國科會專題研究計劃成果報告

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一、計畫中文摘要：請於五百字內就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。

關鍵詞：愛滋病毒, 酵母菌雙混種系統, Vpr, HAX-1

愛滋病已成當代人的一個夢魘。如何由健康的愛滋病毒帶原者進展到嚴重後天免疫不全症候群仍是一個謎，不過關鍵可能在愛滋病毒的輔助基因。愛滋病毒有六個輔佐基因，其中兩個是病毒生命週期中必需者，爲 tat 與 rev。而另外四個輔助基因 (nef、vpr、vpu、vif) 是病毒生命週期中不被使用者。最近的證據指出這四個輔助基因與愛滋病毒在人體內之致病力極具相關性，因此已成爲學界研究重點。本計畫主要是要鑑定出細胞內會與 Vpr 直接作用之蛋白，以進一步了解 Vpr 蛋白的功能。酵母菌雙混種系統是目前鑑定蛋白與蛋白作用之利器。我們已利用酵母菌雙混種系統成功地尋找到一些一些會與 Vpr 蛋白作用之細胞蛋白質，這些蛋白包括 HAX-1, HHR-23A 及 Dnaj，這也暗示 Vpr 可能與 DNA 修補, 細胞訊號傳遞及蛋白傳送成形有關。我們也找到幾個至今功能不明的蛋白。在本研究計劃中我們挑選了 HAX-1 做進一步研究，以了解 Vpr 如何與 HAX-1 作用，及其可能的功能。首先我們利用 GST pull down 的方法驗證 Vpr 與 HAX-1 確實有交互作用。在酵母菌雙混種系統也發現 Vpr 的第一與第二次單元 (domain) 負責與 HAX-1 的交互作用。爲了測試 Vpr 與 HAX-1 交互作用功能的實驗模式已成功建立，包括細胞週期阻斷 (cell cycle arrest) 與異位活化 (transactivation)。我們證實在上述模式中 Vpr 可將細胞週期停止在 G_2/M 階段，也可異位活化 HIV-1 的 long terminal repeat (LTR)。在測試 HAX-1 對 Vpr 此兩種功能是否有任何影響實驗中，已初步發現 HAX-1 細胞蛋白確實參與 Vpr 的異位活化功能。至於 HAX-1 是透過何種機轉來影響 Vpr 的異位活化功能，有待新的年度計劃實驗來進一步闡述證明。

二、計畫英文摘要：請於五百字內就本計畫要點作一概述，並依本計畫性質自訂關鍵詞。

Keywords：HIV-1, Vpr, yeast two-hybrid system

Infection with human immunodeficiency virus (HIV) has become a nightmare for contemporary people. The mechanism underlying the progression from HIV carrier state to frank AIDS probably lies on the auxiliary genes of HIV. HIV-1 has six auxiliary genes; 2 of them are essential (tat and rev) and 4 are accessory (vif, vpr, vpu and nef). These 4 accessory genes have attracted most intense research interest in recent 2 years as we come to know their close relationship with in vivo pathogenicity. This project is aimed to understand the operating mechanism of HIV-1 vpr gene by identification of Vpr-interacting cellular proteins. Yeast two-hybrid system is a powerful tool in disclosing protein-protein interaction. We have used this system to study Vpr function and have thus far identified several cellular proteins that interact with Vpr. Among these, the identification of HAX-1, HHR-23A, Dnaj suggests that Vpr may be involved in DNA repair, signal transduction, and chaperon system. Some novel cellular proteins were identified also and their functions remain elusive. In 1999 we confirmed the interaction between Vpr and HAX-1 using GST pull-down assay. Furthermore, the first and second domains of Vpr mediate the Vpr-HAX-1 interaction. In order to understand the functional significance of this interaction, two experimental models were established, namely, cell cycle arrest and transactivation. Vpr was confirmed to stop cell cycling at the junction of G_2/M phase and to transactivate HIV-1 LTR. According recently experimental data, suggesting that HAX-1 participate in Vpr-induced transactivation function. The further functional signification of Vpr-HAX-1 interaction will be addressed in the coming project. We believe that our study can clarify the pathologic role played by Vpr in AIDS pathogenicity and may help design anti-Vpr strategy that may be beneficial for AIDS management.

