行政院國家科學委員會專題研究計畫成果報告

FMR1 基因調控：細胞內 DNA 足印之研究

FMR1 Regulation: in vivo DNA Footprinting Study

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中文摘要

*FMR1*是引起X染色體脆折症的基因。X染色體脆折症是由染色體Xq27.3位置的脆折點而命名。*FMR1*篩選到以後，只需要去測量基因CGG重覆片段的長度及基因的甲基化，就可以診斷患者、帶原者，及產前診斷。可是由於*FMR1*是直接靠它在染色體上的位置而被選殖到的，所以直到現在它的功能仍不清楚，X染色體脆折症也缺乏有效的治療。

為了能瞭解*FMR1*的功能，我們首先研究其基因的調控。我們以診斷X染色體脆折症的經驗為基礎，首先分析了*FMR1*啟動子的構造，並証明*FMR1*啟動子會被甲基化抑制(Hwu et al.BBRC 1993; 193:324-9)。我們接著以遺傳學(突變株的研究)及生化學(以合成的轉錄因子進行研究)上的証據，指出*FMR1*啟動子可以被cAMP及CREB所活化(Hwu et al. DNA & Cell Biology)。

在本研究中我們試圖用細胞內足印(*in vivo* DNA footprinting)，去觀察 FMR1 啟動子(包括 MSE,methylation sensitive element)在細胞中和轉錄因子的結合，進而瞭解 FMR1 在細胞內的作用及調控。我們並計畫發展 RNase protection assay (RPA)，以便更精確的測定細胞在不同狀況中 *FMR1* 表現的變化。

很不幸的，我們無法突破細胞內足印的技術，因為 FMR1 啟動子 GC 含量極高，更有 CGG 重複序列，導致細胞內足印分析中 primer extension 的部分極為困難。RPA 實驗則無法看到 FMR1 在細胞內的變動，因為 FMR1 的調控其實只局限在少數的細胞，甚至細胞的一部分。所以我們目前已改變研究方法，將直接以動物模式來研究，預期會有突破性的成果。

關鍵詞：*FMR1*, 基因調控, X 染色體脆折症，DNA 足印

Abstract

FMR1 is the gene responsible for the fragile X syndrome. The cloning of *FMR1* make revolutionary changes in the diagnosis of fragile X syndrome. Both the diagnosis of patients and carriers, and even prenatal diagnosis, could be easily and confidently done by the analysis of CGG repeat and gene methylation. However, position cloning did not help the understanding of the function of *FMR1*. Till now, the function of *FMR1* is unknown, and there is no effective treatment for the fragile X syndrome.

We have studied the structure of *FMR1* promoter, and proved its methylation sensitivity (Hwu et al. BBRC 1993; 193:324-9). We also showed by genetic (study of mutants) and biochemical (study with recombinant transcription factor) evidences that, *FMR1* promoter could be activated by cAMP and CREB (Hwu et al. DNA & Cell Biology).

In this study, we want to use in vivo DNA footprinting to study the interactions between FMR1 promoter (including the methylation sensitive element, MSE) and nuclear proteins. These interactions will help the understanding of the regulation and function of FMR1 protein. Beside, we will develop an technique called RNase protection assay (RPA). RPA is a sensitive, stable and reliable method to quantitate RNA. It will be used to detect changes of FMR1 expression in cells under various conditions, to prove the presence of FMR1 regulation.

Unfortunately, we met a great difficulty in the in vivo DNA footprinting assay. The FMR1 promoter is extremely GC rich, and it also contains the CGG repeat. This made primer extension, the critical step in footprinting assay, very inefficiency. RPA assay could not detect any changes in the cellular FMR1 gene, since the regulation of FMR1 is very cell specific, and even subcellular specific. Now we have shifted our method into animal model study which goes smoothly recently.

