行政院國家科學委員會專題研究計畫成果報告

利用基因轉殖魚研究 *FMR1* 啟動子
Transgenic Zebrafish Study of *FMR1* Promoter

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一、中文摘要

X 染色體脆折症是引起遺傳性智能不足的主要原因之一，在 X 染色體脆折症患者，*FMR1* 基因內的 CGG 重覆序列長度會增加，並引起基因的甲基化。*FMR1* 基因的篩選雖然解釋了 X 染色體脆折症特殊的遺傳模式，但是 *FMR1* 的功能至今仍不清楚。

在我們過去的研究中，我們證實甲基化是 *FMR1* 基因表現抑制的直接原因(Hwu et al, BBRC 1993;193:324-9)。我們進一步分析 *FMR1* 啟動子的構造，並發現 *FMR1* 啟動子中有一段序列(MSE)可以被 cAMP 活化(Hwu et al, DNA Cell Biology 16:449-53)。然而過去我們想看細胞株內部 *FMR1* 表現變化之努力卻不順利。最近有人報告 *FMR1* 的變化可能發生在極細微的構造中(如神經之突觸)。因此 *FMR1* 之變化必須直接在生物體中研究，也就是利用轉殖動物。我們要用的則是斑馬魚。我們將在斑馬魚魚卵中注射由 *FMR1* 啟動子驅動的 *LacZ* 基因，再用染色的方法，來判斷 *FMR1* 啟動子表現的位置。啟動子的不同片段經剪裁後接到 *LacZ* 基因的前方，再用來注射魚卵。

在研究的過程當中，我們發現所有 *FMR1* 啟動子片段所驅動的 *LacZ* 基因，都不會在魚卵中表現。在對照組實驗中，以 CMV 啟動子片段所驅動的 *LacZ* 基因可以廣泛的表現在魚的胚胎中，尤其是在 york sac。另外如果用 GTP cyclohydrolase I 的啟動子片段所驅動的 *LacZ* 基因，則可以在腦部看到表現。因此 *FMR1* 啟動子無法以斑馬魚魚卵注射來研究。

關鍵詞：X 染色體脆折症，*FMR1*，基因調控，轉殖動物，斑馬魚

Abstract

Fragile X syndrome is a major cause of hereditary mental retardation. In patients with fragile X syndrome, *FMR1*gene usually has an expanded CGG repeat sequence, and the gene is hypermethylated. Although the position cloning of *FMR1* explained the unusual patterns of inheritance in the fragile X syndrome and helped its DNA diagnosis, the function of *FMR1* is not known.

Our previous work on the fragile X syndrome has shown that methylation is the direct cause of *FMR1* inactivation (Hwu et al. BBRC 1993; 193:324-9). We further showed that that an element (methylation sensitive element, MSE) in *FMR1* promoter could be activated by cAMP (Hwu et al. DNA & Cell Biology 16:449-53). However, our experiments on the regulation of *FMR1* in cultured cells were not successful. Some recent reports hinted that changes in *FMR1* expression happened at subcellular level of specific tissues. Therefore *FMR1* regulation must be studied *in vivo.* In this project, we inject a *FMR1* promoter-*lacZ* gene construct into eggs of zebrafish. Promoter sequences of different lengths were inserted before the *lacZ* gene.

The results showed that none of the *FMR1* promoter-*lacZ* gene construct could be expressed in fish embryo. On the contrary, CMV promoter-*lacZ* gene construct showed extensive expression in the embryo, especially in the york sac. Also *lacZ* gene drived by the GTP cyclohydrolase I promoter was specifically expressed in the brain. Therefore, *FMR1* promoter could not be studied in the zebrafish system.

Keywords: fragile X syndrome, *FMR1*, gene regulation, transgenic animal, zebrafish

二、緣由與目的

Fragile X syndrome (FRAXA) is the most important hereditary form of mental retardation. It is characterized by a Xq27.3 fragile site, macroochidism, and expectation in transmission (Sherman 1985). Its gene - *FMR1* (Fra X Mental Retardation 1) contains an expanded CGG repeat (Verkert 1991, Fu 1991) which may lengthen through female transmission. The gene is heavily methylated at its 5'CpG-island in patients with fragile X syndrome (Oberle 1991, Boyes 1991).

FMR1 encoded a protein (FMRP) of 614 amino acids (Verkerk et al., 1991) which contains two KH domains and an RGG box, and it is also proved to be an RNA binding protein (Ashley et al., 1993). Different proteins may also exist due to alternative splicing of the mRNA (Verkerk et al., 1993). FMRP was recently shown to be associated with ribosomes, and may shuttle between nucleus and cytoplasm (Feng et al., 1997). Although *Fmr1* knockout mice showed similar phenotypes as patients (The Dutch-Belgian fragile X consortium, 1994), the true function of FMRP is still not clear.

