉殖小鼠中，我們發現有一些小鼠在Rota Rod中的表現不佳。這些小鼠有可能是腦中GCH基因的表現受到影響，所以表現出運動上的障礙。然而這些小鼠的子代並沒有辦法穩定的表現出這樣的異常，所以無法證實運動障礙和轉殖基因間的關係。

當轉殖小鼠年齡增大後，我們發現其中有一些有異常的動作。這是一種持續性的沒有目的的旋轉。來自同一祖先的好幾株都可以看到這樣的異常。這可能是一種晚發性的運動障礙，很可能和轉殖基因，也就是突變型的GCH基因有關。這些小鼠的生殖力差，新生小鼠死亡率高，相關組織學分析正在進行中。

關鍵詞：GTP cyclohydrolase I，Dopa-敏感性肌肉張力不全症，基因轉殖小鼠

Abstract

GTP cyclohydrolase I (GCH) is responsible for the first step of tetrahydrobiopterin (BH_4) biosynthesis. BH_4 is an important molecule, because it is the cofactor of phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase and nitric oxide synthase. GCH gene mutations are associated with wither malignant phenylketonuria and dopa-responsive dystonia (DRD). Both of them are responsive to L-dopa treatment. DRD is mostly dominantly inherited, and through the dominant-negative mechanism.

In the past years, we have isolated a 13kb DNA from human genomic library containing the 5' region (including part of exon 1) of GCH gene. We have shown that both a 2.8 and 5kb fragments possessed promoter activity. We connected the 2.8kb fragment to LacZ gene. The construct was injected into mouse fertilized egg. We have screened F0 and F1 for the LacZ mice, but the gene was not expressed. We inserted a GCH cDNA containing the G201E DRD mutation after the 5kb promoter. Both F0 and F1 G201E mice were produced.

The phenotype of the transgenic mice was checked by Rota-Rod. Several mice were found to have poor performance on the Rota. These mice may have decreased GCH activity because of the expression of the dominant negative GCH gene. However, the offspring of these mice could not express stably the same phenotype. Therefore, we can not make conclusion on the association between motor defect and the transgene.

When the mice were getting old, some of them demonstrated behavior problem. They tended to make purposeless circling movement. This could be a late onset motor abnormality, and may be associated with the GCH gene. Some mice were from the same ancestor. These mice have low fertility power, and many of their babies died shortly after birth. Further studies on them are going now.

Keywords: GTP cyclohydrolase I, Dopa-responsive dystonia, transgenic mice

二、緣由與目的

GTP cyclohydrolase I (GTP-CH) catalyzes the rate limiting step of tetrahydrobiopterin (BH_4) biosynthesis (Nichol, 1985). BH_4 is the cofactor of several important human enzymes including phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase (Kaufman 1959, Nagatsu 1964, Lovenberg 1967) and nitric oxide synthase (Griffith and Dennis, 1995). The latter three are responsible for the production of bioactive humeral factors dopamine,

serotonin and nitric oxide. GTP-CH mutations have been found in patients with hyperphenylalaninemia (malignant phenylketonuria), and dopa-responsive dystonia (DRD) (Segawa et al., 1971; Nygaard, 1988).

In hyperphenylalaninemia, patients are not able to metabolize phenylalanine and have multiple neurotransmitter deficiency. In DRD, it looks like only nigro-striatal dopaminergic neurons are affected, and the main symptom is dystonia (Segawa 1976, 1986). The GTP-CH activities in hyperphenylalaninemia patients are very low, while the activities are usually 10 to 20% in HPD/DRD patients (Segawa 1996, Ichinose and Nagatsu, 1997). After the cloning of human GTP-CH gene (Ichinose, 1994), recessive mutations were found in hyperphenylalaninemia and dominant mutations were found in DRD (Ichinose, 1994; Ichinose et al., 1995; Furukawa et al., 1996; Bandmann et al., 1996; Blau et al., 1995). The enzyme is a homodecamer (Nar et al, 1995). In our previous work, we have found a case of DRD who has a homologous GTPCH gene mutation (R249S).