Keywords: *FMR1*, gene regulation, fragile X syndrome, DNA footprinting

Introduction

Fragile X syndrome (FRAXA) is an important, and also very interesting disease. It is the most important hereditary form of mental retardation, and characterized by Xq27.3 fragile site, macroochidism, and expectation in transmission (Sherman 1985). The gene - *FMR1*- for this disease was found by position cloning. *FMR1* (Fra X Mental Retardation 1) contains an expanded CGG repeat (Verkert 1991, Fu 1991). The risk of expansion during oogenesis increases with the number of repeat, thus accounts for a paradox (expectation) that the risk of mental impairment in fragile X pedigree is contingent upon position of individual in the pedigree. The gene is also hypermethylated at a 5'CpG-island in patients (Oberle 1991, Boyes 1991). Because position cloning did not tell the function of genes, the function of *FMR1* is a new challenge to scientists. The study of *Fmr1* knockout mice showed similar phenotypes as patients (The Dutch-Belgian fragile X consortium, 1994), however, the pathological finding was minimal.

The nucleotide sequence of *FMR1* predicts a protein of 614 amino acids which is highly conserved among species (Verkerk et al., 1991). FMR protein (FMRP) contains two KH domains and an RGG box, and has been shown to bind its own messenger as well as 4% of the human fetal brain mRNAs (Ashley et al., 1993). Different proteins may also exist because alternative splicing of *FMR1* generates up to 12 mRNA species (Verkerk et al., 1993).

FMR1 has been thought to be a non-vital house-keeping gene. But recent studies revealed highly enrichments of *FMR1* expression in certain types of cells in both brain and testes (Hinds et al., 1993; Bächner et al., 1993; Abitbol et al., 1993). These specific tissue distributions of *FMR1* provide a functional role for FMRP because brain (Reiss et al., 1991) and testis (Johannisson et al., 1987) are the two organs which show most prominent pathological findings in patients with fragile X syndrome. Recently, *FMR1* gene was further reported to be modulated in proliferating and quiescent cultured cells (Khandjian et al., 1995). Therefore, *FMR1* expression is controlled at various levels by mechanisms linked to cell differentiation and growth.

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 460bp promoter region is GC rich, containing several putative SP1 sites, and a TATA like box 26bp upstream of the major transcription initiation site. Methylation may interfere with transcription by direct preventing the binding of transcriptional factors (Iguchi-Ariga 1989) or by altering chromatin structures (Keshet 1986) through the action of methyl-CpG-binding proteins (Boyes 1991). A genomic fragment containing this region has also been shown to direct tissue-specific distribution in transgenic mice (Hergersberg et al., 1995).

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 was called methylation sensitive element (MSE), and demonstrate enhancer effect in transfection assays. MSE contains a

cAMP-responsive element (CRE)-like sequence. Recombinant CRE binding protein (CREB) bound to MSE, and the activity of CRE could be activated by both CREB and forskolin (cAMP). Evidences shown by us were both genetic (study of mutants) and biochemical (study with recombinant transcription factor).