三、研究計畫之背景及目的：

請詳述本研究計畫之背景、目的、重要性以及國內外有關本計畫之研究情況，重要參考文獻等。本計畫如為整合型計畫之子計畫，請就以上各點分別述明與其他子計畫之相關性。

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS), a pandemic that has affected millions of people worldwide (Greene 1991). The mechanism underlying progression from healthy HIV carrier to symptomatic AIDS remains unknown. Cumulative evidence has suggests that HIV accessory genes may play major roles for the in vivo destruction of human immune system (Subbramanian and Cohen; Huang and Jeang 1995). It is hoped that understanding the functions of HIV genes can help design proper anti-viral strategy to stop or delay the progression of AIDS.

In line with the conventional retroviruses, HIV possesses three major genes, pol, gag, and env (Greene 1991). For HIV-1 there exist 6 additional genes. The six auxiliary/non-structural proteins of HIV can be categorized into 2 groups, essential (Tat and Rev) and accessory (Vpr, Vpx, Vif, Vpu, and Nef). HIV replicates competently in vitro in T-cell lines without the need for accessory gene functions (Subbramanian and Cohen, 1994). However, in primary lymphocytes and macrophages, accessory gene products such as Vpr and Vpx contribute to the efficient progression of the virus lifecycle (Balliet et al., 1994; Balotta et al., 1993; Connor et al., 1995; Kwamura et al., 1994; Park and Sodroski, 1995). Furthermore, in vivo there must be a selection pressure directed towards preserving these accessory genes since many primary HIV-1 isolates maintain these reading frames open.

Vpr is a 96 amino acid protein that is well-conserved between HIV-1,HIV-2 and SIV. The expression of the mRNA for Vpr is Rev-dependent (Schwartz et al., 1991; Arrigo and Chen, 1991). Vpr is localized to the nucleus of infected cells (Lu et al., 1993), and during late stages of the virus lifecycle is incorporated into the virions at an estimated 5-10 copies per viral particle (Cohen et al., 1990). Incorporation of Vpr into virion requires the p55Gag precursor and more specifically its carboxy-p6domain (Lu et al., 1993; Paxton et al., 1993; Zhao et al., 1994; Kondo et al., 1995). This virion-association of Vpr is unique amongst the HIV accessory proteins and suggests that Vpr may play a significant role soon after virus entry into cells. Up to now, several functions have been attributed to HIV-1 Vpr including moderating viral replication, nuclear transport of preintegration complexes, transactivation function, and effects on the host cells (for review see Huang and Jeang, 1995). Although a number of functions have been proposed for Vpr, there are only a limited number of Vpr-associated cellular proteins identified. Three cellular proteins have been reported to form protein-protein complexes with Vpr. These include a 41 kDa cytosolic protein that also complexes with glucocorticoid receptor protein (Refaeli et al., 1995), a 180 kDa protein (Zhao et al., 1994), and the cellular uracil DNA glycosylase DNA repair enzyme (BouHamdan et al., 1996). In the last two cases, amino acids in Vpr spanning positions 60 to 81 and 15 to 77, respectively, were reported to be important for binding to cellular proteins. A firm understanding of the role for Vpr-cellular protein association is yet missing. In order to further understand the biologic role undertaken by Vpr, identification of possible cellular proteins that associate with Vpr should be very valuable.

Yeast two-hybrid system is most suitable for this purpose (Chien et al, 1991; Durfee et al, 1993; Gyuris et al, 1993). This system exploits the finding that a DNA-binding transactivator can be splitted into DNA-binding module and activator module (Ma and Ptashne,1988). With both modules tethered to gene promoter region, the transactivation function can proceed as the original intact

protein does. In practice, the protein of interest (bait protein) is first fused with the DNA-binding domain of LexA or Gal4 (Chien et al, 1991; Durfee et al, 1993; Gyuris et al, 1993) and then transformed to yeast cells together with hybrid gene of Gal4 activator domain and cDNA library. Further selection picks up only yeast cells that contain bait protein and its associated protein, which can then be easily identified. This system has been proven to be very powerful in the study of protein-protein interaction and can be extended to test deletional and site-directed mutations of the bait protein to determine critical residue that mediates the protein-protein interaction. Previous studies picked up Vpr-associated proteins based on immunoprecipitation; however, the versality of immunoprecipitation is much inferior to yeast two-hybrid system. To date, limited success has been achieved by applying yeast two-hybrid system to study Vpr (Bouhamdan et al, 1996; Stark and Hay, 1998; Mahalingam et al, 1998) and we believe it is worth trying this system to study HIV-1 accessory genes.