FMRP has wild distribution, but more enriched in certain types of cells in both brain and testes (Hinds et al., 1993; Bächner et al., 1993; Abitbol et al., 1993). These tissue specific distribution support its functional role in brain and testis, the two organs affected in patients with fragile X syndrome (Johannisson et al., 1987; Reiss et al., 1991). *FMR1* has been reported to be modulated in proliferating and quiescent cultured cells (Khandjian et al., 1995) which suggested a link in function to cell differentiation and growth. *FMR1* mRNA may be transferred to synapses where the translation of FMRP may be stimulated by neurotransmitters (Weiler et al., 1997).

In order to study the control of *FMR1* expression, we have defined the *FMR1* promoter, and showed that it is methylation sensitive (Hwu et al., 1993). The 460 bp promoter region is GC rich, containing several putative SP1 sites, and a TATA like box 26bp upstream of the major transcription initiation site. Our following study further identified a 22bp region of methylation sensitive nuclear protein protection in the promoter by DNaseI footprinting assay (Hwu et al., 1997). This element, called methylation sensitive element (MSE), was an enhancer in transfection assays. MSE contains a cAMP-responsive element (CRE)-like sequence (Lin and Green, 1988). Recombinant CRE binding protein (CREB) bound to MSE, and both CREB and forskolin (cAMP) activated CRE. However, in the past 6 months, when we looked into cultured cells to see changes of FMR1 expression under various conditions, no changes could be found be various methods.

In according to some recent reports, the changes in *FMR1* expression probably happens on subcellular structures of specific cells (Weiler et al., 1997). Very likely, the study of *FMR1* regulation must be performed *in vivo*, that is, in transgenic animals. The zebrafish is an excellent vertebrate system for genetic analysis (Streisinger et al 1981, Kimmel and Warga 1988). The organism is particularly attractive for this purpose because (1) females can produce a large number of eggs, (2) early development is rapid (gastrulation is at 5 hg; somites form between 10-20 hr), (3) the embryo is transparent throughout the early developmental processes, (4) early stages of development can occur in haploid embryos, (5) the generation time is only 60-70 days, and (6) that one can raise large numbers of fish in a relatively small space. Work by Stuart et al has already shown that it is possible to obtain transgenic zebrafish by injecting plasmid DNA into fertilized fish eggs (Stuart et al 1990, Culp et al 1991). Stuart et al. (Stuart et al 1990) has showed that DNA injected into the cytoplasm of fertilized zebrafish eggs could integrate into the fish genome and be inherited in the germ

line. Neuron specific expression of P19 embryonal carcinoma cell necdin and chicken gicerin were also good examples (Kuo et al 1995, Kim et al 1996). The expression of *lacZ* gene could easily stain after fixation or even in living embryos (Lin et al 1994).

In this project, we injected *FMR1* promoter-triggered *lacZ* gene into eggs of zebrafish, and the gene expression was located by the staining of β-galactosidase activity. CMV promoter and another gene with tissue specific localization, the GTP cyclohydrolase I gene, were used as controls.

三、結果與討論

DNA constructions. A genomic fragment containing this region has also been shown to direct tissue-specific distribution in transgenic mice (Hergersberg et al., 1995).

FMR1 promoter constructs have been established previously (Hwu et al., 1993, 1997). All promoter constructs will be inserted into a plasmid before the *lacZ* gene. Control plasmid is CMV-driven *lacZ* gene. Another control is a GTP cyclohydrolase I promoter (5 kb).

Fish maintenance and egg collections. Eggs for injection were obtained by placing about 10-25 6- to 12-month-old fish, in a female/male ration of 2:1, in a 15-gallon tank at least 1 day before eggs are needed. Mating occurred in the morning shortly after the lights come on. Eggs were collected by placing a nuptial chamber in the tank for 10 min.

For injection, eggs were pipetted onto an agarose ramp formed by resting a wide glass slide in the lid of a 60-mm Petri dish containing about 15 ml of molten 1 % agarose. Removing the slide left a ramp terminating in a groove. Eggs rolled down the ramp and lined up in the groove where the majority could be injected into the cytoplasm above the yolk at the one- or two-cell stage. Injection was performed under a dissecting microscope.

Detection of β-galactosidase activity. To determine the patterns of β-galactosidase expression, embryos were fixed at 24 h and assayed for β-galactosidase activity. Embryos were fixed in 4% paraformaldehyde, 4% sucrose, 0.15 mM $CaCl_2$, and 0.1 M sodium phosphate buffer (pH 7.2) for 1 hr at 4^oC. The embryos were then rinsed in 0.1 M sodium phosphate, incubated in 1 mg/ml 5-bromo-4-chloro-indoyl-β-D-galactopyranoside (X-Gal),