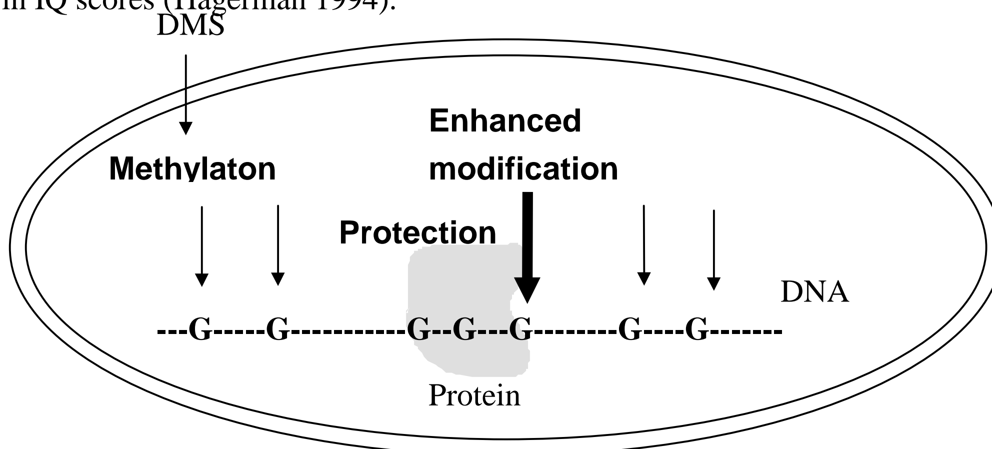
Although our findings give new imaginations about the function of FMR1, therse are all *in vitro* studies. Therefore, currently, we are working on the analysis of FMR protein. We already had a home-made rabbit antibody. So western blot could be done on cultured cell and tissues. Immunofluorescence staining of cultured cells are also going. Besides that, two important experiments are designed for the following year. The first is RNase protection assay (RPA). RPA is a sensitive, stable and reliable method to quantitate RNA. It will be used to detect changes of FMR1 expression in cells under various condition, to prove the presence of FMR1 regulation. The second experiment is *in vivo* DNA footprinting. It could demonstrate the binding of transcription factors to MSE (methylation sensitive element) in intact cells. We hope these two key experiemts could open a new era of study. Hopefully, there will be effective treatment for the fragile X syndrome because these patients are declining in IQ scores (Hagerman 1994).

Materials and Methods:

Preparation of DNA

Lymphoblasts will be grown in RPMI plus 15% FCS. Normal lymphoblasts (GM02010) and lymphoblasts from patient with fragile X syndrome (GM03200) will be used for comparism. 2.5 X 10^8 lymphoblasts will be washed in warm (37°C) PBS and suspended in 1 ml of warm PBS in 50 ml tube. 1 μl (0.1%) of dimethylsulfate (DMS) then will be added to the cells for 5 min. The methylation reaction is stopped by adding 49 ml ice-cold PBS, and washed again with cold PBS. DNA extraction will be performed with a salting-out method. The purified DNA will be dissolved in water (not TE buffer).

As a control for *in vivo* footprinting, untreated purified genomic DNA from the same cells being analyzed is isolated as outlined above, omitting the *in vivo* DMS treatment step. This sample served as a naked DNA control lane in the final DNA sequencing autoradiogram by displaying the guanine-specific cleavage pattern in the absence of bound proteins. The purified genomic DNA is first digested with a restriction enzyme that does not cut within the region of interest in order to reduce viscosity of the DNA solution.



Following digestion, the purified DNA is then chemicallly modified *in vitro* by DMA treatment. The conditions for DMS treatment must be adjusted so the size distribution of the cleaved fragments averages approximately 600 bp.

Piperidine cleavage of chemically modified DNA

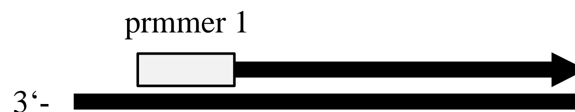
Piperidine cleavage is performed in 1.5-ml tubes. The DNA is resuspended in 90 µl of water and piperidine is added to a final concentration of 1 M (10%). The samples are incubated at 90-95°C for 30 min and then place on ice. Then, a 1/10 vol of 3 M sodium acetate, pH 7, is added and mixed, and the DNA is precipitated with 2 vol of ethanol. The DNA pellets are then resuspended in TE to a concentration of approximately 1 µg/µl.

Ligation-mediated PCR (LMPCR)

The cleaved genomic DNA is denatured and a primer (primer 1) is annealed and extended with a DNA polymerase to form a blunt end. An asymmetric double-stranded linker is then ligated to the blunt end. After ligation of the linker, the nested set of linkered genomic DNA fragments from the region of interest is amplified by PCR using a second (internal) gene-specific primer (primer 2), and the long strand of the linker. PCR amplification is then performed for 19-20 cycles. Following PCR, the amplified DNA sequencing ladder can be visualized by primer extension of another radiolabeled (internal) primer (primer 3).