In our study, the whole coding region of Vpr (291 b.p.) was cloned to pBTM116, a plasmid containing LexA DNA binding domain. This hybrid pLexA-Vpr plasmid was thrown into yeast two-hybrid system together with cDNA library of bone marrow cells (Human bone marrow 5'-stretch plus cDNA library, Clontech, USA). We have successfully identified several cellular proteins that interact firmly with Vpr using the yeast two-hybrid system (Table1). These proteins included, among others, HHR23A, HAX-1, MSJ-1, SMA (spinal muscular atrophy), and Dnaj protein. HHR23A is involved in DNA repair enzyme system and is part of nucleotide excision repair complex. Bouhamdan et al. in 1996 found that UNG (uracil DNA glycosylate DNA repaid enzyme) interacts with Vpr using yeast two-hybrid system (Bouhamdan et al. 1996). Hence, one of the functions of Vpr may be related to DNA repair, and interaction between HHR23A and Vpr deserves further scrutiny. HAX-1 has been found to be associated with HS1, a substrate of Src family tyrosine kinase (Suzuki Y, et al, 1997). Interaction of Vpr with HAX-1 implicated that Vpr may affect signal transduction and may thus contribute to pathogenicity of HIV-1. Dnaj may be involved in protein folding and assembly together with Hsp70 (Cyr DM et al, 1994). Our yeast system also identifies some cellular proteins, whose functions are still elusive.

The preliminary results so far have shown that our yeast two-hybrid system using pLexA and bone marrow cDNA library has yielded interesting and important information. This three-year project also has addressed the subsequent issues of Vpr/HAX-1 association. These include verification of the interaction by independent biochemical assay (GST pull down assay) and transactivation functional analysis of Vpr as well as HAX-1. The further functional signification of Vpr-HAX-1 interaction will be addressed to figure out the functions of Vpr in vivo and the impact of this interaction at cellular level.

四、研究計畫之結果及討論：

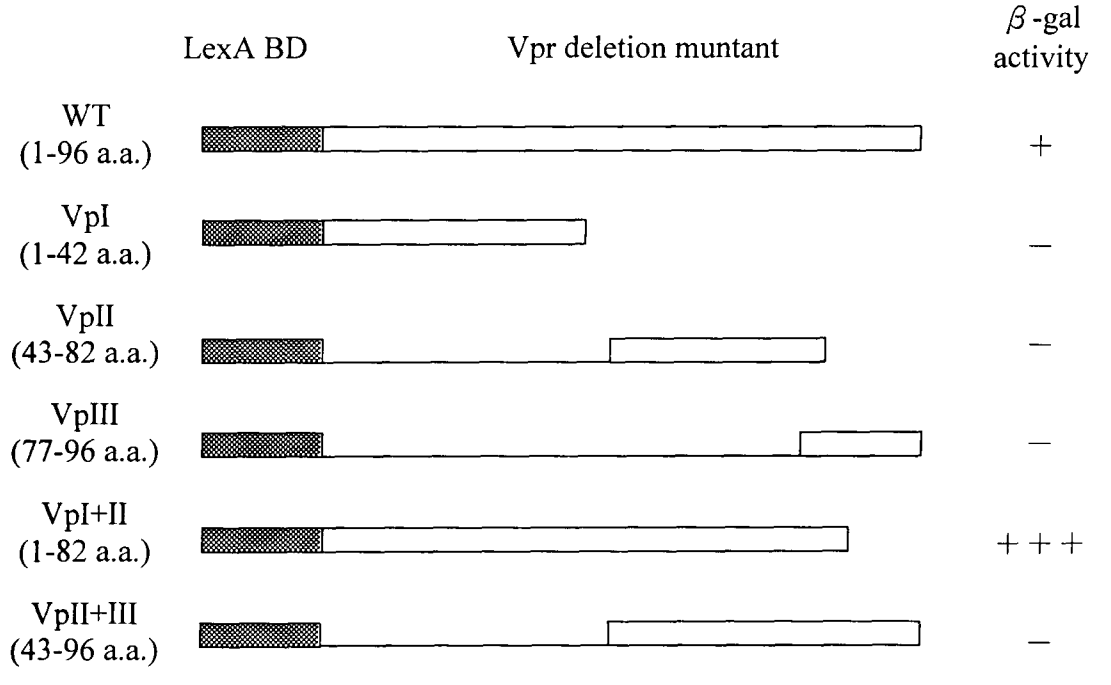
Table 1: Candidates of HIV-1 Vpr-associated proteins by two-hybrid screening in yeast