It will be very useful if there is an animal model of GTPCH deficiency to solve the unusual inheritance in GTPCH deficiency, explore the disease mechanism and improve the treatment, and understand more about Dopa metabolism. Our hypothesis is that residual enzyme activity (gene dosage) determines the phenotype, and inheritance depends on the mechanisms of mutations (loss-of-function or dominant-negative).

The *hph-1* mouse has BH4 deficiency and reduced levels of dopamine, norepinephrine and serotonin (Hyland, 1996), however, the underlying defect of the *hph-1* mouse is still not known (Gutlich et al., 1994). Transgenic mice expressing *COLIA1* gene with Gly859Cys mutation died intrauterine or shortly after birth (Stacey et al., 1988). Both the endogenous and mutant pro-α1(I) collagen was expressed, but a striking reduction of the total collagen I content was proportional to the level of mutant gene expression. Mutations in the p53 tumor suppressor gene are found at high frequency in a wide range of cancers (Hollstein et al., 1991). Wild-type p53 acts as a negative regulator of cell growth (Kuerbitz et al., 1992). Mutant forms of p53 no longer possess the ability to arrest cell growth and are unable to bind to specific DNA response sequences (Michalovitz et al., 1990). Mutant p53 actually inhibit wild-type p53 in a “dominant negative” manner through a complex formation between the two forms (Milner et al., 1991).

In this study, we tried to make a mouse model for GCH deficiency. Our strategy is to make transgenic mice with a dominant negative GCH mutant cDNA.

三、成果及討論

材料及方法

Tissue section preparation

The animal is killed by CO2. It is first perfused with warm normal saline, followed by 2% paraformaldehyde. Organs are dissected and fixed for another two hours. The organs are then soaked in 30% sucrose in PBS with 2 mM MgCl2. Brain and organs are then embed in OCT compound and ready for frozen section.

Beta-galactosidase staining

To determine the patterns of β-galactosidase expression, tissue sections will be fixed and stained for β-galactosidase activity.

Fixation and section as described in previous section

The slides are rinsed in PBS with 2 mM MgCl2

Staining buffer

PBS

1 mM $MgCl_2$

1.5 mM $K_4[Fe_3(CN)_6]$

1.5 mM $K_3[Fe_2(CN)_6]$

Substrate: 1 mg/ml

5-bromo-4-chloro-indoyl- -D-galactopyranoside (X-Gal)

Incubation at 37^{o}C for 3 hr

Counter stain with hematoxylin and mount

PCR detection

PCR will be done by primer pair P1F/P4R. This pair of primer specifically amplify human GCH promoter sequence with no cross amplification of the mouse DNA.

Southern blot analysis

Southern blot will be performed with a 700 bp DNA fragment located at the proximal human GCH promoter. This probe detects no cross-hybridization signal with mouse DNA. A non-radioisotope method will be used in the study. DNA will be cut by different enzyme for the purposes of both verification of integration and estimation of copy number.