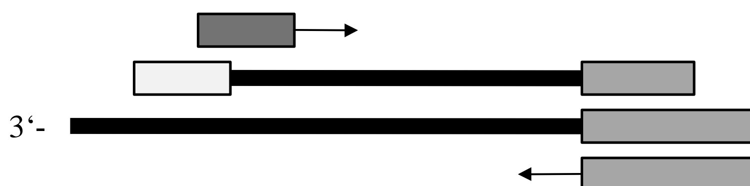
First strand primer extension: LMPCR is initiated by annealing of primer 1 to the modified and cleaved genomic DNA, folloed by extension of primer 1 by *pfu* polymerase.

Step 1: primer extension



Step 2: Ligation

Step 3: PCR



Step 4: Primer extension

pfu polymerase will be used in this study because it give blunted product. This will first facilitate the ligation reaction. Second, in PCR, *pfu* polymerase does not give extra terminal A nucleotide, thus afford more accurate length estimation. Two µg cleaved genomic DNA, 0.6 pmol (4ng) primer 1, 1x *pfu* polymerase buffer, and 250 µM dNTP in 30 µl will be heated in 98°C 5min, shift to 60°C in 10 min, 60°C 30min, and 76°C 10min. After the reaction, DNA will be purified by desaulting.

Ligation of the linker primer: Ligation will be performed in 1x ligase buffer, 1.66 mM ATP, 100 pmol linker (1.2 ug), 4.5U ligase in 75µl, 15°C overnight. After ligation, the DNA will be purify by desaulting kit.

PCR amplification: PCR will be performed with 10 pmol (100ng) each primer and 250 µM dNTPs with *pfu* polymerase. Cycling as 98°C 1min and 66°C 2min for 20 cycles. The PCR product will be purified by desaulting.

Final primer extension: Final extension will be performed using end-labeled primer 3 with *pfu* polymerase at 69°C. The extension product will be separated by 6% sequencing gel.

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In vitro transcription

Preparation of template DNA: For synthesis of run-off transcripts of defined length, the template DNA should be linearized by digestion to completion with a suitable restriction enzyme that cleaved distal to the promoter.

1. Amplification and cloning of cDNA sequences from PC12 cells (Rat) and BHK cells (Hamster). RNA was extracted from PC12 and BHK cells by acid-phenol-quanidium method. Reverse transcription was performed by AMV reverse transcriptase and a specific primer (mfmr-2) designed from a sequence completely identical between human and mouse *FMR1* gene. PCR was performed by primer set mfmr-3 (5'-TTCTCAACCTAACAGTACA) and mfmr-2 (5'-GTCTACTACCTCGACCCATTC). Two PCR products (330 & 270bp) were found because of the alternative splicing of the *FMR1* gene. The shorter product was cloned into pBluescript II vector. Several clones were sequenced, and were found to be different by only one of two nucleotides. Therefore one clone (P8) was selected to be used as the template.