Protein	SMN1	HHR23A	HAX-1	MRJ	Unknown
clone	1-2 3-4 1-4 4-6 1-6 4-8 1-8 4-15 1-12 4-23	1-5 1-16 3-11 3-30	1-30 2-3	3-13 3-25 4-18 4-21	1-3 1-20 1-22 1-25 2-4 3-16 4-4

* SMN1, human spinal muscular atrophy gene product, survival motor neuron protein
* HHR23A protein, a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein
* HAX-1, a novel intracellular protein, directly associates with HS1, a substrate of Src family tyrosine kinases.
* MRJ, a new member of human Dnaj-related gene family, Danj-Hsp70 association involved in protein assembling, folding, transport.

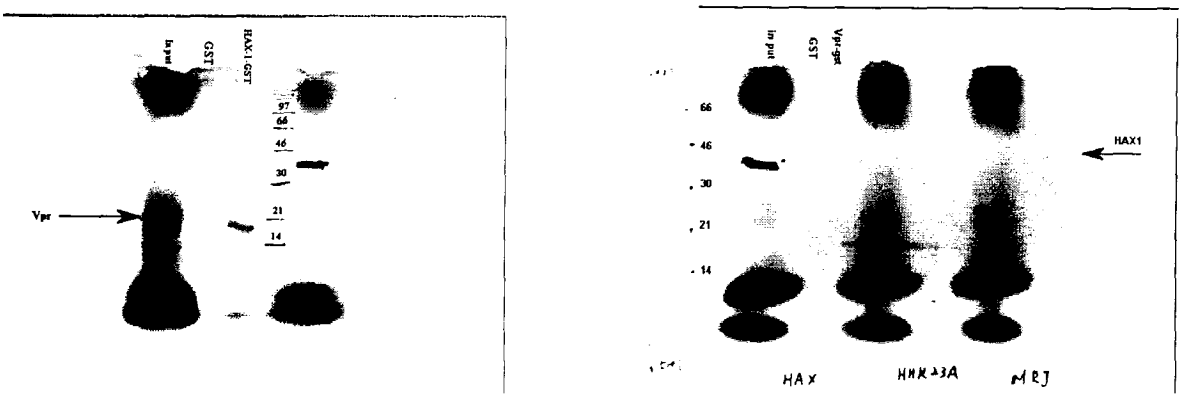
In this study, we have successfully identified several cellular proteins that interact with Vpr using the yeast two-hybrid system. These proteins included, HHR23A, HAX-1, MRJ-1, SMN1 (spinal muscular atrophy determine gene), Dnaj-like protein(MRJ protein), and some unknown protein. (see Table1)

Fig. 1. Interaction of Vpr deletion mutant with HAX-1 in the two-hybrid system



The result of interaction of Vpr deletion mutants with HAX-1 in the two-hybrid system, indicate that first and second domains of Vpr(1-82 a.a.) necessary for binding to HAX-1.Within this region, first domain(1-42 a.a.) seem to be essential for Vpr-HAX-1 interaction(see Fig. 1).