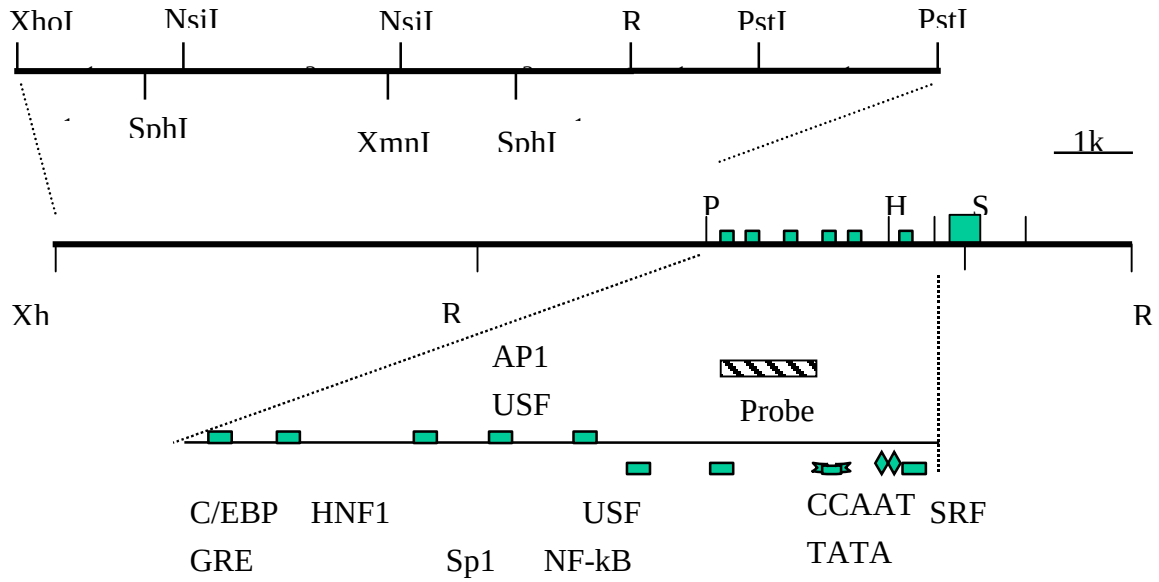
Map of human GCH promoter.

One positive phage clone was obtained from Human genomic library (ClonTech, USA). A XhoI/EcoRI 13kb fragment was subcloned into pBluescriptII vector.

Promoter activity

Promoter fragments of different lengths were ligated before the CAT reporter gene. The vectors were transfected into BHK cells, and CAT activity was assayed by standard method.

Map of the promoter region



PCR and Southern analyses of Lacz-mice

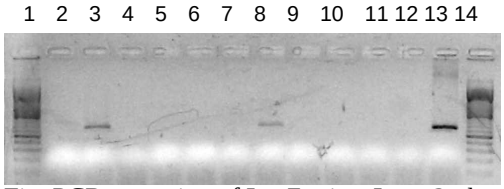


Fig. PCR screening of LacZ mice. Lane 2: the G2 mouse, lane 12: negative control, lane 13: positive control, lanes 1 and 14: marker

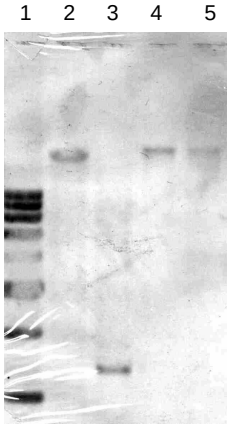


Fig. Southern blot analysis of LacZ mice. Lane 1: marker, lane 2: human DNA EcoRI cut, lane 3: human DNA PstI cut, lane 4, 5: LacZ mice number 2 and 7, EcoRI cut. The probe is a human promoter fragment

Rota Rod assay

	8 rpm			16 rpm		
ID	1	2	3	1	2	3
137	58	120	77	42	120	120
151	36	120	120	28	13	20
11	49	120	61	56	23	10
1	120	51	120	22	119	36
23	68	120	120	38	120	95
N	120	120	90	120		
N	120	120	120	120		
N	81	75	36	120		
139	120	120	120	120	120	60
90	89	120	120	90	92	120
76	49	120	43	32	47	97
75	20	46	36	33	67	65
61	120	120	120	120	120	120
159	120	120	120	54	112	30
156	86	120	120	120	120	31
155	50	58	16	120	120	120
108	37	58	120	18	120	120
117	27	35	49	10	10	10

These results are still under calculation and analysis. The phenotype of the transgenic mice was checked by Rota-Rod. Several mice were found to have poor performance on the Rota. These mice may have decreased GCH activity because of the expression of the dominant negative GCH gene. However, the offspring of these mice could not express stably the same phenotype. Therefore, we can not make conclusion on the association between motor defect and the transgene.

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