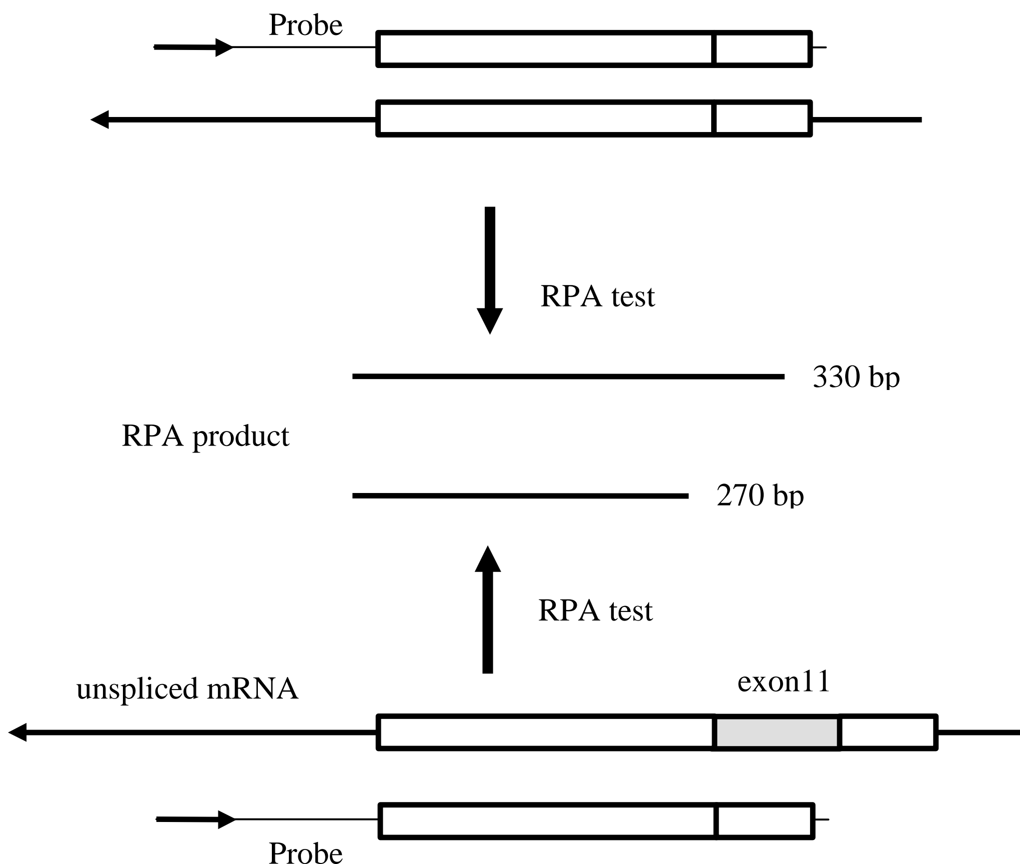
2. Linearization of template DNA. The plasmid will be linearized by XhoI which is distal (relative to the T3 promoter) to the cloning site (EcoRV). Linearized DNA will be purified by phenol-chloroform extraction and prepared as 0.5 µg/µl. The transcipt will include a piece of vector sequence flanking the FMR1 seuqence. These sequences will be cut in the RPA assay, thus give a difference between the uncut probe and the protected

product. Two protected product will be expected caused by the alternatively spliced RNA species. Unspliced RNA (containing exon 11) will give a shorter product (270bp), and the spliced RNA (without exon 11) will give a longer product (330bp).

Assembly of the transcription reaction **:** In final volume of 20µl, add 2 µl 10X transcription buffer, 1 µl each of 10mM ATP, CTP and GTP, 2 µl (1µg) linearized template DNA, 1 µl 0.05mM UTP, 5 µl [α-^{32}P] UTP 800 Ci/mmol, and 2 µl T3 RNA polymerase (MAXIscript, Ambion USA). The mixture will be incubated for 30 min at 37°C.

Removal of template DNA: Add 1 µl of Rnase-free Dnase I (2 U/ µl) to the reaction, and incubate at 37 ^{o}C for 15 min. The labeled probe will be diluted in lysis buffer and used in the RPA test. The length of the probe will be checked by 5% sequencing gel.

Two kinds of RPA product due to mRNA altanative splicing



Preparation of cultured cell lysate

1. PC12 cells in 6 cm dishes will be treated by 1μM forskolin for 8 or 24 hours. After treatment, cell will be lyzed in 100-250 μl lysis/denaturation solution (1-2 x 10^7 cells/ml) (Direct Protect, Ambion, USA). Vortex the lysate vigorously or passing the lysate through a 25G needle ten times to break the DNA (or the solution will be too viscous to handle). Store the lysate at -70°C until used.

2. BHK cells will be transfected with CREB expression vector. Six cm dishes will be transfected with 30 ng pRSV-CREB and 2 μg pGERM4 DNA (carrier). Cell will be harvested 24 and 48 hours after transfected as decribed for PC12 cells.