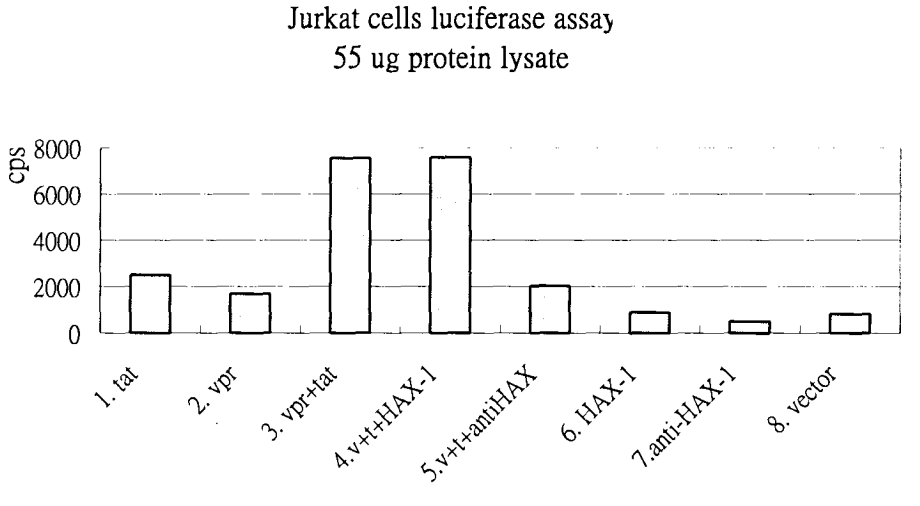
Fig.2. Interaction of Vpr with HAX-1 in vitro by GST pull down assay



a. Binding of ^{35}S-Vpr to GST-HAX-1 **b. Binding of ^{35}S-HAX-1 to GST-Vpr**

In vitro experiments using GST-HAX-1(or GST-Vpr) fusion protein and in vitro-translated ^{35}S-Vpr(or^{35}S-HAX-1) were performed to demonstrate the direct interaction between Vpr and HAX-1(see Fig. 2). These results of binding of Vpr to GST-HAX-1 (or HAX-1 to GST-Vpr) are in agreement with those interaction of the yeast two-hybrid system .

Fig.3. Functional analysis of the effect of Vpr on LTR transcription by over-express sense or antisense HAX-1



To verify that Vpr-HAX-1 interaction takes place in mammalian cells and has biological significance, we co-transfected of CMV-HAX-1 plasmid or anti-sense HAX-1 plasmid to human Jurkat T cells together with Vpr and tat plasmid. Anti-sense HAX-1 plasmid significantly reduced Vpr and tat induced transactivation effect of HIV-1 LTR promoter by LTR-luciferase reporter assay. Our analysis showed that blocking of HAX-1 by excess of anti-sense HAX-1 plasmid indeed affect the coactivator function of Vpr(see Fig. 3).

十八、研究計畫之參考文獻：

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附表一:

BLAST results of Vpr-associated candidates proteins by yeast two-hybrid screening

Clone	β-galactosidase activity	BLAST result
1-2	+++	Human spinal muscular atrophy gene
1-3	+++	unknown
1-4	+++	Human spinal muscular atrophy gene
1-5	+	Human mRNA for HHR23A protein
1-6	++	Human spinal muscular atrophy gene
1-8	++	Human spinal muscular atrophy gene
1-12	++	Human spinal muscular atrophy gene
1-16	+	Human mRNA for HHR23A protein
1-20	+++	unknown
1-22	+	unknown
1-25	±	unknown
1-30	+	Human HS1 binding protein HAX-1 mRNA
2-3	++	Human HS1 binding protein HAX-1 mRNA
2-4	++	unknown
3-4	+++	Human spinal muscular atrophy gene
3-11	+	Human mRNA for HHR23A protein
3-13	++++	Homo sapienes mRNA for MSJ-1
3-16	+++	unknown
3-25	++	Homo sapienes mRNA for MSJ-1
3-30	±	Human mRNA for HHR23A protein
4-4	+++	unknown
4-6	++	Human spinal muscular atrophy gene
4-8	++	Human spinal muscular atrophy gene
4-15	±	Human spinal muscular atrophy gene
4-18	+	Homo sapienes mRNA for MSJ-1
4-21	++	Homo sapienes mRNA for MSJ-1
4-23	++++	Human spinal muscular atrophy gene

P.S.1. HHR23A protein, a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein

P.S.2. HAX-1, a novel intracellular protein, directly associates with HS1, a substrate of Src family tyrosine kinases.

P.S.3. MSJ-1, a new member of human Dnaj-related gene family