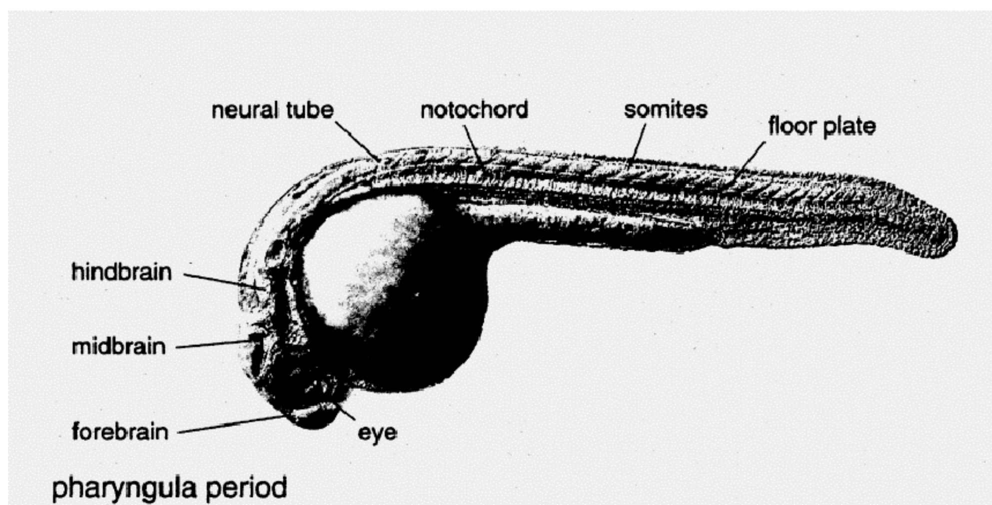

Fig.1. Anatomy of zebrafish.

150 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $K_4[Fe_3(CN)_6]$, 1.5 mM $K_3[Fe_2(CN)_6]$, in 5 mM sodium phosphate buffer (pH 7.2), at 37^oC for 3 hr, fixed again, and then ,mounted in 50% glycerol and 0.1 M phosphate buffer.

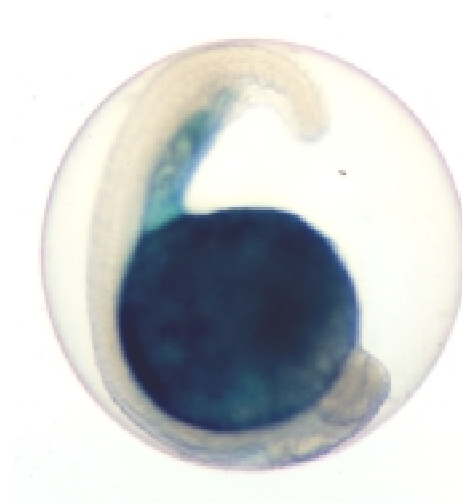

Fig.2 (right). β-galactosidase staining of zebrafish embryo injected with CMV promoter driven *lacZ* gene

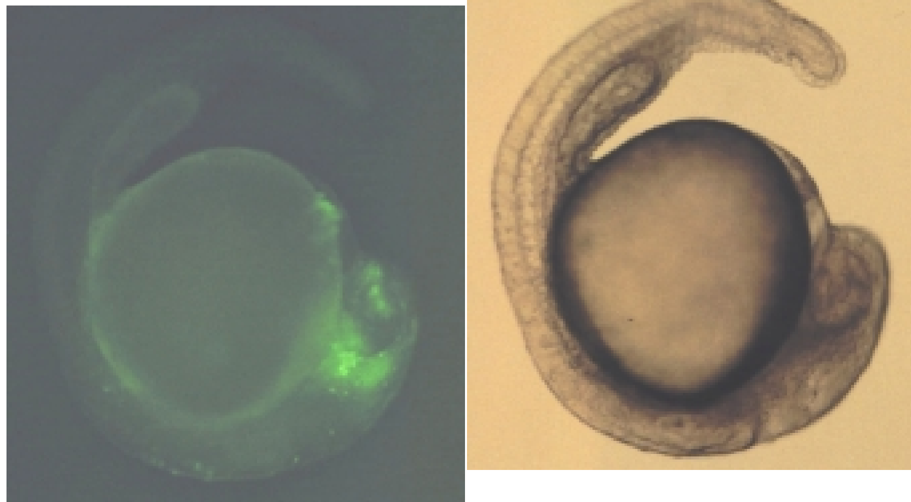

Fig.3. Fluorescence of zebrafish embryo injected with GTP cyclohydrolase I promoter driven green fluorescence GFP gene. Left: fluorescence; right: phase contrast.

However, none of the *FMR1* promoter driven constructs expressed in the zebrafish after injection of fish eggs.

四、計畫成果自評

本計劃讓我們對於實驗動物模式有了深刻的體認。斑馬魚因為某種原因不表現我們的啟動子，卻可以表現另一個我們感興趣的基因-GTP cyclohydrolase I。本計劃除了建立我們斑馬魚實驗的技術外，也驅使我們走向更複雜的動物模式-小鼠的實驗。

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