Hybridization of probe and lysate:

For quantitative detection of RNA it is important that the labeled probe be present in excess over the target RNA. Ideally one should have a 3 to 10 fold molar excess of probe over the target RNA. Generally 1 x 10^5 cpm of a probe with a specific activity of 1-10 x 10^8 cpm/μg gives acceptable results when using 45 μl of lysate to detect a rare to moderately abundant message.

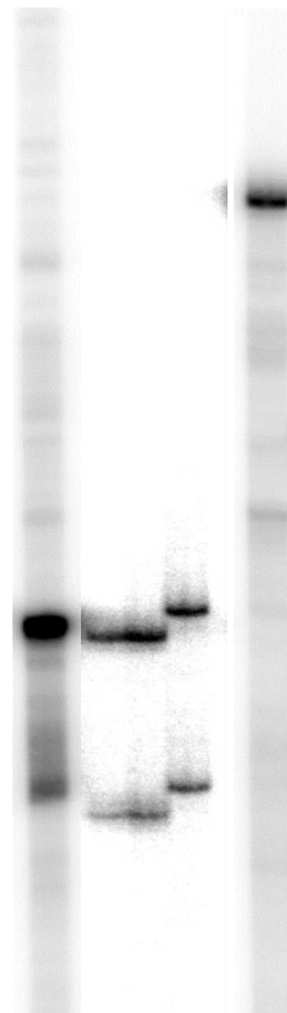
RNase digestion of hybridized probe and lysate:

RNase cocktail containing both RNase A and RNase T1. RNase T1 (alone) is often substituted for RNase cocktail when the probe being used is from a different species than the RNA sample, or when the target sequence is very "AU rich". RNase T1 only cuts after G residues, and then only if several bases in a row are not paired.

Protease digestion of RNase A/T1 and separation and detection of protected fragments

Results and Discussion

The RPA test

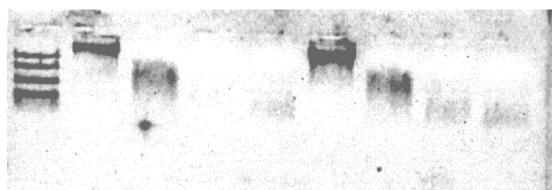


1 2 3 4 5 6

Direct protect RNase protection assay (RPA). Either 20 (lane 2) r 45 ml (lanes 3, 4) BHK cell lysate were assayed. RNase cocktail was added to lanes 2, 3, and 5. RNase T1 was added to lane 4. There are 2 protected products in lanes 2 to 4. This are due to the presence of 2 species of mRNA products due to alternative splicing of the *FMR1* mRNA. The RPA products in lane 4 was shorter than lane 2 and 3, because RNase

Piperidine cleavage

M 1 2 3 4 5 6 7 8



Lymphoblasts treated by 0% (lanes 1, 5), 0.1% (lanes 2, 6), 0.25% (lanes 3, 7), or 0.5% (lanes 4, 8) DMS at 37°C for 5 min. Purified DNA was then modified by 10% (lanes 1-4), or 2.5% (lanes 5-8) piperidine at 80°C. The resulting DNA was electerophoresed in 1.5% agarose gel. The DNA size markers were 5, 2, 1, 0.5/0.45 kb.

計畫成果自評

本實驗的困難應該是因為無法突破細胞內足印的技術，因為 FMR1 啟動子 GC 含量極高，更有 CGG 重複序列，導致細胞內足印分析中 primer extension 的部分極為困難。RPA 實驗則無法看到 FMR1 在細胞內的變動。其實在我們執行計畫的過程中，已有研究發現因為 FMR1 的調控其實只局限在少數的細胞，甚至細胞的一部分，如神經的樹突。因為不論是細胞內足印，或是 RPA 實驗, 看的都是細胞株或一定量的組織細胞。這樣的方法是沒有辦法顯現出 FMR1 的調控的。我們目前已改變研究方法，將直接以動物模式來研究，預期會有突破性的成果。

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